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Research Paper

Methylisothiazolinone toxicity and inhibition of wound healing and regeneration in planaria

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ABSTRACT

Methylisothiazolinone (MIT) is a common biocide used in cosmetic and industrial settings. Studies have demonstrated that MIT is a human sensitizer, to the extent that in 2013 MIT was named allergen of the year. Recently, we showed that MIT exposure in *Xenopus laevis* (the African clawed frog) inhibits wound healing and tail regeneration. However, it is unknown whether MIT affects these processes in other animals. Here, we investigated the effects of MIT exposure in planaria—non-parasitic freshwater flatworms able to regenerate all tissues after injury. Using a common research strain of *Dugesia japonica*, we determined that intact planarians exposed to 15 μM MIT displayed both neuromuscular and epithelial-integrity defects. Furthermore, regenerating (head and tail) fragments exposed to 15 μM MIT failed to close wounds or had significantly delayed wound healing. Planarian wounds normally close within 1 h after injury. However, most MIT-exposed animals retained open wounds at 24 h and subsequently died, and those few animals that were able to undergo delayed wound healing without dying exhibited abnormal regeneration. For instance, head regeneration was severely delayed or inhibited, with anterior structures such as eyes failing to form in newly produced tissues. These data suggest that MIT directly affects both wound healing and regeneration in planarians. Next, we investigated the ability of thiol-containing antioxidants to rescue planarian wound closure during MIT exposure. The data reveal both *n*-acetyl cysteine and glutathione were each able to fully rescue MIT inhibition of wound healing. Lastly, we established MIT toxicity levels by determining the LC_{50} of 5 different planarian species: *D. japonica, Schmidtea mediterranea*, *Girardia tigrina, Girardia dorotocephala,* and *Phagocata gracilis*. Our LC_{50} data revealed that concentrations as low as 39 μM (4.5 ppm) are lethal to planarians, with concentrations of just 5 μM inhibiting wound healing, and suggest that phylogeny is predictive of species toxicity levels. Together these results indicate MIT may have broad wound healing effects on aquatic species in general and are not limited to *X. laevis* alone. Future studies should investigate the impact of MIT on wound healing in other organisms, including non-aquatic organisms and mammals.

1. Introduction

Methylisothiazolinone (MIT) is a common antifungal found in cosmetics, household products, pesticides, water storage and cooling units. In use since the 1980s, MIT was initially combined with methylchloroisothiazolinone (MCI) in a 3:1 MCI/MIT ratio (Lundov et al., 2011b). After a combination of reports and studies showing MCI/MIT was a sensitizer (Bruze et al., 1987a; Bjorkner et al., 1986; De Groot et al., 1985), another study found MIT to be the weaker sensitizer (Bruze et al., 1987b). Approved for use by itself in 2005, MIT is now one of the leading preservatives in both cosmetic and industrial products. MIT's permitted concentration in cosmetics is 100 ppm, while there are no limits on industrial products (Lundov et al., 2011a). Investigations into its mechanism of action show that MIT contains an active thiol moiety that interacts with free thiols oxidatively to form disulphides (Collier et al., 1990), and thus it interacts with available enzymatic cysteines *in vitro* (Du et al., 2002). Interactions destroy protein thiols and lead to the production of free radicals and ultimately cell death (Williams, 2007), making MIT commercially and industrially ideal for the prevention of bacterial and fungal growth. Since its incorporation into cosmetics in 2005, reports of contact allergy have risen dramatically (Lundov et al., 2011a; Aerts et al., 2015; Wilford and De Gannes, 2017; Uter et al., 2013; Thyssen et al., 2006), with human sensitization below permitted concentrations (Yazar et al., 2015). After rising reports, MIT was named allergen of the year in 2013 (Castanedo-Tardana and Zug, 2013).

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Currently, not much is known about other effects of MIT exposure, particularly in animal models. A study in cultured rat cortical neurons demonstrated that MIT reduced the size and complexity of axonal and dendritic outgrowth as well as cell-to-cell contact in developing neurons (He et al., 2006). Furthermore, a 10-min exposure of cultured cortical neurons to 100 μM MIT resulted in cell death over a 24 h period (Du et al., 2002). In this study, neurons exhibited cell death while surrounding glial cells did not, suggesting a high level of MIT sensitivity specific to neurons compared to glial cells (Du et al., 2002). Further investigations into human sensitization have focused on studying how immune cells are activated during immune responses (Böttcher et al., 2008; Sakaguchi et al., 2007). One study using cultured human monocytes and dendritic cells showed protein thiol groups, as opposed to amino groups, are key to activation of human immune cells during allergic reactions (Becker et al., 2003).

