BEHAVIOURAL RESPONSES OF WOOD FROG (*LITHOBATES SYLVATICUS)* TADPOLES TO DILUTED BITUMEN

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Abstract

The study of behaviour in ecotoxicology allows for a broader understanding of the effects of pollution in an ecosystem. Amphibians are vulnerable to contaminant exposure, especially during early life stages when they are restricted to a waterbody. Since wood frog (*Lithobates sylvaticus)* tadpoles are exposed to contamination during diluted bitumen spills, an assessment of the impact of exposure was needed for freshwater systems. As part of the Boreal lake Oil Release Experiment by Additions to Limnocorrals (BOREAL) experiment, diluted bitumen was added to lake mesocosms at the International Institute for Sustainable Development's Experimental Lake Area near Kenora, Ontario. Water from these mesocosms was transported to separate microcosms in which wood frog (*Lithobates sylvaticus*) tadpoles were reared from Gosner stage 25. Behavioural assays were conducted every three to four days in a separate arena and video recorded. Space-use, sociality, and activity were quantified across a gradient of exposure to diluted bitumen infused lake water. No relationship between sociality and space use metrics and diluted bitumen concentrations were observed. There was a decrease in activity as diluted bitumen concentration increased, however the relationship between activity and diluted bitumen exposure should be further investigated to determine the physiological basis for this decrease in activity.

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Introduction

The interaction between hydrocarbons and the marine environment is consequential for both water quality and for the quality of life of marine organisms. It is known that petroleum exposure in marine environments is harmful to both water quality (Paul *et al.* 2013), as well as marine biota (Brussaard *et al.* 2016, Nelson *et al.* 2016). Research into the effects of hydrocarbons in freshwater environments and biota has been given much less attention in comparison, however (McKnight *et al.* 2016). Diluted bitumen (dilbit) is composed mainly of crude petroleum and diluents. Crude petroleum is typically obtained from bituminous tar sands, whereas diluents are composed of natural gas condensates, added to liquefy the crude petroleum (Alsaadi *et al.* 2018). Diluents allow for transport through pipelines by reducing viscosity of the tar-like crude petroleum. Diluents include compounds such as benzene, toluene, ethylbenzene and xylenes, a mixture often referred to as BTEX (Alsaadi *et al.* 2018). Polyaromatic compounds found in tar sands bitumen, such as phenanthrene, are typically the focus of dilbit exposure experiments; these are referred to as polycyclic aromatic hydrocarbons (PAHs) or polycyclic aromatic compounds (PACs), interchangeably (Alsaadi *et al.* 2018, Harner *et al.* 2018). Diluents and other contaminants contained in dilbit, including naphthenic acids, are of concern for animals in aqueous environments due to their toxicity and physiological effects. Naphthenic acids have been shown to decrease growth and alter development in amphibian larvae (Melvin and Trudeau 2012), and PAC concentrations have been linked to changes in genetic expression of *cyp1a* (cytochrome P4501A encoding gene) and alteration of DNA methylation in yellow perch (McDonnell *et al.* 2019). The toxicity of dilbit and its components to aquatic life is a cause for concern for exposed freshwater ecosystems.

The accidental spill of dilbit into the freshwater environment could potentially have harmful effects on freshwater biota. Toxicity of dilbit to aquatic organisms in freshwater has been demonstrated experimentally (Dew *et al.* 2015). While there has not been sufficient research into toxicity in aquatic biota, recent studies illustrate contamination effects across various trophic levels: in microbes, the toxicity of diluted bitumen has been observed with lower biomass and activity, due to a decrease in photosynthetic microbe population with exposure (Yergeau *et al.* 2013). In invertebrates, dilbit exposure has led to observed mortality and decreased reproductive success in *Ceriodaphnia dubia* (Robidoux *et al.* 2018). Fish populations also suffer from dilbit exposure in freshwater, as it can cause reduced hatching success, embryonic malformations, and reduced size in fathead minnows (Colavecchia *et al.*2004). With increasing focus on the method of transport of dilbit through pipelines, the need for an assessment of the effects of dilbit on freshwater environments and biota has become more urgent.

Quantifying effects of dilbit is particularly important for amphibian species because they are confined to waterbodies in early life stages (i.e., eggs and tadpoles), before becoming metamorphs around Gosner stage 42 (Gosner 1960). Furthermore, amphibians at large are at risk from habitat loss (Ficetola *et al*. 2015) and are often used as indicators of ecosystem stress (Welsh and Ollivier 1998). Thus, knowing how dilbit affects amphibians may play a role in conservation efforts. *Lithobates sylvaticus*, or Wood Frog tadpoles, were chosen as a model for amphibian species as they have a broad geographical and ecological range with habitats ranging across North America (Dodd 2013). Furthermore, it has already been established that PAH compounds are absorbed into wood frog tadpole tissues with exposure to dilbit (Mundy *et al.* 2019). Knowing how wood frogs respond to dilbit exposure would be useful for determining how amphibians might respond to dilbit across a range of biomes.

One understudied aspect of wood frog biology, with respect to dilbit and other contaminants, is behaviour. Animal behaviour as a discipline has sought to understand both at an evolutionary and mechanistic level why animals exhibit specific behaviours when interacting with each other and the environments in which they live. Animal behaviour is not purely shaped by only genetics or only environmental circumstance, but it is an interaction between the two. Interactions with the environment can shape behaviour tendencies over time, such as in the adaptation of great tit (*Parus major*) dialect in woodland or plains habitat (Hunter and Krebs 1979). In this way, behaviour is central to the evolutionary framework, as the reproductive fitness and success of animals relies heavily on behavioural tendencies. This framework is illustrated by Careau and Garland (2012) as they include behaviour as central not only to the energetics of individuals, but also to the resulting Darwinian fitness of individuals. Animals that are more suited to adapting to new environmental circumstances, such as contamination, will pass on their traits and those behavioural responses to future generations.

The link between behavioural tendencies and contamination has been explored previously in tadpoles. Pesticides, such as carbaryl, have been observed to alter response to predator cues in leopard plains tadpoles (Bridges 1997). Escape behaviours may also be altered upon exposure to neonicotinoids in wood frog tadpoles (Lee-Jenkins and

Robinson 2018). As there is a link between contamination and behaviour in tadpoles, behavioural tendencies with respect to dilbit exposure needs to be explored.

The objective of this experiment was to determine whether dilbit exposure, in field conditions, would affect wood frog tadpole behaviour. I hypothesized that behavioural change would occur, and that behavioural effects would increase with increasing concentration of dilbit. Specifically, I predicted that activity would decrease, and that sociality and space use would change because of that decreased activity in individuals.

Methods

Study Area

All experimental procedures were performed at the International Institute for Sustainable Development’s Experimental Lake Area (IISD-ELA) in Northwestern Ontario, Canada. The Boreal lake Oil Release Experiment by Additions to Limnocorrals or BOREAL project was initiated in the summer of 2017 to provide assessment of the effects of dilbit on a boreal lake. The BOREAL project has four main areas of inquiry, with experiments focused at assessing the fate, toxicity, ecosystem structure and function, and bioaccumulation of dilbit in the freshwater environment. Briefly, nine 10-m diameter decagonal mesocosms were constructed in Lake 260 (49°41'56.0"N, 93°45'57.9"W) at IISD-ELA and were subsequently pinned to the sediment floor. Eight mesocosms were contained within booming to separate experimental mesocosms from the surrounding lake waters. A far-field control was constructed outside the booming (Appendix 1). Inside a mesocosm, the depth was approximately 2 m (depending on depth of sediment). Water chemistry and other physical factors were monitored in each mesocosm prior to dilbit addition. Mesocosms were established two weeks prior to dilbit addition to allow for re-sedimentation and stabilization of the mesocosm post-construction. Sampling ports were installed for water sampling, with the water intake approximately at one-meter depth for extraction with diaphragm pumps.

Rearing Procedure

One *Lithobates sylvaticus* egg mass was removed from Lake 227 at the IISD-ELA on May 5, 2018 and transferred to rearing facilities. A 600 L tank was partitioned into three separate rearing pools and equipped with aerators and predator meshes (Appendix 2). The egg mass was split into three equal parts, with each reared in a separate partition. The rearing tanks were surrounded by a water bath to keep temperature consistent and contained water from Lake 260. Water changes were performed with pristine water from Lake 260 regularly (approximately every three to four days) on each of the partitions in the rearing setup to ensure suitable water quality. During early life stages, eggs were suspended in fishnets below the surface of the partitioned sections., Tadpoles hatched on approximately May 15th, 2018. During rearing, tadpoles were fed frozen spinach liberally, and were reared until approximately Gosner Stage 25 (Gosner 1960). After reaching this developmental stage, 360 tadpoles were transferred to the field setup.

Microcosms and tadpole husbandry

Transferred tadpoles were held in 16 L stainless steel tanks filled with water taken from the BOREAL mesocosms. Twenty-four stainless steel tanks were first soaked in water from Lake 260 and potential contaminants rinsed out with lake water. The tanks were organized into three groups of eight and were filled with water taken from the mesocosm that corresponded to their respective treatment (Appendix 3). Each dilbit treatment had three replicates which were assigned with stratified randomization.

Fifteen tadpoles were placed in each microcosm on June 7, 2018. From June 7-20th, 2018, tadpoles in each microcosm were fed 10 g of frozen spinach every other day. Residual waste was cleaned from microcosms daily and water changes, morphological assays (data not included), and behavioural assays were performed every three-four days on a rotating schedule. Water changes involved the use of 24 L portable water jugs assigned to each treatment, which could be filled by diaphragm pump at each mesocosm's sampling port. Fifty percent of the microcosm volume (8 L) was removed and stored in wastewater jugs, before being replenished with 8 L of fresh mesocosm water. Wastewater was returned to the mesocosm of origin. Mesocosm four (M4), which was within booming, was used for the control treatment water changes until dilbit exposure, after which point the far-field control (M9) was used for the control replicates to reduce risk of atmospheric deposition. In analyses, the control treatment is referred to as M4.

