Chemosensory avoidance behaviors of marine amphipods *Allorchestes compressa* revealed using a millifluidic perfusion technology

Cite as: Biomicrofluidics 14, 014110 (2020); doi: 10.1063/1.5131187

Submitted: 13 October 2019 · Accepted: 10 January 2020 ·

Published Online: 22 January 2020

Yutao Bai, Jason Henry, and Donald Wlodkowic ^{a)}

AFFILIATIONS

The Phenomics Laboratory, School of Science, RMIT University, Melbourne VIC 3083, Australia

^{a)}Author to whom correspondence should be addressed: donald.wlodkowic@rmit.edu.au

ABSTRACT

Chemosensory avoidance behaviors of aquatic invertebrates provide a functional link between early responses to pollutants at the infraorganismal and ecologically relevant supraorganismal levels. Despite significant importance, there is, however, a notable lack of user-friendly laboratory techniques. Here, we demonstrate a scalable millifluidic platform for higher throughput quantitative chemobehavioral studies. With a proof-of-concept application of this technology, we discovered that native Australian marine amphipods *Allorchestes compressa* exhibit rapid avoidance behaviors against a panel of environmental stressors. This work provides a novel avenue for the development of quantitative neurobehavioral systems applicable in diverse environmental risk assessment studies.

Published under license by AIP Publishing. <https://doi.org/10.1063/1.5131187>

INTRODUCTION

Neurotoxic effects of industrial pollutants are currently under-investigated and urgently require new testing methods that employ integrative and functional endpoints.¹ Behavior represents an innate response to internal (physiological) and external (environmental) factors. It integrates multiple levels of biological organization and is ecologically relevant end point against many anthropogenic toxicants.² The chemosensory avoidance responses, in particular, represent a rapid defense mechanism against unfavored chemical stimuli³ and were proven to be a highly sensitive indicator of environmental stress in diverse aquatic species.^{4–6}

Despite a significant potential to apply behavioral biometrics in predictive aquatic toxicology, analyzing chemotactic traits of marine invertebrates has received little attention.⁷ This is mainly because of the notable unavailability of feasible technologies designed specifically to study chemotaxis behaviors of small aquatic invertebrates.

To address this gap, we developed a scalable millifluidic perfusion system for quantitative chemobehavioral studies. As a proof-of-concept, we demonstrate that the integration of fluidic and video-based animal tracking techniques can reveal previously unknown behavioral traits such as avoidance behaviors of native Australian marine amphipods *Allorchestes compressa* against a

panel of environmental stressors.⁸ This species has been investigated as a promising sentinel organism with high relevance to Australian estuarine ecosystems, straightforward culture due to rapid reproduction, and acclimation to laboratory conditions. Although testing criteria for many native species such as *A. compressa* have not been standardized yet, they follow adaptations of existing testing guidelines that predominantly use freshwater amphipods such as *Hyalella azteca* or *Gammarus pulex*.^{2,9–11}

MATERIALS AND METHODS

Test organisms and chemicals

Wild type adult marine amphipods *Allorchestes compressa* with a length of 8 ± 1 mm and older than four weeks were used in all experiments. All specimens were collected at Swan Bay (Victoria, Australia). Animals were cultured in a glass aquarium with filtered natural seawater ($\text{pH } 8.2 \pm 0.1$) and fed on native seagrass. Filtered natural seawater used in cultures and testing was collected from Port Phillip Bay near the town of Sorrento, Victoria, Australia. The culture was kept at a constant temperature ($18 \pm 1^\circ\text{C}$) under 12 h:12 h light:dark cycle. After 72 h of acclimation to laboratory conditions, randomly selected amphipods with a length of 5 ± 1 mm were used in all experiments, utilizing in-house methods.²

Copper chloride and zinc chloride were purchased from Sigma-Aldrich (Sigma-Aldrich, Australia) and prepared by dissolving the chemicals in the filtered natural seawater in volumetric flasks, followed by serial dilutions.