Recently we demonstrated that in *Xenopus laevis* MIT exposure effects extend to inhibition of both embryonic wound healing and tail regeneration following injury (Delos Santos et al., 2016). Our data showed that 75 μM MIT is sufficient to inhibit normal tail regeneration following amputation. Immunohistochemistry data revealed nerve, muscle, notochord and spinal outgrowth were all lacking in MIT-exposed tadpoles. This study also showed that MIT acts during the first day of wound healing, with later exposure failing to impact the regenerative process. While normal *Xenopus laevis* embryonic wound healing occurs within 6 h of injury, we found that 50 μM MIT inhibited wound healing significantly. However, it is not clear whether other organisms are similarly impacted by MIT, particularly in aquatic environments where exposure to biocides are most likely gained via commercial and industrial runoff and wastewater.

Planarians are free-living freshwater flatworms that are an ideal model in which to investigate the effects of MIT. There are several hundred planarian species (Oviedo et al., 2008), and they are found almost ubiquitously in freshwater streams, rivers, ponds, and lakes (Reddien and Sanchez Alvarado, 2004). Planaria are highly sensitive to chemicals (Hagstrom et al., 2015; Raffa and Rawls, 2009) and have been well-established as a pharmacological and toxicological model (Prá et al., 2005; Balestrini et al., 2017; Raffa and Valdez, 2001; Horvat et al., 2005; Thume and Frizzo, 2017; Rawls et al., 2011; Grebe and Schaeffer, 1991). Planaria provide both behavioral and morphological readouts, including neurological effects (Hagstrom et al., 2016; Pagán et al., 2009; Buttarelli et al., 2008). Neurological readouts are especially useful as planarians possess a true central nervous system with bi-lobed cephalic ganglia, making them a suitable model for studying the evolutionary history of the brain. Planarians also possess the unique ability to regenerate any and all tissues after injury (Rink, 2013). Wounds close within 1 h of injury (Sanchez Alvarado and Newmark, 1998), while regeneration occurs over ~2 weeks in most species (Lobo et al., 2012).

The aim of this study is to determine the effects of MIT exposure on wound healing and regeneration in planarians. Here we observe phenotypes in whole planaria at varied MIT concentrations, using the common model organism *Dugesia japonica*. To assess the effects of MIT exposure on wound healing and regeneration, we amputate planarians (by bisection) and observe both wound closure and regenerative morphology following MIT exposure (in comparison to untreated controls). Next, we hypothesize the addition of thiol-containing antioxidants glutathione (GSH) and *n*-acetyl cysteine (NAC) may rescue wound healing by occupying MIT and inhibiting its mechanism of action in planarians. Lastly, we establish LC_{50} concentrations for 5 different species of planaria.

2. Materials and methods

2.1. Animal care

A clonal line of *Dugesia japonica* (GI) and an asexual clonal line of *Schmidtea mediterranea* (CIW4) were maintained at 20 °C in the dark. *Girardia tigrina* (Ward's Science, Rochester, NY), *Girardia dorotocephala* (Carolina Biological Supply, Burlington, NC), and *Phagocata gracilis* (Ward's Science) were purchased commercially and used to establish colonies that were kept at room temperature in the dark. All planarians were kept in worm water comprised of 0.5 g/L of Instant Ocean salts. Animals were fed no more than 1X week with "natural" (no antibiotics or hormones) liver paste made from whole calf's liver (Creekstone Farms, Arkansas City, KS). Liver was frozen and thawed only once prior to feeding animals. Worms 5–7 mm in length were starved at least one week prior to experimentation.

2.2. Reagents

Chemicals used include: methylisothiazolinone (MIT, Sigma Aldrich, St. Louis, MO), glutathione (GSH, TCI America, Portland, OR) and *n*-acetyl cysteine (NAC, Sigma Aldrich). Stock solutions of MIT, GSH, and NAC were made with deionized water and stored at 4 °C. Working concentrations were made by diluting stock concentrations with worm water. Directly following surgery, animals were transferred into each chemical; solutions were not changed over the course of the experiment.