Dilbit at various concentrations was added to the mesocosms in a simulated spill by Environment Canada employees and trained personnel on June 20th, 2018 (Table 1). From June 21–July 9, 2018, microcosm water changes used contaminated water from the various mesocosms (i.e., each microcosm received water from their respective treated mesocosm). The first water changes of the post-exposure period (June 21) used mesocosm water obtained 36 h after initial mesocosm exposure. Behavioural assays, morphological assays (not examined in this experiment), and water changes occurred on a rotating schedule for the duration of sampling. Dissolved oxygen (DO) and temperature were monitored daily with a handheld probe (Hach LDO10105 IntelliCAL LDO Rugged Probe) throughout the experiment (see Appendix 8-11). pH was monitored daily with an IntelliCAL PHC101 Field Low Maintenance Gel Filled pH Electrode (see Appendix 8-11). Each microcosm continued to be given 10 g of frozen spinach every other day throughout the duration of the experiment, and residual waste was cleaned from microcosms daily. Floats were added to the tanks when tadpoles began to metamorphize to higher Gosner Stages, as they required room to breathe and rest above water.

Table 1: Dilbit dosage data of experimental mesocosms and microcosms. Volume of dilbit added to each experimental mesocosm on Lake 260, with the approximate concentrations of total petroleum hydrocarbons (TPH) for each treatment in both mesocosms and microcosms are detailed. TPH measurements for mesocosms were collected on June 28, 2018. TPH measurements for microcosms were collected on July 9, 2018. Total petroleum hydrocarbons present in mesocosms significantly differ between control and treated mesocosms. Microcosm TPHs are not quite as variable between control and treatments in all (n) microcosm replicates (Stoyanovich *et al.* 2019, Patterson *et al*. 2019, unpublished data).

Mesocosm	Treatment (Dilbit:Water)	Mesocosm Dilbit Dose (L)	Mesocosm TPH (μg/L)	Microcosm Mean TPH (μg/L)	n
M1	1:1,000	179.78	1191 ± 109	1017.73 ± 48.24	3
M8	1:2,100	81.83	743 ± 139.26	697.24 ± 67.22	3
M3	1:4,600	42.34	533 ± 35.63	654.03 ± 94.85	3
M7	1:10,000	18.13	223 ± 75.16	468.08 ± 36.25	3
M2	1:21,000	5.51	223 ± 6.22	552.82 ± 260.64	3
M5	1:46,000	2.87	199 ± 0.06	416.79 ± 3.17	2
M6	1:100,000	1.45	185 ± 28.69	319.46 ± 26.64	3
M4	Control	0.00	80 ± 59.55	436.9 ± 56.89	3

Behavioural Assays

Behavioural assays, consisting of an open-arena test (Carlson and Langkilde 2013), were performed on five individuals randomly selected from each microcosm and transferred to separate behavioural arenas (24 cm x 28 cm x 14 cm; Appendix 4). Tadpoles were acclimated in their behavioural arenas for at least 10 min before being moved to a tabletop surface and filmed for 10 min. Transfer and design in an open-field setup was performed as described by Carlson and Langkilde (2013). All filming was done with five Activeon CX Gold Plus action cameras at a resolution of 1080P at 60 frames per second, with a narrow field of view. All animal collection and handling procedures were done in accordance with Queen's University and University of Winnipeg Animal Care Committee guidelines.

Video Analysis

Three behavioural metrics were analyzed in this experiment: activity, sociality, and space-use. Activity, or total movement within a behavioural arena, was observed through counting lines crossed on a grid overlay within a thirty second interval. Sociality was measured as the likelihood of individuals to aggregate with other individuals, exhibiting social behaviour. Like fish, tadpoles are more exposed to potential predators in open water, so frequenting open spaces (i.e., the center) in a behavioural arena indicates bolder individuals than those that remain close to edges (Carlson and Langkilde 2013). In this way, space use in the behavioural arena was used as a measure of boldness.

The 10 min videos were split into 600 photos (one frame per second) with the *pathtrackr* R package. Twenty photos representing every 30s interval were selected for analysis. These twenty photos began at 30 seconds and concluded with the frame at ten minutes (frame 600). If a video did not have 600 frames, the number of frames needed for a ten-minute analysis was subtracted from the initial frame used. For example, a video with 599 frames would begin analysis on frame 29 and proceed every thirty frames. Each of the five tadpoles present in the behavioural arena was analyzed individually. Location of the tadpole was quantified through use of a 6 x 7 grid overlay (Appendix 5) over the video frames observed. An individual was recorded as being within a square on the grid overlay if a majority of the tadpole's body, excluding the tail, was within the square's borders. The tadpole's location was also denoted as being in an outer, middle, or inner square. Activity was measured by comparing the locations of each individual after each 30s interval. The number of lines crossed, over the shortest distance to reach one grid location from a tadpole's previous location, was counted (Appendix 6). The first frame observed (e.g. Frame 30) always had 0 lines crossed in activity, as there was no previously observed frame, so it was removed from the calculation of mean activity. Sociality was quantified as the number of tadpoles within one grid unit of the center of an individual's body at each frame (Appendix 7).

Data Analysis

Activity and sociality were plotted against dilbit concentration in a linear mixed effects (LME) model to determine whether there was any relationship between the two factors, with sampling day as a random effect. Sampling day was chosen as a random effect because a developmental effect was observed during initial data analysis. For this reason, day 18 was removed from all analysis after a significant decrease in activity was observed. Individual tadpoles had metamorphosized to grow large limbs and were less likely to swim at day 18.

To create a LME model with activity, the mean number of lines crossed per individual was averaged. These values were added together for each of the five individuals present in a replicate and averaged for each video recorded. This mean number of lines crossed per video was compared to mesocosm dilbit dose in the LME model.

To create a LME model with sociality, the mean number of proximal tadpoles observed per individual was averaged. These values were added together for each of the five individuals present in a replicate and averaged for each video recorded. This mean number of proximal tadpoles per video was compared to mesocosm dilbit dose in the LME model.

To analyze space use, the percentage of frames spent in outer squares compared to middle and inner squares was calculated for each individual. Those percentages were averaged per five individuals in a replicate, for each video recorded. Percentage of time spent in the outer arena was plotted against mesocosm dilbit dose.

When collecting observations after dilbit exposure, replicate M5B experienced significant mortality and thus was eliminated from further analysis.

Results

While experimental microcosms were not in Lake 260, much of the same environmental variation was present throughout replicates. Results from water quality monitoring in microcosms indicate that temperature, pH, and DO conditions were quite variable among the different days when behavioural sampling took place (Appendix 8-11). When comparing microcosms measured on the same day however, readings were relatively consistent between treatments, except for the control (with no dilbit added), as seen in Tables 2-5.

Table 2: Water quality measurements of microcosms from the first day post-exposure, on which behavioural sampling took place. Mean pH, temperature, and DO (mg/L and percent saturation) were observed for replicates of all treatments. (Patterson *et al*. 2019, unpublished data)

Days Post-Exposure	Treatment	Mesocosm Dilbit Dose (L)	pH	D.O. (mg/L)	D.O. (%)	Temp. (˚Celsius)
1	M4	0.00	7.6 ± 0.5	7.6 ± 1.0	98.5 ± 13.6	25.9 ± 0.3
1	M6	1.45	8.6 ± 0.7	9.2 ± 0.6	118.7 ± 7.5	25.3 ± 0.2
1	M5	2.87	9.1 ± 0.5	9.4 ± 0.7	122.1 ± 8.8	25.7 ± 0.3
1	M2	5.51	9.2 ± 0.3	9.6 ± 0.5	124.5 ± 6.4	25.5 ± 0.2
1	M7	18.13	8.6 ± 0.4	8.9 ± 0.4	115.5 ± 6.3	25.5 ± 0.5
1	M3	42.34	9.3 ± 0.2	9.5 ± 0.3	123.7 ± 3.4	25.6 ± 0.3
1	M8	81.83	8.9 ± 0.3	9.1 ± 0.5	118.4 ± 6.7	25.3 ± 0.05
1	M1	179.78	8.9 ± 0.5	9.1 ± 0.7	117.5 ± 9.3	25.5 ± 0.09

Table 3: Water quality measurements of microcosms from the fifth day post-exposure, on which behavioural sampling took place. Mean pH, temperature, and DO (mg/L and percent saturation) were observed for replicates of all treatments. (Patterson *et al*. 2019, unpublished data)

Days Post-Exposure	**Treatment**	**Mesocosm Dilbit Dose (L)**	**pH**	**D.O. (mg/L)**	**D.O. (%)**	**Temp. (˚Celsius)**
5	M4	0.00	6.7 ± 0.1	4.4 ± 0.4	50.3 ± 4.8	18.7 ± 0.5
5	M6	1.45	7.3 ± 0.04	6.1 ± 0.3	68.5 ± 2.9	18.8 ± 0.2
5	M5	2.87	7.3 ± 0.09	6.3 ± 0.5	70.6 ± 4.6	18.4 ± 0.3
5	M2	5.51	7.2 ± 0.005	6.8 ± 0.2	76.7 ± 2.3	18.5 ± 0.05
5	M7	18.13	7.4 ± 0.4	6.6 ± 0.2	75.5 ± 1.5	18.4 ± 0.08
5	M3	42.34	7.1 ± 0.02	6.6 ± 0.3	73.8 ± 3.5	18.4 ± 0.09
5	M8	81.83	7.1 ± 0.05	6.6 ± 0.5	75.2 ± 6.2	18.4 ± 0.09
5	M1	179.78	7.1 ± 0.08	6.4 ± 0.6	72.3 ± 6.7	18.5

Table 4: Water quality measurements of microcosms from the ninth day post-exposure, on which behavioural sampling took place. Mean pH, temperature, and DO (mg/L and percent saturation) were observed for replicates of all treatments. (Patterson *et al*. 2019, unpublished data)

Days Post-Exposure	Treatment	Mesocosm Dilbit Dose (L)	pH	D.O. (mg/L)	D.O. (%)	Temp. (˚Celsius)
9	M4	0.00	7.1 ± 0.08	3.5 ± 0.4	41.1 ± 4.4	19.8 ± 0.2
9	M6	1.45	7.5 ± 0.09	4.9 ± 0.5	57.2 ± 5.7	20.1 ± 0.3
9	M5	2.87	7.3 ± 0.07	5.0 ± 0.6	58.5 ± 6.9	19.7 ± 0.09
9	M2	5.51	7.1 ± 0.05	4.7 ± 0.7	54.9 ± 8.1	19.5 ± 0.09
9	M7	18.13	7.1 ± 0.04	4.8 ± 0.5	54.7 ± 5.5	19.5 ± 0.09
9	M3	42.34	7.1 ± 0.04	4.4 ± 0.8	51.3 ± 8.7	19.6 ± 0.05
9	M8	81.83	7.1 ± 0.07	5.2 ± 1.0	59.9 ± 12.0	19.6 ± 0.05
9	M1	179.78	7.1 ± 0.04	4.5 ± 0.6	52.3 ± 7.1	19.6 ± 0.05