Chlorpyrifos was prepared by directly dissolving the stock solutions in the seawater and diluted to specified concentrations. Considering the short half-life of chlorpyrifos (<1 d),¹² freshly prepared aliquots chlorpyrifos were used in all experiments.

Design and fabrication

The millifluidic devices were designed using Solidworks 2016 (Dassault Systemes SolidWorks Corp, Concord, MA, USA). Biocompatible poly(methyl methacrylate) (PMMA) thermoplastic sheets cut using an infrared laser system were used as a substratum, utilizing in-house methods.²

The poly(dimethylsiloxane) (PDMS; Sylgard 184; DowCorning Corp.; Midland, MI, USA) was used to fabricate custom sealing gaskets, utilizing previously described methods.¹³

Computational fluid dynamics (CFD) simulations

Finite volume simulations were performed in COMSOL 4.4 (Comsol, Burlington, MA, USA) software to solve the associated differential equations governing the balance of mass, momentum, and chemical species, utilizing in-house methods.² The design optimization in the virtual space ensured the formation of consistently uniform binary zones within the test chambers.

Fluidic and illumination manifolds

The millifluidic perfusion system was designed as a plug and play and easily scalable chipboard module. Each chipboard was mounted on a custom stage with a 5° incline to assist purging of air bubbles and fluidic perfusion. The drainage system was integrated within the stage. Transmitted light illumination used an array of light-emitting diode (5500 K, 820 lx) designed for shadow-free imaging. MINIPUS Evolution peristaltic pumps and polyvinyl chloride (PVC) tubing (Gilson, Inc., Middleton, Wisconsin, USA) were used for fluidic actuation.

Videomicroscopy and data acquisition

Videos were acquired using a Panasonic Lumix G7 digital camera (Panasonic Australia Pty Ltd) equipped with a 30 mm macro lens (Olympus Corp., Tokyo, Japan). The camera was mounted on a vibration-less column (Polaroid M3, Polaroid Inc., USA) for simultaneous imaging of the entire chipboard. Time-resolved video files at a resolution of 1080p and a rate of 24 frames per second (fps) were automatically acquired using a programmable external High-Definition Multimedia Interface (HDMI) recorder (Atomos Shogun).

Animal tracking and data analysis

Ethovision XT 14 (Noldus Information Technology) software suite was used to detect, track, and quantify the movement of test animals, as described previously.¹⁴ The software employed the contrast detection principle to automatically assign x-y coordinate

pairs to centroids of each animal. It simultaneously recognized and tracked up to 16 animals per defined arena. This enabled automatic calculation of behavioral parameters and data visualization, such as reconstruction of trajectories and occupancy heatmaps. At every 10-min segment of the experiment, the cumulative time animals spent in each zone was calculated and averaged across all replicates. Unless otherwise stated, all experiments contain data from at least 12 replicates (six chips per group). For data analysis, a standard one-way ANOVA with Dunnett's test was applied to perform a comparison between selected data group with significance set at $p < 0.05$.

To quantify the sensitivity of the organism to exposure, time-to-reaction index (TTRI) was used. TTRI was defined as a time length required to elicit a statistically significant change in the occupation time in the defined zone ($p < 0.05$; Dunnett's test), comparative to the last time interval before each exposure. Furthermore, to describe the overall reaction tendency to the chemical stimuli, we propose another dimensionless parameter chemotaxis tendency index (CTI),

$$CTI = \Delta \bar{t}_s \times \frac{n_s}{N}, \quad (1)$$

where $\Delta \bar{t}_s$ was averaged occupation proportion value changes over the time intervals with significance to control ($p < 0.05$; Dunnett's test), n_s was the number of time intervals with significance, and N was the total number of analyzed exposure intervals.