2.3. Phenotype assay

D. japonica were exposed to varied MIT concentrations (as stated) and observed for phenotypes at multiple time points after initial exposure. Phenotypic categories included: wild-type (where animals were undistinguishable from untreated controls), lesions (loss of epithelial integrity), head regression (a unique but common planarian strategy where animals in a toxic environment lyse head structures prior to regenerating them), and phenotypes indicating neuromuscular inhibition (c-shape and screw-like). In addition, contracted phenotypes (where the soft-bodied animals scrunch to reduce total surface area) were observed, as well as animals which crawled out of the media and died (from dehydration) rather than remain exposed to the drug. This latter phenotype is commonly observed with highly toxic exposures. For this and all other experiments, negative controls were performed with animals exposed to worm water only.

2.4. Wound healing assay

D. japonica were amputated midway between the head and tail (bisection) under a dissecting microscope, as previously described (Beane et al., 2012). The resultant head and tail fragments were exposed to 15 μM MIT and compared to untreated controls. Animals were scored and imaged 1 and 6 h post amputation (hpa), and then were subsequently rescored at 1, 2, 3, and 7 days. Wound healing was defined as the lack of any observable tissue emerging from the wound site. No difference was found between head fragments (with posterior wounds) and tail fragments (with anterior wounds), and thus the data was combined. Untreated controls were followed every 5 min for 30 min to establish a normal wound healing timeline.

2.5. Regeneration assay

D. japonica were bisected (as above) to produce tail fragments and placed in 15 μM MIT in 12-well untreated culture plates immediately after microsurgery. Water was not changed over the course of 14 days. Anterior (head) regeneration was followed over the course of 14 days, with the same animals scored and imaged on days 4, 7, and 14 post amputation.

2.6. Antioxidant treatment

D. japonica were bisected (as above) and individual head and tail fragments were placed into wells in a 24-well plate (one fragment per

well). Thiol-containing GSH and NAC were added to either worm water or 15 μM MIT as stated. Antioxidants were added at a 1:1 molar ratio with MIT. Animals were observed and imaged for wound healing at 1 hpa. No difference was found between head and tail fragments and thus the data was combined.

2.7. $LC_{50,}$ $EC_{50,}$ and NOEC/LOEC

LC_{50} and EC_{50} were calculated using probit analysis (Finney, 1952) for intact (whole) animals of five planarian species: *D. japonica, S. mediterranea, G. tigrina, G. dorotocephala* and *P. gracilis*; and for head and tail fragments (resulting from bisection) for *D. japonica.* Whole animals and fragments were exposed to given concentrations of MIT for 6 h and then scored for survival (LC_{50}). For $EC_{50,}$ no observed effect concentration (NOEC), and lowest observed effect concentration (LOEC), fragments were scored at 6 h of exposure (which also corresponded to 6 hpa) for the presence or absence of wound closure (as defined above). No difference was found between head and tail fragments and thus the data was combined.

2.8. Image collection and statistical analyses

Images were taken using a Zeiss V20 fluorescent stereomicroscope with AxioCam MRc camera and Zen Lite software (Zeiss, Oberkochen, Germany). Adobe Photoshop was used to orient and scale images, and improve clarity. Data were neither added nor subtracted; original images available upon request. Data from the wound healing assay and antioxidant treatment were analyzed by a two sample *t*-test between percents using the Statistics Calculator software (StatPac, V. 4.0, StatPac Inc., Northfield, MN, USA). Error bars are standard error of the proportion.

3. Results

3.1. Phenotypic effects of MIT in planarians

Dugesia japonica planaria were examined for MIT-specific effects at 6 h (Fig. 1B) and at 24 h (Fig. 1C), as well as at 3, 7, and 14 days after MIT exposure. The portion of animals exhibiting each phenotype showed little change after 24 h, so data is not shown. The observed defects included: contracted, c-shape, screw-like, lesions, and head regression (Fig. 1A). In addition, there was a significant portion that crawled out of the media and died due to dehydration (not shown in figure). The contracted phenotype (Fig. 1A2) occurs when planaria "scrunch" body wall muscles, reducing surface area and resulting in ruffled edges at the lateral margin (Hagstrom et al., 2016). Both c-shape (Fig. 1A3) and screw-like (Fig. 1A4) are hyperkinesia phenotypes representing a disruption in normal neuromuscular function (Hagstrom et al., 2016; Venturini et al., 1989). Both lesions (Fig. 1A5) and head regression (Fig. 1A6) represent a loss of epithelial integrity, resulting in the extrusion of parenchymal tissues through the lysing epithelium. Head regression, where planaria lyse anterior structures prior to regenerating them, is often seen following toxic exposure.