Table 5: Water quality measurements of microcosms from the twelfth day post-exposure, on which behavioural sampling took place. Mean pH, temperature, and DO (mg/L and percent saturation) were observed for replicates of all treatments. (Patterson *et al*. 2019, unpublished data)

Days Post-Exposure	Treatment	Mesocosm Dilbit Dose (L)	pH	D.O. (mg/L)	D.O. (%)	Temp. (˚Celsius)
12	M4	0.00	8.9 ± 0.4	8.2 ± 2.6	98 ± 30.6	21.8 ± 0.2
12	M6	1.45	9.4 ± 1.0	11.0 ± 1.2	131.4 ± 13.7	21.2 ± 0.4
12	M5	2.87	9.9 ± 0.05	11.1 ± 0.4	132.2 ± 3.9	20.9 ± 0.05
12	M2	5.51	9.6 ± 0.7	10.7 ± 1.4	127.7 ± 16.5	20.8 ± 0.2
12	M7	18.13	10.09 ± 0.4	11.9 ± 1.1	141.2 ± 14.2	21.0 ± 0.3
12	M3	42.34	10.1 ± 0.04	11.5 ± 0.3	135.8 ± 3.9	21.1 ± 0.09
12	M8	81.83	10.0 ± 0.3	11.3 ± 0.6	134.3 ± 7.3	21.1 ± 0.1
12	M1	179.78	10.2 ± 0.2	12.6 ± 0.7	149.6 ± 8.5	21.3 ± 0.2

There was a significant decrease in mean activity per behavioural arena observed at increased mesocosm dosage ($p= 0.045$, $n= 16$; Figure 1). The mean activity, or lines crossed each 30s, for all five individuals, per microcosm (video) analyzed was 4.27± 1.02 (Appendix 18-25). The mean activity for replicates analyzed at the highest treatment, M1 (179.78 L mesocosm dilbit dose), was 4.18± 0.75 lines crossed (Appendix 25). In the control treatment, M4 (0.00 L mesocosm dilbit dose), the mean activity was 4.59± 1.04 lines crossed (Appendix 18). There was no relationship between mean proximate tadpoles and concentration of dilbit across all individuals in a particular microcosm ($p= 0.85$, $n= 16$) (Figure 2), with a mean of 8.04± 1.82 proximal tadpoles per microcosm (see Appendix 14-15). Space use within the behavioural arena was consistent throughout trials and did not change with increasing dilbit concentration (Figure 3). Individuals spent nearly 100 % of the time around the outer edges of the arena, with few individuals venturing towards the open water in the center (see Appendix 16-17).

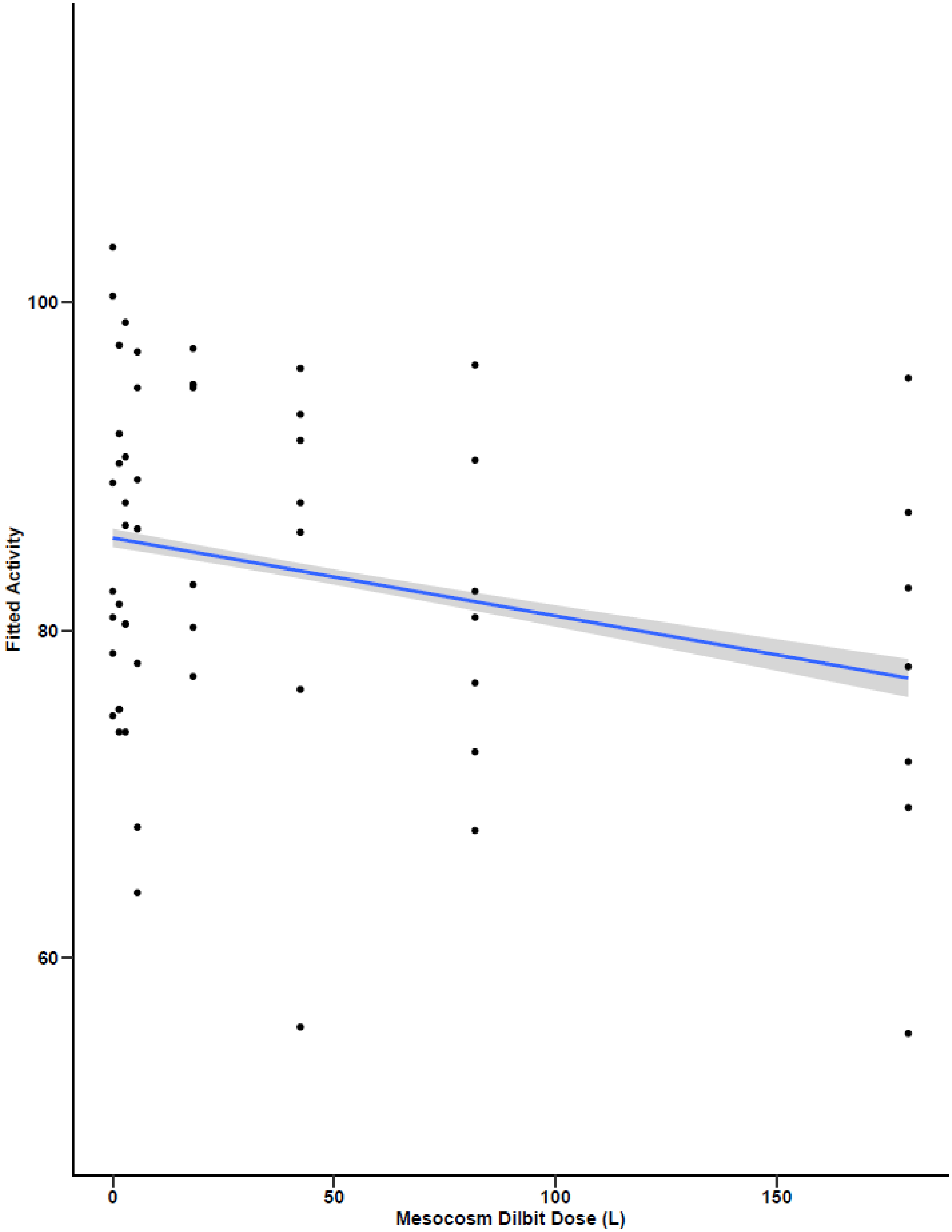

Figure 1: Mean activity index for each video plotted against mesocosm dosage from the water transferred to microcosms in a linear mixed effects model. A significant decrease is seen as the mesocosm dilbit dose increases (p= 0.045, n= 16).

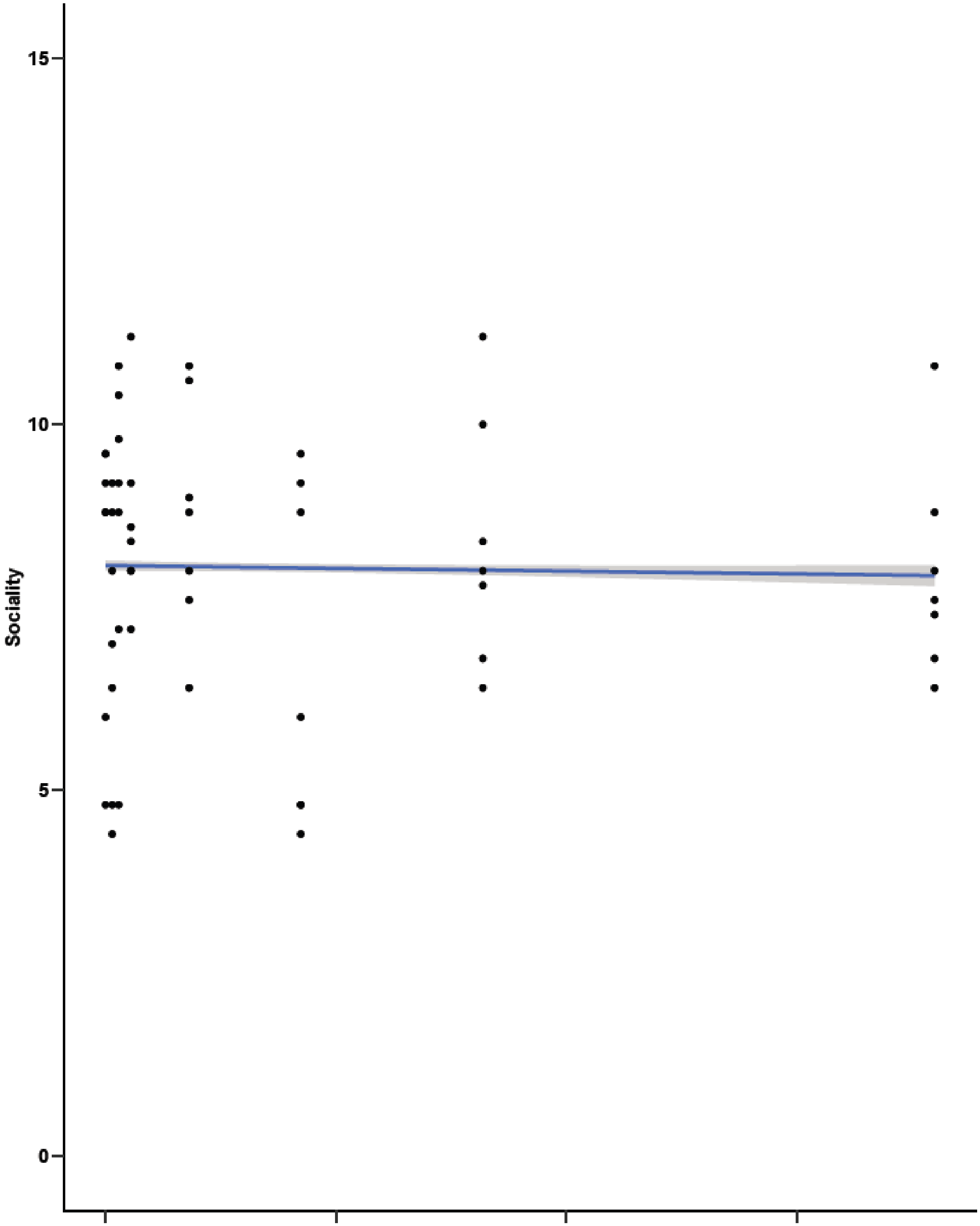

Figure 2: Mean social index, or the number of proximal tadpoles per video, plotted against mesocosm dosage from the water transferred to microcosms in a linear mixed effects model. No correlation between mean proximal tadpoles and dilbit level was observed (p= 0.85, n= 16).