RESULTS AND DISCUSSION

The device design consisted of two modules: (i) the multilayer 3D chip body ($66 \times 44 \times 9.5$ mm, each) with an optically-transparent top layer equipped with integrated inlet ports. Each circular main chamber ($\phi 35 \times 8$ mm) had an internal volume with approximately 14.6 ml and was connected with inlet and outlet circuitry through an array of microchannels ($6 \times 1 \times 0.5$ mm). The microchannels allowed for a uniform microperfusion as well as efficient "caging" of the specimens inside the chamber and (ii) the chip holder with engravings of 3.5 mm in depth that mounted the chip body to an outlet manifold via a PDMS gasket [Fig. 1(a)]. Mating of two reusable modules utilized stainless steel M4 bolts for a complete fluidic seal [Fig. 1(a)].

Since the parallel fluidic actuation was independent of the chipboard system geometry, it provided a modular design that is scalable toward higher throughput applications. The system expansion is simply achieved by adding new actuated chipboard units in parallel with identical functionality to existing ones. In the current setup, up to 12 chips can be recorded simultaneously with one camera, this number can be further increased with multiple cameras.

CFD simulations indicated that at a flow rate of 0.8 ml/min, the specimen will experience a low impact artificial millifluidic environment with a constant pressure of 0.19 Pa and a peak velocity of 0.45 mm/s within the main chamber. A range of flow rates up to 4.5 ml/min was examined. As described in the previous study,² the condition under selected flow rates did not affect survival nor inhibit the innate swimming behaviors of animals. Turbulent flow was only identified in the area immediately

FIG. 1. The 3D CAD model (a) showing the layers of the millifluidic chip. CFD simulation (b) allows for the optimization of fluid flow inside the device chamber. The laminar zone formed inside the chip chamber (c) was experimentally validated using Trypan Blue dye. LOC system schematics (d) highlight the modular chip components capable of higher throughput by the expansion of the tubing network. The drainage and illumination subsystem (e) enable behavioral assays to be completed and a quantitative occupation map to be generated.

surrounding the inlet port and transitioned to the laminar flow after passing the microchannels. The *in silico* modeling indicated that the laminar fluid flow was maintained and two distinct fluidic domains with uniform mass distribution could be successfully formed within 2 min [Fig. 1(b)]. The theoretical assumptions were validated experimentally using 0.04% trypan blue dye [Fig. 1(c)].

The proof-of-concept chemotaxis behavioral studies were performed using heavy metals like copper and zinc and the organophosphate chlorpyrifos. All chemicals selected were ranked as major anthropogenic pollutants in the aquatic risk assessment³ and commonly used in the ecotoxicity tests with amphipod species.⁶ It was hypothesized that the avoidance responses are more sensitive than mortality and could be triggered at low concentrations of toxicants. Initially, the 48 h median lethal concentrations (LC_{50}) range² of copper chloride at 5.0 mg/l was tested [Fig. 2(a)]. The video recording commenced immediately after loading the specimens and filling the chamber with clean seawater. During the pre-exposure stage, the amphipods were allowed to acclimate for up to 20 min in clean seawater. Subsequently, the randomly selected inlet ports were actuated with the toxicant to form a binary mixture. The exposure was performed for 2 h (Exposure I), followed by a reverse actuation (Exposure II) of the same duration [Fig. 2(a)].

In the exposure stage I, the TTRI was 30 min and the CTI was 62.8, indicating that the treatment with copper chloride invoked strong and persistent avoidance behaviors (negative chemotaxis)

within a very short time period. After reversal actuation (exposure stage II), the zone preference was altered progressively with the TTRI of 50 min and the CTI of 28.3. The elongation of TTRI with simultaneous reduction of the CTI could be explained as an adaptation to unfavorable chemical stimuli or as an overall anesthetic effect due to neuromuscular or metabolic toxicity.

Next, we investigated the avoidance behaviors to 1.0 mg/l zinc chloride (48 h LC_{50}) under shorter exposure regimens of up to 60 min [Fig. 2(b)].^{2,8} The TTRI in each exposure stage was 30 min and 20 min, and the CTI was 55.4 and 23.0, respectively. Similar to the copper chloride test, the avoidance behavior was rapid and strong, highlighting the value of chemotaxis assays as a fast and effective tool in behavioral ecotoxicology.