A greater range of phenotypes was observed at 6 h (Fig. 1B) as compared to 24 h (Fig. 1C) of exposure. Low MIT concentrations at both the 6 and 24 h marks showed no phenotype (similar to untreated controls), with ≥97% of animals appearing wildtype at or below 5 μM MIT at 6 h and at or below 10 μM at 24 h. The contracted phenotype was only found at 6 h, at concentrations between 10 and 30 μM. Neuromuscular impairments (c-shape/screw-like) were observed in a broad range of concentrations from 10 to 45 μM at 6 h, but were only seen at 10 and 20 μM at 24 h. This pattern was similar for epithelial defects (lesions/head regression), which at 6 h were widely observed at 20 to 45 μM concentrations but at 24 h were only seen at 10 and 20 μM. Animals that crawled out of the media and subsequently died were mainly observed at the 20 μM concentration (30%) at both 6 and 24 h following exposure, but were also seen occasionally at 5 μM (3%) and 30 μM (10–20%) for both time points. Lastly, death was only observed at higher concentrations. At 6 h of exposure, death occurred at 40 μM (25%) and 45 μM (80%). However, by 24 h the amount of death significantly increased and was seen in concentrations as low as 20 μM (35%), with most animals exposed at higher concentrations dying: 90% of animals at 30 μM and 100% of animals at 35, 40 and 45 μM died (Fig. 1B and C, $n \geq 19$ for each concentration).

3.2. MIT effects on planarian wound healing

After injury, the majority of untreated control planarians closed their wounds within 30 min (Fig. 2A). We followed the time course of wound healing in amputated (bisected) *D. japonica* fragments that were immediately exposed to 15 μM MIT after surgery, observing animals at 1 and 6 h (Fig. 2B and C), and at 1–3 and 7 days after injury (Fig. 2C). At all time points, the majority of MIT-exposed animals failed to close their wounds. Wound closure was scored as the absence of any tissues coming out of the wound site, with the presence of any escaping tissue considered an open wound (see Fig. 2B3-4). 73% of untreated control animals had undergone wound closure by 1 h, with 82% by 6 h and 100% by 1 day post amputation (Fig. 2C); in contrast, MIT-exposed animals had significantly lower levels of wound closure, with 6% at 1 h, 11% at 6 h, and only 21% after 1 day post amputation ($n \geq 21$ for each condition, $p \leq 0.0001$). To determine the toxicity of MIT in *D. japonica* fragments, we measured the LC_{50}, $EC_{50,}$ lowest observed effect concentration (LOEC), and no observed effect concentration (NOEC) after 6 h of exposure (Fig. 2D). The LC_{50} of fragments exposed to MIT during the wound healing period was found to be 33 μM—with an EC_{50} of 9.33 μM, an LOEC of 5 μM ($p = 0.0354$), an NOEC of 1 μM ($p = 1$) for observed effects on wound closure ($n \geq 20$).

3.3. Effects of MIT on planarian regeneration

When treated with 15 μM MIT, 50% of amputated (bisected) *D. japonica* died after failing to undergo wound healing, while 38% of animals eventually closed their wounds but still died post wound healing (Fig. 3A). Only 13% of animals exposed to MIT were able to survive after delayed wound healing occurred (Fig. 3A). Observation of *D. japonica* tail fragments (which need to regenerate a new head) that survived 15 μM MIT exposure ($n = 7$) revealed abnormal regeneration in all animals as compared to untreated controls (Fig. 3B and C). While all control tail fragments were able to regrow heads prior to 14 days post amputation (Fig. 3B), the majority of MIT-exposed tail fragments that survived (57.1%, 4 out of 7) failed to undergo head regeneration by day 14 (Fig. 3C3). The remaining MIT-exposed animals (3 out of 7) were able to regenerate some anterior structures, such as the eyes; however, the new eyes did not occur in the newly produced (unpigmented) tissues but rather in the preexisting (pigmented) tissues (Fig. 3C3'). For comparison, Fig. 3B2 shows day 7 controls with eyes regrowing within the white region that comprises the new tissues of the regeneration blastema.