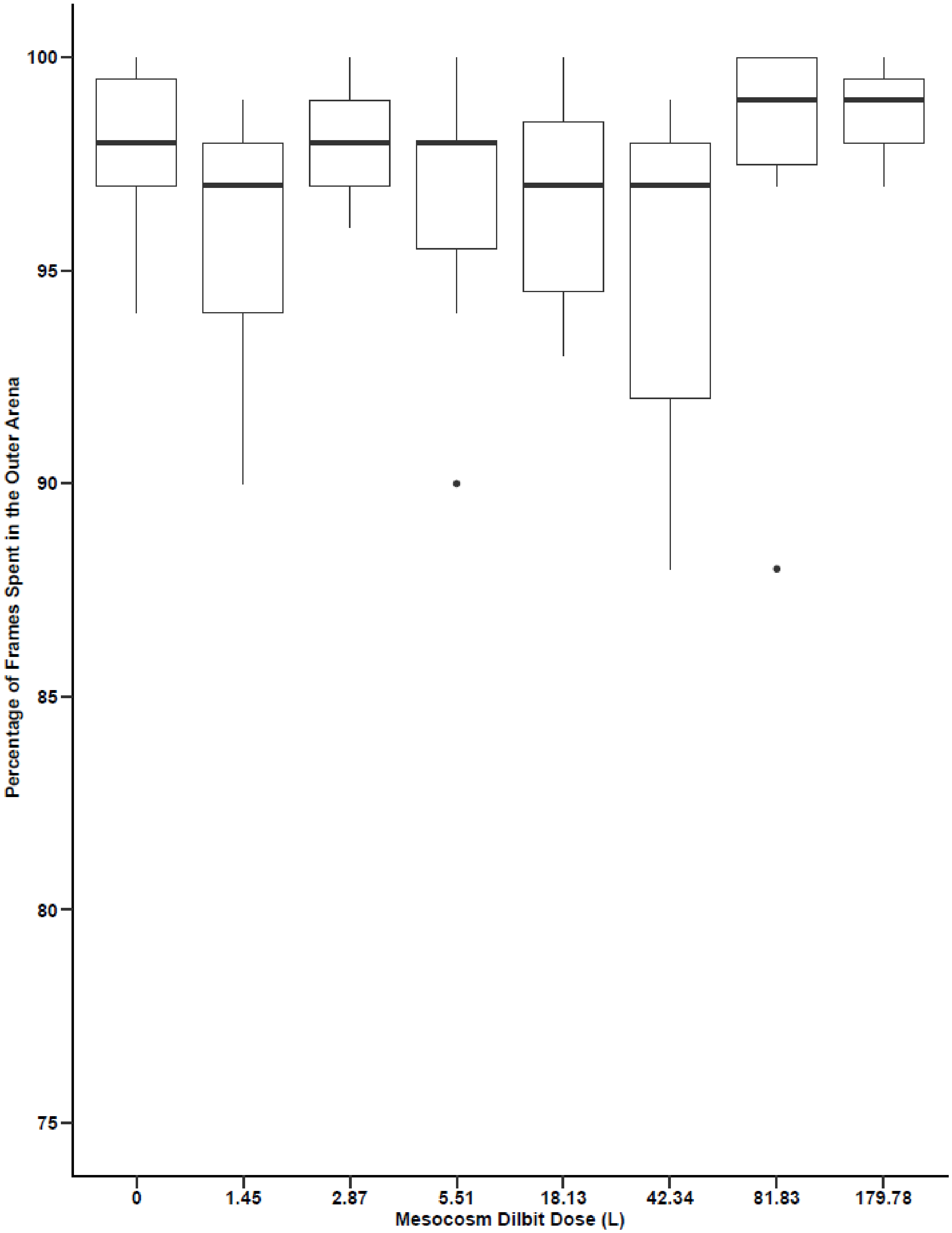

Figure 3: Percentage of frames observed where individuals are within the outer squares of the grid overlay plotted against mesocosm dilbit dose. Individuals spent almost all of the frames observed within the outer edges of the behavioural arena, with no change in space use with respect to increasing dilbit concentration in the mesocosm water transferred to microcosms.

Discussion

There was a decrease in mean activity in replicates with increased dilbit dosage to mesocosms. While a definite cause for the decrease is unknown, elsewhere it has been suggested that dilbit exposure can adversely affect cardiac muscle in fish species (Alderman *et al.* 2016, Nelson *et al.* 2016). A decrease in cardiac function could lead to loss of activity in tadpoles with high PAH concentration in tissue. Further research is needed on the physiological effects of PAH retention in amphibian tissues before correlations between exposure and behavioural syndromes can be made, however. This decrease in mean activity is notable because there is inherent variability in conditions and individuals in any field-based experiment. Ecology experiments done in the field do not have controlled weather conditions, and changes in lake water characteristics (i.e. pH, DO, etc.) are not under the restrictions of a lab-based setup. For this reason, lab-based and field experiments often have differing results (Mikó *et al.* 2015, Saura-Mas 2002). To see any effects of exposure across replicates in such a dynamic system, like that of an actual lake environment, is worthy of further investigation.

Contaminants have previously been shown to cause changes in activity, or swimming behaviour, in tadpoles. Lajmanovich *et al.* (2019) performed an experiment in which *Odontophrynus americanus* tadpoles were exposed to pyriproxyfen pesticide, resulting in a multitude of physiological changes, such as a decrease in cardiac activity, as well as a decrease in swimming activity. As acetylcholinesterase activity was also measured to have increased significantly with exposure, acetylcholinesterase activity was negatively correlated with their swimming behaviour endpoints. Acetylcholinesterase is used at neuromuscular junctions to inhibit the activity of acetylcholine (Colović *et al.*

2013), thus placing a control over muscular, and thus swimming activity. The observation of contaminants such as pyriproxyfen in changing acetylcholinesterase activity, and therefore neuromuscular activity and subsequent behaviour is significant. This finding suggests that neuromuscular functions can influence the activity of tadpoles, but also that contaminants and their physiological effects can be linked to behavioural change. It is plausible that dilbit exposure in this experiment affected similar physiological pathways, leading to reduced activity. Integrative questions such as these however were beyond the scope of this experiment. That is why it is crucial to research the connection between PAH retention and physiological change, with respect to activity in future.

There are several possible explanations as to why there was not a significant behavioural change in sociality and space use in wood frog tadpoles with dilbit exposure. Firstly, the concentrations chosen to use in the BOREAL experiment were not very high, with the highest concentration having a dilbit to water ratio of 1:1000. It is important to note that the dilbit concentrations chosen were more relevant to concentrations in a potential spill, and thus were more relevant to wood frog exposure. A positive control where dilbit concentration could be highly exaggerated may have given more definitive trends in activity. This additional replicate would have shown what higher concentrations could be capable of in terms of behavioural change. Another possibility as to why changes in space use and sociality were not observed would be the variability of the mesocosm water change itself. The sampling port for water samples which were used for water changes was located at the midpoint, at approximately a one-meter depth, below the surface. It is unlikely that contaminants moved down the water column uniformly, and the window for ideal PAH exposure could have been missed within the timeframe of this experiment. Unfortunately, variability in the distribution of contaminants could not have been prevented with the weathering of the dilbit. The dynamic nature of the surface skim itself causes further uncertainty about the distribution of contaminants in the water column. It is possible therefore that contaminant exposure within the duration of the experiment was not uniform in exposure length and intensity, which may have influenced contaminant uptake into tissue. There is higher exposure to volatile compounds such as benzene before volatilization, earlier in a dilbit spill. Because of the immense sampling that took place immediately after the simulated spill, as well as safety concerns, the mesocosm water was added to microcosms 36h after initial exposure. It is possible that if these contaminants cause immediate behavioural change in spill sites, that water changes starting closer to initial exposure (2h, 5h, 10h, 24h, etc.) may have shown greater effects on behaviour.

With a recent study demonstrating PAC retention in tissues of wood frog tadpoles (Mundy *et al.* 2019), it is clear that with dilbit exposure, PACs will be retained in tissues. While the main focus of Mundy *et al.* was not the effects of exposure, it also demonstrated one component of the lake ecosystem which was not present within the microcosms used in this experiment. The microcosms did not incorporate sediment from Lake 260, thus tissue retention may not have occurred to the extent it might have otherwise. Without significant PAH retention in tissues, potential physiological changes and behavioural changes caused by the contaminants also would not occur to the extent that it might have.

Metamorphic staging and the timeframe under which exposure took place may have influenced the results of this experiment. Diluted bitumen and its related contaminants have been tied to changes in development in aquatic vertebrates (Colavecchia *et al.* 2004, Colavecchia *et al.* 2007, Alsaadi *et al.* 2018, McDonnell *et al.* 2019, Melvin and Trudeau 2012,), ranging from decreased hatching success, embryonic malformations, to outright mortality. It is possible that tadpoles may have shown greater behavioural change if tadpoles were hatched and reared in contaminated water. However, the time at which egg masses had to be collected and reared did not correspond to the exposure period for BOREAL. Exposure of tadpole eggs during rearing and throughout early life stages may have yielded different results.