Chlorpyrifos is known to be highly hazardous to aquatic life by interfering with the organism's nervous system.¹⁵ Despite those characteristics, no chemotaxis studies on marine amphipods have been previously reported thus two arbitrary concentrations were selected [Figs. 2(c) and 2(d)]. The selected concentrations reflect upper and lower 48 h LC_{50} values for acute toxicity tests on amphipod *Gammarus palustris*.¹⁶ At 10.0 μ g/l, the CTI was 4.1 and 0 in two exposure stages, respectively. This indicated despite the sensitivity to the chemical (TTRI = 30 min), the concentration range was not sufficient to promote persistent negative chemotaxis. These data were further supported by tests using 2.0 μ g/l, where the TTRI and CTI in all stages were 0. Interestingly, despite lack of

¹J. B. Legradi, C. Di Paolo, M. H. S. Kraak, H. G. van der Geest, E. L. Schymanski, A. J. Williams, M. M. L. Dingemans, R. Massei, W. Brack, X. Cousin, M. L. Begout, R. van der Oost, A. Carion, V. Suarez-Ulloa, F. Silvestre, B. I. Escher, M. Engwall, G. Nilén, S. H. Keiter, D. Pollet, P. Waldmann, C. Kienle, I. Werner, A. C. Haigis, D. Knapen, L. Vergauwen, M. Spehr, W. Schulz, W. Busch, D. Leuthold, S. Scholz, C. M. vom Berg, N. Basu, C. A. Murphy, A. Lampert, J. Kunkelkorn, T. Grummt, and H. Hollert, *Environ. Sci. Eur.* **30**(1), 46 (2018).

- ²R. Cartlidge, D. Nugegoda, and D. Wlodkowic, *Sens. Actuators B* **239**, 660–670 (2017).
- ³D. Guillén, A. Ginebreda, M. Farré, R. M. Darbra, M. Petrovic, M. Gros, and D. Barceló, *Sci. Total Environ.* **440**(Suppl. C), 236–252 (2012).
- ⁴Y. Huang, G. Persoone, D. Nugegoda, and D. Wlodkowic, *Sens. Actuators B* **226**(Suppl. C), 289–298 (2016).
- ⁵J. Hellou, *Environ. Sci. Pollut. Res.* **18**(1), 1–11 (2011).
- ⁶P. Y. Kunz, C. Kienle, and A. Gerhardt, *Rev. Environ. Contam. Toxicol.* **205**, 1–76 (2010).
- ⁷A. Gerhardt, *Hum. Ecol. Risk Assess.* **13**(3), 481–491 (2007).
- ⁸M. Ahsanullah and A. R. Williams, *Mar. Biol.* **108**(1), 59–65 (1991).
- ⁹C. K. King, S. A. Gale, R. V. Hyne, J. L. Stauber, S. L. Simpson, and C. W. Hickey, *Chemosphere* **63**(9), 1466–1476 (2006).
- ¹⁰H. T. Vu, M. J. Keough, S. M. Long, and V. J. Pettigrove, *Environ. Toxicol. Chem.* **35**(5), 1130–1137 (2016).
- ¹¹P. Y. Kunz, C. Kienle, and A. Gerhardt, in *Reviews of Environmental Contamination and Toxicology*, edited by D. M. Whitacre (Springer, New York, NY, 2010), Vol. 205, pp. 1–76.
- ¹²National Registration Authority of Australia, in *The NRA Review of Chlorpyrifos*, NRA Review Series (NRA, 2000).
- ¹³N. M. Fuad, J. Kaslin, and D. Wlodkowic, *Biomicrofluidics* **11**(5), 051101 (2017).
- ¹⁴J. Henry, A. Rodriguez, and D. Wlodkowic, *PeerJ* **7**, e7367–e7367 (2019).
- ¹⁵L. Guzzella, A. Gronda, and L. Colombo, *Bull. Environ. Contam. Toxicol.* **59**(2), 313–320 (1997).
- ¹⁶A. K. Leight and R. F. Van Dolah, *Environ. Toxicol. Chem.* **18**(5), 958–964 (1999).