3.4. MIT and antioxidants

MIT has an active thiol moiety that interacts with free thiol groups. We used two thiol-containing antioxidants – *n*-acetyl cysteine (NAC) and glutathione (GSH) – to determine if MIT interacts with thiol groups in planarians (Fig. 4). In contrast to 15 μM MIT alone, which inhibited wound closure, amputated *D. japonica* animals exposed to 15 μM MIT in combination with either 15 μM NAC or 15 μM GSH exhibited normal wound healing (Fig. 4A). The majority of NAC/MIT (79%) and GSH/MIT (62.5%) treated animals were able to close their wounds by 1 h post amputation, similar to untreated controls ($p = 0.5253$ and $p = 0.2281$, respectively); additionally, there was no significant effect on wound healing ability following exposure to GSH ($p = 0.2281$) or

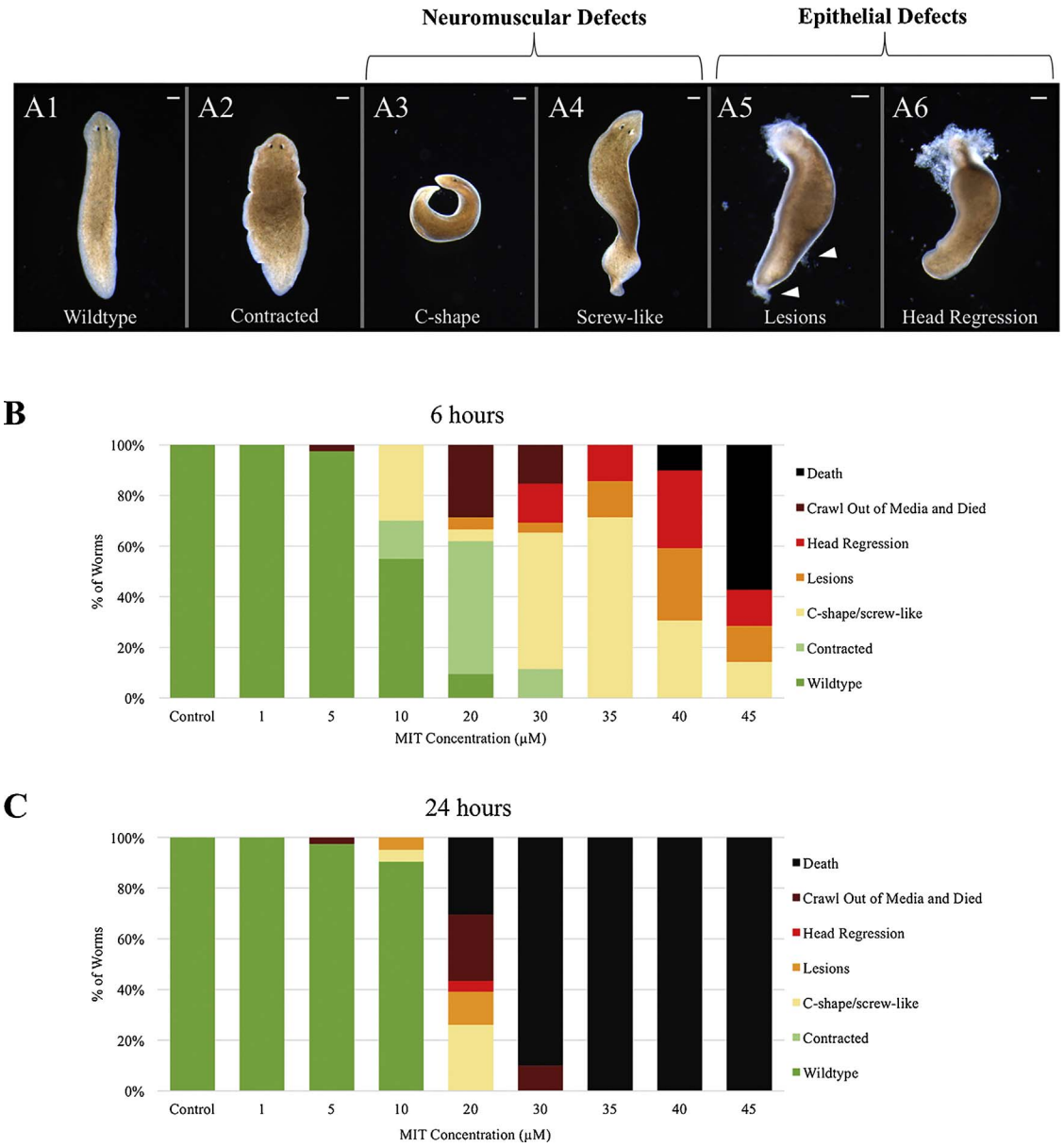


Fig. 1. Phenotypic Effects of Methylisothiazolinone (MIT) Exposure on Planarians.
(A) Representative images of observed phenotypes in MIT-exposed *Dugesia japonica* planarian flatworms. Phenotypes of whole (intact) animals: (A1) Wildtype (similar to untreated controls), (A2) contracted (muscle contraction), (A3) c-shape (neuromuscular defect), (A4) screw-like (neuromuscular defect), (A5) lesions (epithelial defect), and (A6) head regression (epithelial defect). Arrows = lesions. Note that the animal in A5 is also beginning to undergo head regression. Anterior is up, scale bars = 250 µm. (B and C) Quantification of phenotypic percentages by concentration after (B) 6 h and (C) 24 h of MIT exposure. n ≥ 19 for each concentration.

NAC ($p = 0.7564$) alone as compared to controls (Fig. 4B-C, n = 24 for each condition).

3.5. MIT toxicity across planarian species