One of the advantages to ecosystem-based ecotoxicology and the use of field-based experiments for projects such as these is that conditions, while variable, will more closely resemble natural ecosystems. While pH and DO were not identical in each microcosm replicate analyzed, this is similar to changing conditions in a waterbody. pH and dissolved oxygen between microcosm replicates were relatively consistent, however these values changed significantly by sampling day. The changes in pH and DO may be explained by the water change schedule, as dissolved oxygen and pH usually increased on sampling days closer to water changes (Patterson *et al*. 2019, unpublished data). The differences in pH and DO between behavioural sampling days may have also been a result of dilbit addition, as the control treatment, M4, typically had differing measurements in pH and DO from the dilbit-treated mesocosm water. The contribution of dilbit to water quality will be clearer with further data from the BOREAL project. The change in pH and DO may have affected behavioural results, but since water quality measurements were relatively consistent by sampling day, if there was a significant change to behavioural metrics from these factors they would have been present across each replicate on a particular sampling day, which was not the case.

Unfortunately, the TPH levels between microcosms was not highly variable between treatment and control replicates. This is concerning, as the exposure to contaminants, namely hydrocarbons, may not have been similar between the mesocosm and microcosm environment. Uptake of contaminants into tissue may have also affected this reading. Sampling of microcosm TPH levels occurred days after the final water change, so it is possible that these measurements do not accurately represent the TPH levels seen immediately after a water change. Eutrophication was also variable between microcosms, which might have influenced the water quality. It must be acknowledged that regardless of precautions taken, field-based studies of tadpole behaviour will always differ from the results of those conducted in a lab setting (Mikó *et al.* 2015). Like within a lake, competition for food was present among tadpoles in each replicate. While each replicate was fed the same mass of frozen spinach, competition for food resources most likely led to the size discrepancy seen in individuals, even with individuals transferred to the microcosms initially having similar Gosner staging. Difference in staging and mass within the timeframe of the exposure may have influenced results. Mass and other physiological parameters will be incorporated into the statistical model in future.

Overall this study supports the view that dilbit exposure likely decreases activity in wood frog tadpoles. At the concentrations used, exposure does not adversely affect their space use and sociality, however. This decrease in activity is concerning, considering its importance for the survival of wood frog tadpoles in their early life stages. As behavioural change could have ecosystem-wide implications (Sih *et al.* 2004), any behavioural affects from exposure is disadvantageous for conservation of amphibian species at risk.

Conclusions

1. Mean tadpole activity (lines crossed) decreased across microcosms in tadpoles with increasing concentration of dilbit during exposure.
2. Sociality, or the likelihood of individual tadpoles to aggregate in groups, was not influenced by increased dilbit concentrations.
3. Space-use in tadpoles focused on swimming around the outer edges of the behavioural arenas. This trend was not influenced by increased dilbit concentrations.

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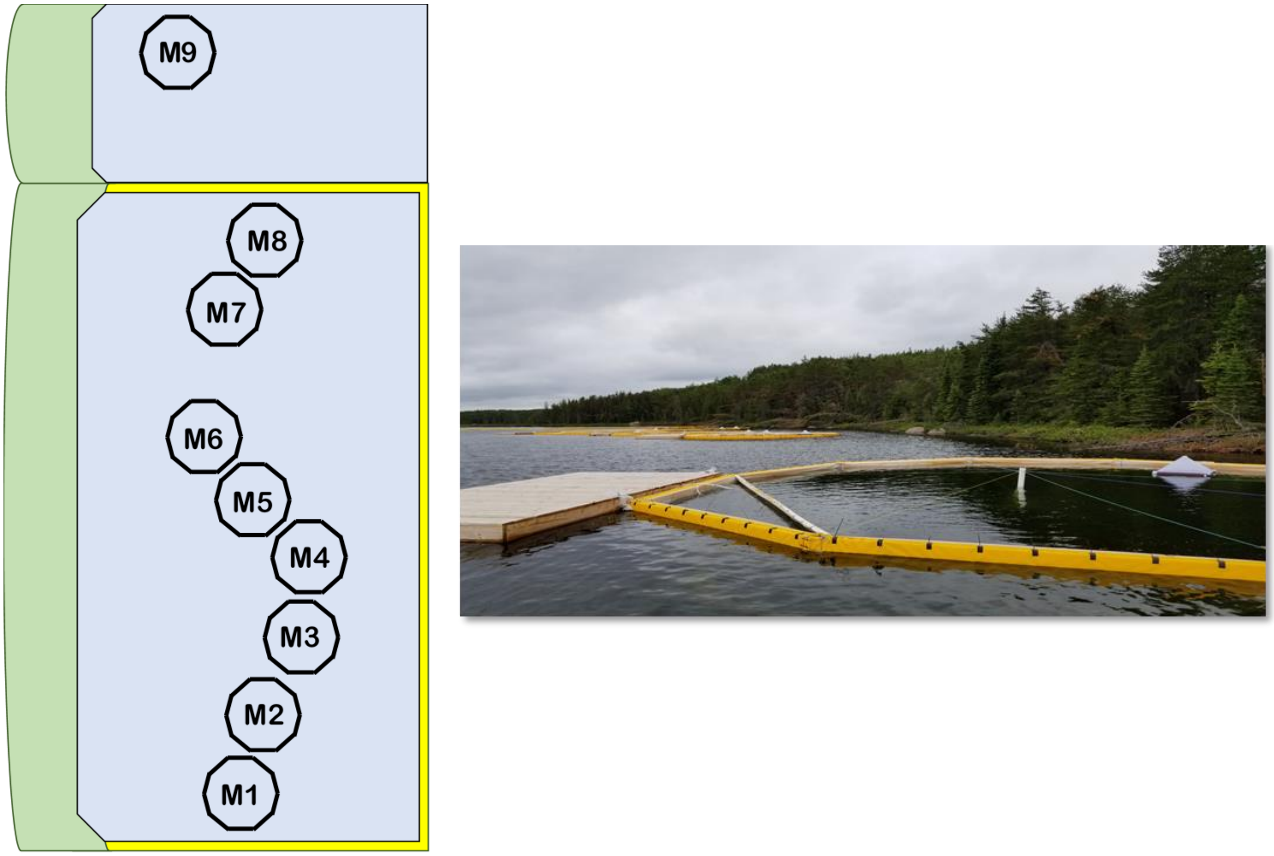
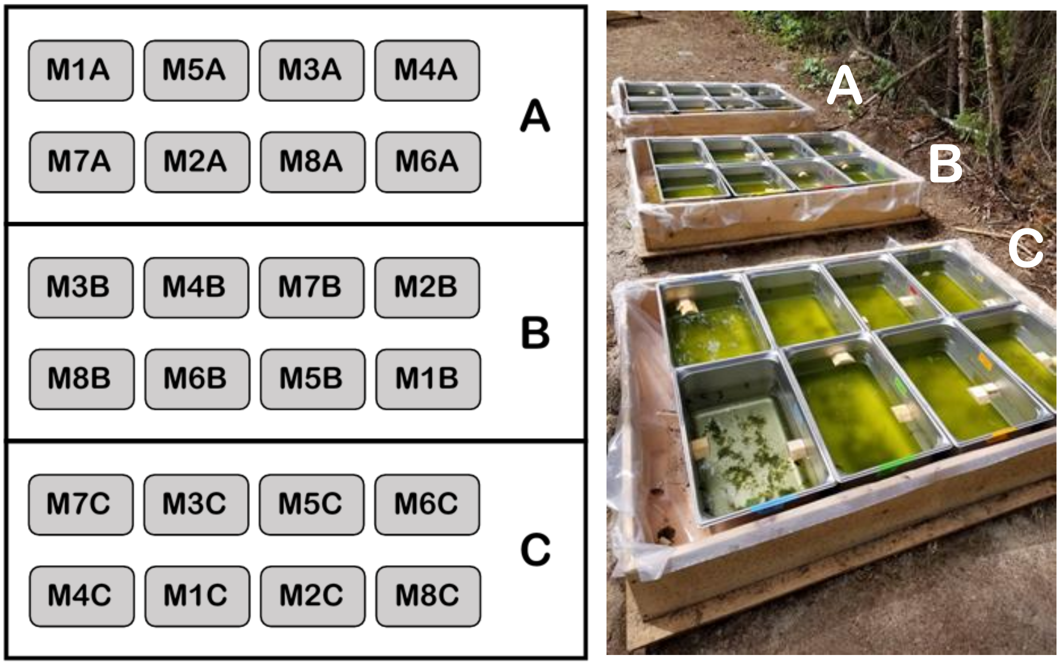
Appendix



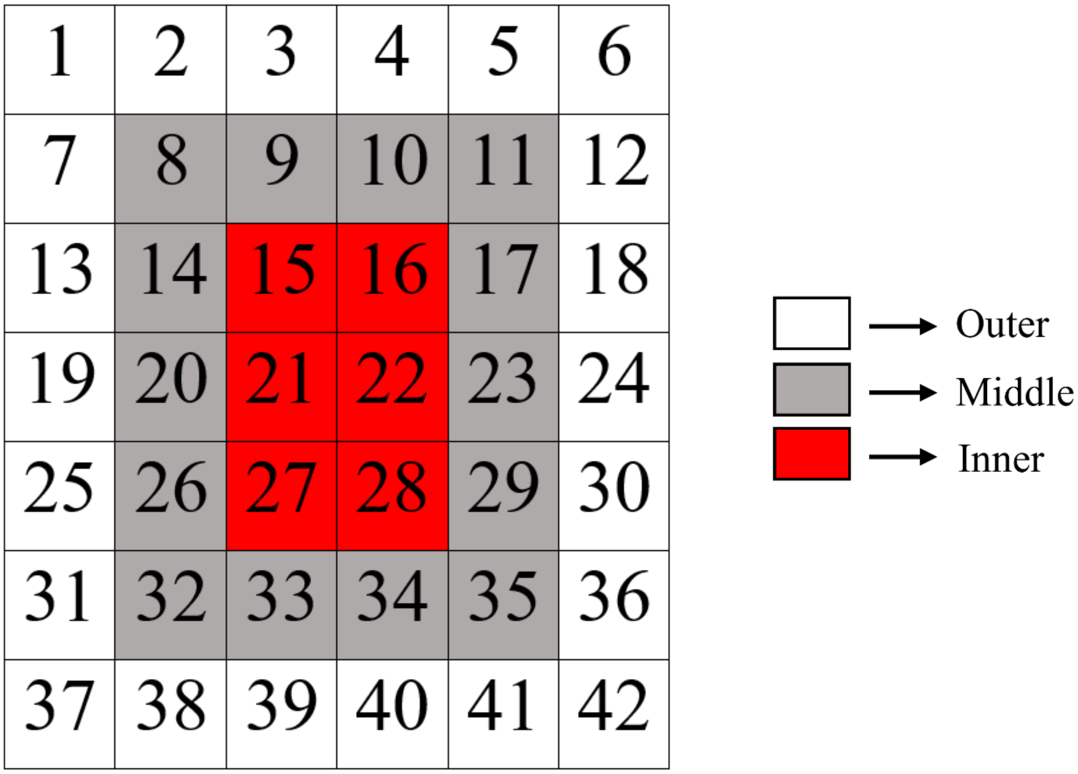
Appendix 1: Mesocosm setup on Lake 260 (left). Mesocosms 1-8 (M1-M8) were within booming to contain the dilbit exposure, and Mesocosm 9 (M9) outside the booming, to act as a far-field control. Lakeside view of a mesocosm prior to exposure (right).

Appendix 2: Rearing setup used to hatch and raise tadpoles from the partitioned egg mass.



Appendix 3: Organization of replicates in microcosm setup (left). Microcosms after tadpole addition and dilbit exposure (right).

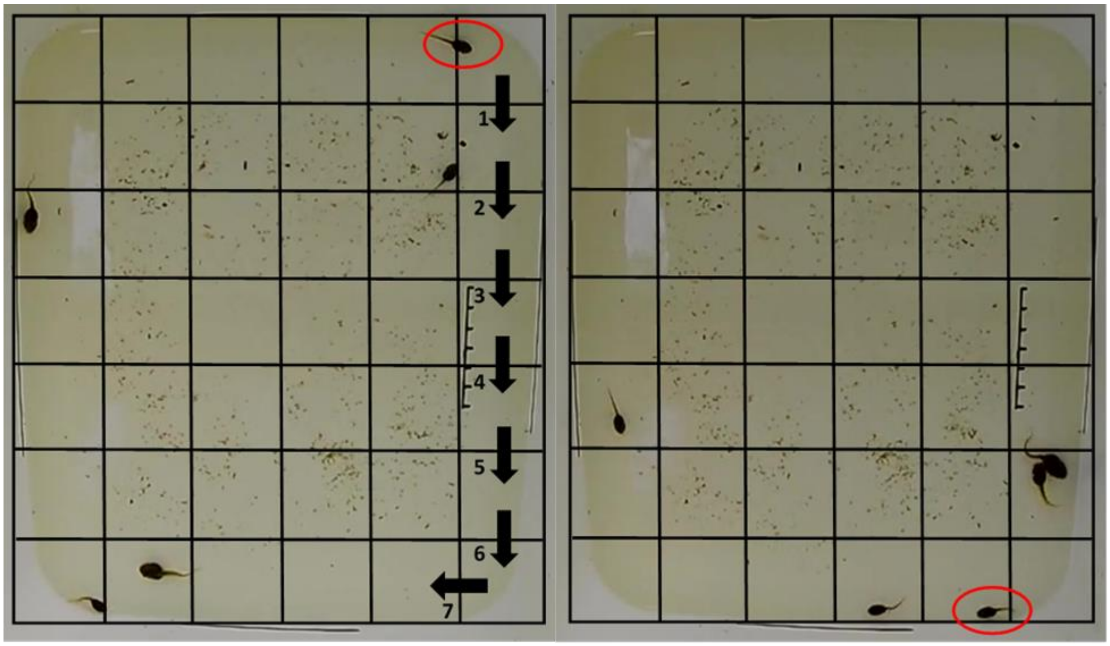
Appendix 4: Behavioural assay configuration for sampling. Behavioural arenas were organized horizontally on a level tabletop surface, with cameras one through five (right to left) positioned directly above for filming.



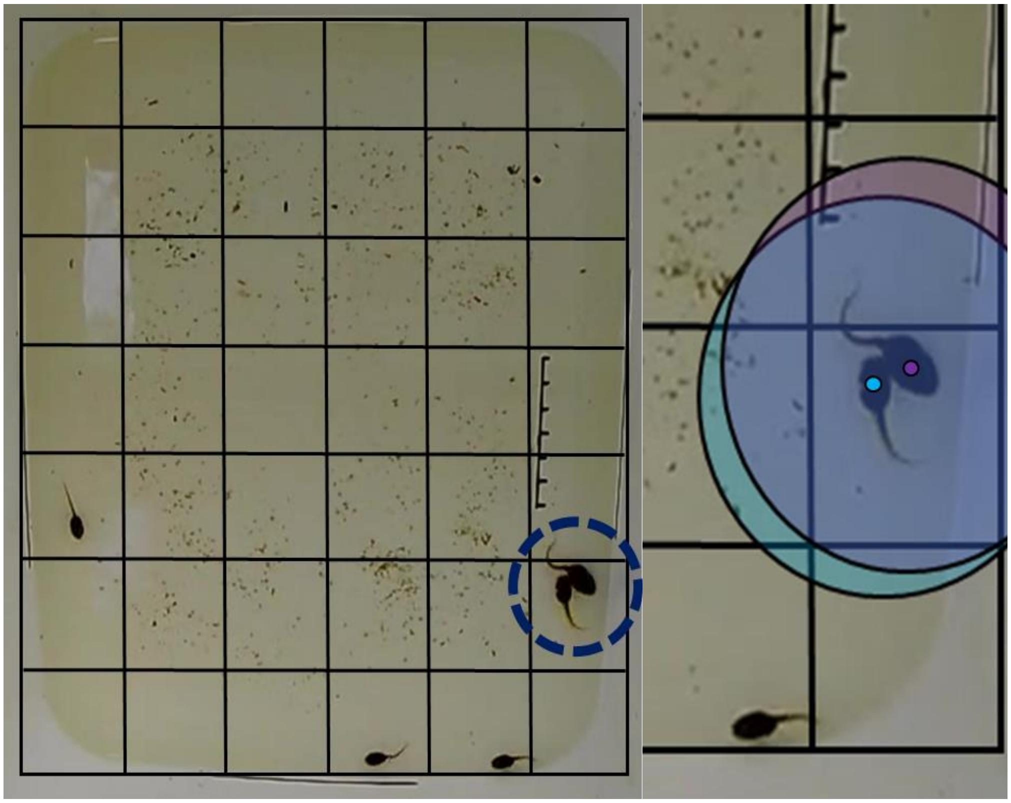
1	2	3	4	5	6
7	8	9	10	11	12
13	14	15	16	17	18
19	20	21	22	23	24
25	26	27	28	29	30
31	32	33	34	35	36
37	38	39	40	41	42

☐ → Outer
▨ → Middle
🟥 → Inner

Appendix 5: Grid overlay used in space use analysis, indicating both the square number and classification as an inner, middle, or outer square on the grid.



Appendix 6: A demonstration of space use and activity in two frames of video with its grid overlay. The first frame includes the original position of the individual being observed (left), circled in red, and the number of lines on the grid these individual crosses to reach its next position thirty seconds later (right). The individual crosses seven lines in total, which would be recorded as its activity for that 30 sec interval, and its grid location (Appendix 5) recorded for its space use.



Appendix 7: Method for determination of proximal tadpoles. When two or more tadpole bodies are within one grid length from each other (left), two circles, whose radius is equivalent to one grid length, centered on the tadpole's body, is compared between the individuals. If the radii overlap with the center of another tadpole's body (right), the tadpoles were considered proximal.

Appendix 8: Microcosm water quality data recorded on June 22, 2018, or one day after dilbit exposure had begun, with respective averages. (Patterson *et al*. 2019, unpublished data)

Tank	Treatment Replicate	pH	D.O. (mg/L)	D.O. %	Temp. (˚Celsius)
M1A	1A	9.51	9.95	129.4	25.6
M1B	1B	8.94	9.00	116.6	25.4
M1C	1C	8.22	8.20	106.6	25.6
M2A	2A	9.17	9.62	125.7	25.8
M2B	2B	8.81	9.00	116.2	25.2
M2C	2C	9.55	10.16	131.7	25.4
M3A	3A	9.02	9.15	119.3	25.7
M3B	3B	9.46	9.90	127.7	25.1
M3C	3C	9.41	9.49	124.1	25.9
M4A	4A	8.32	8.79	115.2	26.0
M4B	4B	7.22	6.37	82.0	25.5
M4C	4C	7.40	7.51	98.4	26.1
M5A	5A	9.56	10.12	132.6	26.0
M5B	5B	9.31	9.49	122.8	25.3
M5C	5C	8.50	8.50	111.0	25.8
M6A	6A	7.62	8.44	109.3	25.3
M6B	6B	9.19	9.91	127.6	25.1
M6C	6C	9.02	9.19	119.3	25.5
M7A	7A	8.65	8.66	113.0	25.8
M7B	7B	8.17	8.53	109.4	24.8
M7C	7C	9.12	9.49	124.1	25.9
M8A	8A	8.58	8.68	112.5	25.4
M8B	8B	9.40	9.87	127.7	25.3
M8C	8C	8.82	8.89	114.9	25.3
Mean		**8.79**	**9.04**	**117.4**	**25.5**
Standard Deviation (+/-)		**0.66**	**0.86**	**11.27**	**0.33**

Appendix 9: Microcosm water quality data recorded on June 26, 2018, or five days after dilbit exposure had begun, with respective averages. (Patterson *et al*. 2019, unpublished data)

Tank	**Treatment Replicate**	**pH**	**D.O. (mg/L)**	**D.O. %**	**Temp. (˚Celsius)**
M1A	1A	7.18	7.01	78.9	18.5
M1B	1B	7.16	6.63	74.9	18.5
M1C	1C	7.01	5.63	63.1	18.5
M2A	2A	7.25	6.93	78.1	18.5
M2B	2B	7.24	6.56	73.5	18.4
M2C	2C	7.25	7.00	78.6	18.5
M3A	3A	7.15	6.89	77.4	18.5
M3B	3B	7.15	6.70	75.0	18.3
M3C	3C	7.10	6.16	69.1	18.5
M4A	4A	6.48	4.95	56.4	19.3
M4B	4B	6.78	3.98	44.8	18.0
M4C	4C	6.78	4.36	49.8	18.7
M5A	5A	7.34	6.81	75.1	18.6
M5B	5B	7.30	6.46	72.5	18.0
M5C	5C	7.14	5.71	64.2	18.6
M6A	6A	7.27	5.80	66.0	19.1
M6B	6B	7.23	6.44	72.6	18.6
M6C	6C	7.32	5.94	66.9	18.6
M7A	7A	7.11	6.34	75.8	18.3
M7B	7B	7.91	6.88	77.1	18.4
M7C	7C	7.15	6.55	73.5	18.5
M8A	8A	7.15	6.89	78.7	18.5
M8B	8B	7.19	7.17	80.5	18.3
M8C	8C	7.08	5.89	66.5	18.5
Mean		**7.16**	**6.24**	**70.4**	**18.5**
Standard Deviation (+/-)		**0.25**	**0.82**	**9.13**	**0.27**