To ascertain the toxicity of MIT in whole (intact) planarians, the LC_{50} was measured after 6 h of exposure (Fig. 5). The MIT LC_{50} for *Dugesia japonica*, the species used in this study, was determined to be 42 µM (Fig. 5B1). To establish whether MIT toxicity was similar in other planarian species, we determined the LC_{50} for four other planaria: one other commonly used research species (*Schmidtea mediterranea*), and three species that are all commercially available (*Girardia tigrina*, *Girardia dorotocephala*, and *Phagocata gracilis*). *S. mediterranea* (Fig. 5B2) is closely related to *D. japonica* and was found to have a similar LC_{50} of 50 µM; the two *Girardia* species (Fig. 5B3-4) are even more closely related and had the same calculated LC_{50} of 39 µM; while the phylogenetic outlier *P. gracilis* (Fig. 5B5) was found to have an LC_{50} of 70 µM (n ≥ 10 for each concentration for all species).

4. Discussion

Since the introduction of MIT into cosmetic products in 2005 (Leiva-Salinas et al., 2014), there have been increasing reports of dermatitis in human studies (Lundov et al., 2011a; Aerts et al., 2015; Wilford and De Gannes, 2017; Uter et al., 2013; Thyssen et al., 2006; Yazar et al., 2015). More recently, research has expanded to aquatic toxicology studies in order to understand the effects of MIT on aquatic environments and organisms. Our previous study in *Xenopus laevis* (Delos Santos et al., 2016) showed that MIT results in wound healing and regeneration defects following exposure. Yet, the few number of extant studies are not enough to clarify if MIT similarly affects other aquatic species. Here, we show that MIT is also toxic to both intact and injured