Appendix 10: Microcosm water quality data recorded on June 30, 2018, or nine days after dilbit exposure had begun, with respective averages. (Patterson *et al*. 2019, unpublished data)

Tank	**Treatment Replicate**	**pH**	**D.O. (mg/L)**	**D.O. %**	**Temp. (˚Celsius)**
M1A	1A	7.11	5.18	59.9	19.6
M1B	1B	7.14	4.69	54.2	19.6
M1C	1C	7.04	3.70	42.9	19.7
M2A	2A	7.08	3.77	43.6	19.6
M2B	2B	7.16	5.07	58.5	19.4
M2C	2C	7.19	5.40	62.5	19.6
M3A	3A	7.04	3.57	41.3	19.6
M3B	3B	7.10	4.34	50.1	19.5
M3C	3C	7.14	5.42	62.6	19.6
M4A	4A	7.16	3.14	36.8	20.1
M4B	4B	7.00	3.41	39.5	19.6
M4C	4C	6.99	4.05	47.1	19.8
M5A	5A	7.36	5.22	60.6	19.8
M5B	5B	7.37	5.68	65.7	19.6
M5C	5C	7.22	4.24	49.1	19.6
M6A	6A	7.60	4.17	49.1	20.5
M6B	6B	7.43	5.20	60.7	20.0
M6C	6C	7.39	5.31	61.7	19.8
M7A	7A	7.09	4.41	50.1	19.6
M7B	7B	7.17	5.41	62.4	19.4
M7C	7C	7.10	4.47	51.7	19.6
M8A	8A	7.06	4.14	47.9	19.6
M8B	8B	7.22	6.59	76.2	19.5
M8C	8C	7.14	4.80	55.5	19.6
Mean		**7.18**	**4.64**	**53.7**	**19.7**
Standard Deviation (+/-)		**0.15**	**0.82**	**9.39**	**0.23**

Appendix 11: Microcosm water quality data recorded on July 3, 2018, or twelve days after dilbit exposure had begun, with respective averages. (Patterson *et al*. 2019, unpublished data)

Tank	Treatment Replicate	pH	D.O. (mg/L)	D.O. %	Temp. (˚Celsius)
M1A	1A	10.46	13.54	161.6	21.5
M1B	1B	10.26	12.22	144.7	21.1
M1C	1C	9.86	12.01	142.6	21.2
M2A	2A	9.79	10.49	129.5	21.0
M2B	2B	8.75	9.08	106.6	20.5
M2C	2C	10.32	12.43	146.9	20.9
M3A	3A	10.03	11.03	130.5	21.0
M3B	3B	10.02	11.57	137.0	21.0
M3C	3C	10.12	11.79	139.8	21.2
M4A	4A	9.30	9.33	112.5	22.0
M4B	4B	9.11	10.56	126.0	21.5
M4C	4C	8.32	4.61	55.4	21.8
M5A	5A	9.91	11.05	131.1	21.0
M5B	5B	9.86	10.74	128.1	20.9
M5C	5C	9.98	11.60	137.4	20.9
M6A	6A	7.96	9.38	112.8	21.8
M6B	6B	10.21	12.26	145.3	20.8
M6C	6C	10.09	11.48	136.1	21.0
M7A	7A	9.68	10.80	127.9	21.0
M7B	7B	10.00	11.48	134.8	20.6
M7C	7C	10.59	13.50	160.9	21.4
M8A	8A	9.85	11.04	130.5	21.0
M8B	8B	10.33	12.22	144.5	21.0
M8C	8C	9.73	10.77	128.0	21.3
Mean		**9.77**	**11.04**	**131.3**	**21.1**
Standard Deviation (+/-)		**0.64**	**1.74**	**20.4**	**0.4**

Appendix 12: Reported statistics for the LME model of mean activity with respect to mesocosm dilbit dosage in replicates.

	Estimate	**Standard Error**	**t value**	**p value**
Intercept	85.41038386	2.65928378	32.117815	0.000000
With Dilbit Addition	-0.04758399	0.02378293	-2.000762	**0.045418**

Appendix 13: Reported statistics for the LME model of mean proximal tadpoles (sociality index) with respect to mesocosm dilbit dosage in replicates.

	Estimate	**Standard Error**	**t value**	**p value**
Intercept	8.0419184567	0.407888942	19.7159512	0.0000000
With Dilbit Addition	-0.0007655969	0.004187878	-0.1828126	0.8549451

Appendix 14: Mean proximal tadpoles (sociality index) in a replicate for the five lowest concentration treatments, on each sampling day, with respect to the first (1) and second (2) sample of replicates analyzed.

Replicate Set	Post-Exposure Day	Mesocosm Treatment	Mean Proximal Tadpoles
1	1	M4	9.6
1	5	M4	8.8
1	9	M4	9.6
1	12	M4	8.8
2	1	M4	4.8
2	5	M4	6.0
2	9	M4	9.2
1	1	M6	9.2
1	5	M6	8.8
1	9	M6	8.0
1	12	M6	4.4
2	1	M6	7.0
2	5	M6	4.8
2	9	M6	6.4
1	1	M5	10.8
1	5	M5	8.8
1	9	M5	10.4
1	12	M5	9.2
2	1	M5	4.8
2	5	M5	7.2
2	9	M5	9.8
1	1	M2	8.4
1	5	M2	11.2
1	9	M2	8.0
1	12	M2	8.0
2	1	M2	8.6
2	5	M2	9.2
2	9	M2	7.2
1	1	M7	9.0
1	5	M7	6.4
1	9	M7	8.0
1	12	M7	7.6
2	1	M7	10.6
2	5	M7	10.8
2	9	M7	8.8

Appendix 15: Mean proximal tadpoles (sociality index) in a replicate for the three highest concentration treatments, on each sampling day, with respect to the first (1) and second (2) sample of replicates analyzed.

Replicate Set	Post-Exposure Day	Mesocosm Treatment	Mean Proximal Tadpoles
1	1	M3	8.8
1	5	M3	4.4
1	9	M3	9.2
1	12	M3	4.8
2	1	M3	9.6
2	5	M3	6.0
2	9	M3	4.8
1	1	M8	11.2
1	5	M8	10.0
1	9	M8	7.8
1	12	M8	8.0
2	1	M8	6.4
2	5	M8	6.8
2	9	M8	8.4
1	1	M1	6.4
1	5	M1	7.4
1	9	M1	7.6
1	12	M1	8.8
2	1	M1	8.0
2	5	M1	10.8
2	9	M1	6.8

Appendix 16: Percentage of frames observed with individuals in outer, middle, and inner squares on the grid overlay, for the five lowest concentration treatments. Space use data was reported for each replicate and sampling day, with respect to the first (1) and second (2) sample of replicates analyzed.

Replicate Set	Post-Exposure Day	Mesocosm Treatment	Inner Frame (%)	Middle Frame (%)	Outer Frame (%)
1	1	M4	0	2	98
1	5	M4	1	5	94
1	9	M4	0	1	99
1	12	M4	1	2	97
2	1	M4	0	3	97
2	5	M4	0	0	100
2	9	M4	0	0	100
1	1	M6	0	1	99
1	5	M6	0	3	97
1	9	M6	0	1	99
1	12	M6	2	8	90
2	1	M6	1	2	97
2	5	M6	0	3	97
2	9	M6	1	8	91
1	1	M5	0	1	99
1	5	M5	2	2	96
1	9	M5	0	0	100
1	12	M5	1	2	97
2	1	M5	1	0	99
2	5	M5	1	2	97
2	9	M5	0	2	98
1	1	M2	3	7	90
1	5	M2	0	2	98
1	9	M2	0	6	94
1	12	M2	1	2	97
2	1	M2	0	2	98
2	5	M2	0	2	98
2	9	M2	0	0	100
1	1	M7	0	7	93
1	5	M7	1	3	96
1	9	M7	0	7	93
1	12	M7	0	1	99
2	1	M7	1	2	97
2	5	M7	0	0	100
2	9	M7	0	2	98

Appendix 17: Percentage of frames observed with individuals in outer, middle, and inner squares on the grid overlay, for the three highest concentration treatments. Space use data was reported for each replicate and sampling day, with respect to the first (1) and second (2) sample of replicates analyzed.

Replicate Set	Post-Exposure Day	Mesocosm Treatment	Inner Frame (%)	Middle Frame (%)	Outer Frame (%)
1	1	M3	1	1	98
1	5	M3	0	2	98
1	9	M3	0	10	90
1	12	M3	0	1	99
2	1	M3	1	5	94
2	5	M3	4	8	88
2	9	M3	2	1	97
1	1	M8	1	2	97
1	5	M8	0	0	100
1	9	M8	0	0	100
1	12	M8	0	0	100
2	1	M8	0	1	99
2	5	M8	2	10	88
2	9	M8	1	1	98
1	1	M1	1	2	97
1	5	M1	0	1	99
1	9	M1	1	0	99
1	12	M1	2	0	98
2	1	M1	0	0	100
2	5	M1	0	0	100
2	9	M1	1	1	98

Appendix 18: Mean lines crossed (activity) per tadpole on each sampling day, from replicates with water from mesocosm four.

Animal ID	Mesocosm Treatment	Post-Exposure Day	Mean Activity (Lines Crossed)
004-M4A-1	M4	1	6.65
004-M4A-2	M4	1	5.15
004-M4A-3	M4	1	4.25
004-M4A-4	M4	1	4.35
004-M4A-5	M4	1	4.7
005-M4A-151	M4	5	5.0
005-M4A-152	M4	5	4.4
005-M4A-153	M4	5	4.7
005-M4A-154	M4	5	2.9
005-M4A-155	M4	5	3.2
006-M4C-91	M4	9	6.2
006-M4C-92	M4	9	4.7
006-M4C-93	M4	9	5.55
006-M4C-94	M4	9	5.1
006-M4C-95	M4	9	4.3
007-M4A-191	M4	12	2.85
007-M4A-192	M4	12	5.3
007-M4A-193	M4	12	3.9
007-M4A-194	M4	12	5.65
007-M4A-195	M4	12	2.9
008-M4A-41	M4	18	4.5
008-M4A-42	M4	18	3.35
008-M4A-43	M4	18	2.35
008-M4A-44	M4	18	2.8
008-M4A-45	M4	18	3.6

Appendix 19: Mean lines crossed (activity) per tadpole on each sampling day, from replicates with water from mesocosm six.

Animal ID	Mesocosm Treatment	Post-Exposure Day	Mean Activity (Lines Crossed)
004-M6B-10	M6	1	4.0
004-M6B-6	M6	1	4.25
004-M6B-7	M6	1	4.15
004-M6B-8	M6	1	5.2
004-M6B-9	M6	1	2.8
005-M6A-216	M6	5	3.75
005-M6A-217	M6	5	2.4
005-M6A-218	M6	5	3.25
005-M6A-219	M6	5	4.0
005-M6A-220	M6	5	5.4
005-M6C-131	M6	5	4.75
005-M6C-132	M6	5	4.5
005-M6C-133	M6	5	4.25
005-M6C-134	M6	5	3.55
005-M6C-135	M6	5	5.5
006-M6B-100	M6	9	5.0
006-M6B-96	M6	9	4.9
006-M6B-97	M6	9	3.6
006-M6B-98	M6	9	5.9
006-M6B-99	M6	9	4.95
007-M6B-171	M6	12	2.85
007-M6B-172	M6	12	3.15
007-M6B-173	M6	12	4.75
007-M6B-174	M6	12	4.0
007-M6B-175	M6	12	3.7
008-M6C-46	M6	18	2.75
008-M6C-47	M6	18	1.45
008-M6C-48	M6	18	5.1
008-M6C-49	M6	18	4.45
008-M6C-50	M6	18	3.8

Appendix 20: Mean lines crossed (activity) per tadpole on each sampling day, from replicates with water from mesocosm five.

Animal ID	Mesocosm Treatment	Post-Exposure Day	Mean Activity (Lines Crossed)
004-M5A-11	M5	1	4.35
004-M5A-12	M5	1	1.0
004-M5A-13	M5	1	5.45
004-M5A-14	M5	1	4.95
004-M5A-15	M5	1	4.35
005-M5A-156	M5	5	5.05
005-M5A-157	M5	5	5.05
005-M5A-158	M5	5	3.65
005-M5A-159	M5	5	3.45
005-M5A-160	M5	5	4.4
005-M5C-206	M5	5	3.95
005-M5C-207	M5	5	3.5
005-M5C-208	M5	5	3.25
005-M5C-209	M5	5	3.95
005-M5C-210	M5	5	3.8
006-M5A-111	M5	9	3.75
006-M5A-112	M5	9	3.75
006-M5A-113	M5	9	6.2
006-M5A-114	M5	9	4.8
006-M5A-115	M5	9	4.15
007-M5C-181	M5	12	4.95
007-M5C-182	M5	12	5.25
007-M5C-183	M5	12	2.35
007-M5C-184	M5	12	4.6
007-M5C-185	M5	12	4.8
008-M5C-51	M5	18	4.2
008-M5C-52	M5	18	4.8
008-M5C-53	M5	18	2.55
008-M5C-54	M5	18	3.1
008-M5C-55	M5	18	2.8

Appendix 21: Mean lines crossed (activity) per tadpole on each sampling day, from replicates with water from mesocosm two.

Animal ID	Mesocosm Treatment	Post-Exposure Day	Mean Activity (Lines Crossed)
004-M2B-16	M2	1	3.9
004-M2B-17	M2	1	3.55
004-M2B-18	M2	1	4.0
004-M2B-19	M2	1	5.75
004-M2B-20	M2	1	5.1
005-M2B-121	M2	5	5.55
005-M2B-122	M2	5	3.1
005-M2B-123	M2	5	3.65
005-M2B-124	M2	5	3.95
005-M2B-125	M2	5	3.25
005-M2C-211	M2	5	4.3
005-M2C-212	M2	5	3.85
005-M2C-213	M2	5	4.1
005-M2C-214	M2	5	5.8
005-M2C-215	M2	5	3.5
006-M2A-101	M2	9	4.2
006-M2A-102	M2	9	2.15
006-M2A-103	M2	9	2.9
006-M2A-104	M2	9	3.4
006-M2A-105	M2	9	3.35
007-M2B-186	M2	12	3.9
007-M2B-187	M2	12	7.0
007-M2B-188	M2	12	4.2
007-M2B-189	M2	12	4.35
007-M2B-190	M2	12	4.25
008-M2C-56	M2	18	5.25
008-M2C-57	M2	18	0.35
008-M2C-58	M2	18	5.25
008-M2C-59	M2	18	4.35
008-M2C-60	M2	18	2.75

Appendix 22: Mean lines crossed (activity) per tadpole on each sampling day, from replicates with water from mesocosm seven.

Animal ID	Mesocosm Treatment	Post-Exposure Day	Mean Activity (Lines Crossed)
004-M7C-21	M7	1	5.0
004-M7C-22	M7	1	5.75
004-M7C-23	M7	1	2.5
004-M7C-24	M7	1	4.5
004-M7C-25	M7	1	2.95
005-M7B-141	M7	5	3.65
005-M7B-142	M7	5	5.65
005-M7B-143	M7	5	4.85
005-M7B-144	M7	5	3.7
005-M7B-145	M7	5	5.85
005-M7C-201	M7	5	4.2
005-M7C-202	M7	5	5.4
005-M7C-203	M7	5	3.3
005-M7C-204	M7	5	4.65
005-M7C-205	M7	5	6.2
006-M7C-86	M7	9	4.15
006-M7C-87	M7	9	5.15
006-M7C-88	M7	9	3.65
006-M7C-89	M7	9	5.6
006-M7C-90	M7	9	1.5
007-M7C-166	M7	12	4.1
007-M7C-167	M7	12	5.7
007-M7C-168	M7	12	2.25
007-M7C-169	M7	12	2.65
007-M7C-170	M7	12	4.6
008-M7A-61	M7	18	3.4
008-M7A-62	M7	18	3.3
008-M7A-63	M7	18	4.4
008-M7A-64	M7	18	3.1
008-M7A-65	M7	18	2.75

Appendix 23: Mean lines crossed (activity) per tadpole on each sampling day, from replicates with water from mesocosm three.

Animal ID	Mesocosm Treatment	Post-Exposure Day	Mean Activity (Lines Crossed)
004-M3C-26	M3	1	4.2
004-M3C-27	M3	1	5.4
004-M3C-28	M3	1	5.0
004-M3C-29	M3	1	4.5
004-M3C-30	M3	1	4.9
005-M3A-126	M3	5	4.25
005-M3A-127	M3	5	5.3
005-M3A-128	M3	5	4.5
005-M3A-129	M3	5	4.1
005-M3A-130	M3	5	4.75
006-M3B-106	M3	9	4.25
006-M3B-107	M3	9	4.8
006-M3B-108	M3	9	4.55
006-M3B-109	M3	9	4.7
006-M3B-110	M3	9	5.0
007-M3B-161	M3	12	5.15
007-M3B-162	M3	12	2.05
007-M3B-163	M3	12	3.95
007-M3B-164	M3	12	6.1
007-M3B-165	M3	12	4.25
008-M3B-66	M3	18	1.95
008-M3B-67	M3	18	4.05
008-M3B-68	M3	18	4.6
008-M3B-69	M3	18	2.65
008-M3B-70	M3	18	2.7

Appendix 24: Mean lines crossed (activity) per tadpole on each sampling day, from replicates with water from mesocosm eight.

Animal ID	Mesocosm Treatment	Post-Exposure Day	Mean Activity (Lines Crossed)
004-M8A-31	M8	1	3.15
004-M8A-32	M8	1	5.2
004-M8A-33	M8	1	3.35
004-M8A-34	M8	1	3.3
004-M8A-35	M8	1	4.2
005-M8C-146	M8	5	4.6
005-M8C-147	M8	5	2.6
005-M8C-148	M8	5	0.6
005-M8C-149	M8	5	5.55
005-M8C-150	M8	5	4.8
006-M8C-116	M8	9	3.8
006-M8C-117	M8	9	3.8
006-M8C-118	M8	9	4.3
006-M8C-119	M8	9	4.35
006-M8C-120	M8	9	4.35
007-M8A-176	M8	12	4.65
007-M8A-177	M8	12	5.1
007-M8A-178	M8	12	4.15
007-M8A-179	M8	12	5.45
007-M8A-180	M8	12	4.7
008-M8B-71	M8	18	2.95
008-M8B-72	M8	18	4.0
008-M8B-73	M8	18	4.5
008-M8B-74	M8	18	2.15
008-M8B-75	M8	18	3.7

Appendix 25: Mean lines crossed (activity) per tadpole on each sampling day, from replicates with water from mesocosm one.

Animal ID	Mesocosm Treatment	Post-Exposure Day	Mean Activity (Lines Crossed)
004-M1B-36	M1	1	4.85
004-M1B-37	M1	1	4.4
004-M1B-38	M1	1	4.2
004-M1B-39	M1	1	3.8
004-M1B-40	M1	1	3.4
005-M1C-136	M1	5	4.55
005-M1C-137	M1	5	4.85
005-M1C-138	M1	5	3.65
005-M1C-139	M1	5	5.55
005-M1C-140	M1	5	5.25
006-M1B-81	M1	9	4.75
006-M1B-82	M1	9	3.5
006-M1B-83	M1	9	4.5
006-M1B-84	M1	9	5.0
006-M1B-85	M1	9	4.05
007-M1C-196	M1	12	3.45
007-M1C-197	M1	12	3.05
007-M1C-198	M1	12	4.15
007-M1C-199	M1	12	4.1
007-M1C-200	M1	12	2.55
008-M1C-76	M1	18	4.75
008-M1C-77	M1	18	3.75
008-M1C-78	M1	18	1.0
008-M1C-79	M1	18	2.65
008-M1C-80	M1	18	0.55