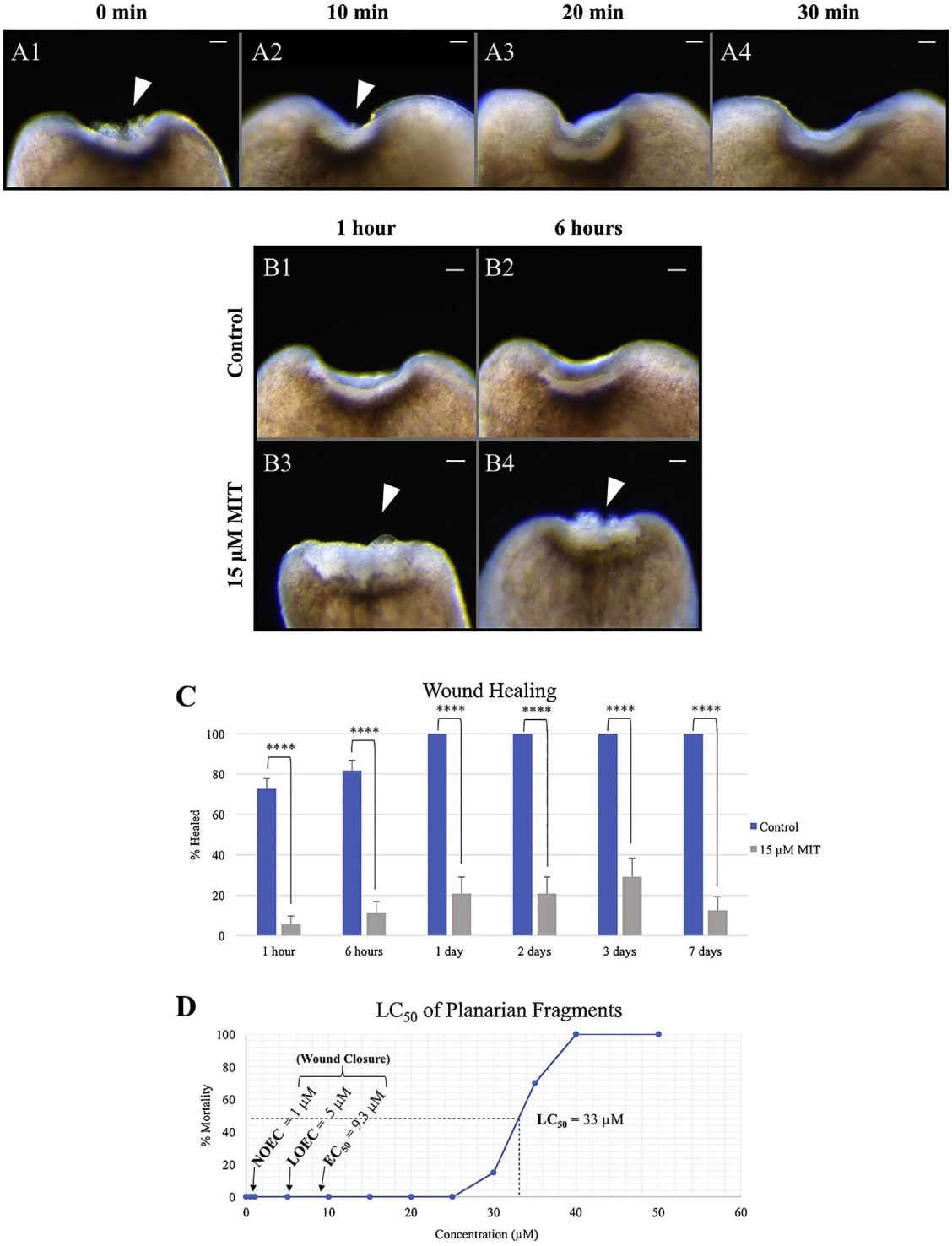


Fig. 2. MIT Exposure Blocks Wound Healing in Planarians.
(A) Timecourse of normal wound healing in untreated *D. japonica* following head amputation. Most planarian wounds close within 30 min post amputation. (B) Images of MIT effects on *D. japonica* wound healing following head amputation. (B1-2) Untreated controls and (B3-4) 15 µM MIT-treated animals at 1 and 6 h post amputation (hpa). Note that following MIT exposure, the initial muscle contraction of the wound site (visible as a dark ring of pigment adjacent to the wound site in B1) fails to occur by 1 h (B3), but does occur by 6 h even though the wound still fails to close (B4). (C) Quantification of wound healing over 7 days post amputation. Note that at day 7 post amputation, 15 µM MIT-exposed planarians show a decrease in percent healed due to death post wound closure. (D) LC_{50} graph of MIT for *D. japonica* head and tail fragments at 6 h of exposure (also 6 hpa). No observed effect (NOEC) and lowest observed effect (LOEC) concentrations, as well as EC_{50}, are indicated for wound closure phenotype. Arrows = unclosed wounds, anterior is up, scale bars = 50 µm. Error bars = standard error of the proportion, n ≥ 20 for each condition, **** $p \leq 0.0001$.

planaria at low concentrations (39 µM and 33 µM respectively), and its sub-lethal effects include neuromuscular defects as well as inhibited wound healing and regeneration.

While the main source of biocide exposure for aquatic organisms is likely to be via industrial wastewater, the existing reports are conflicted. Studies have reported that MIT both was and was not detected in storm wastewater and sewage influent, while artificial wall rainwater assays have recorded run off concentrations ranging from 100 to

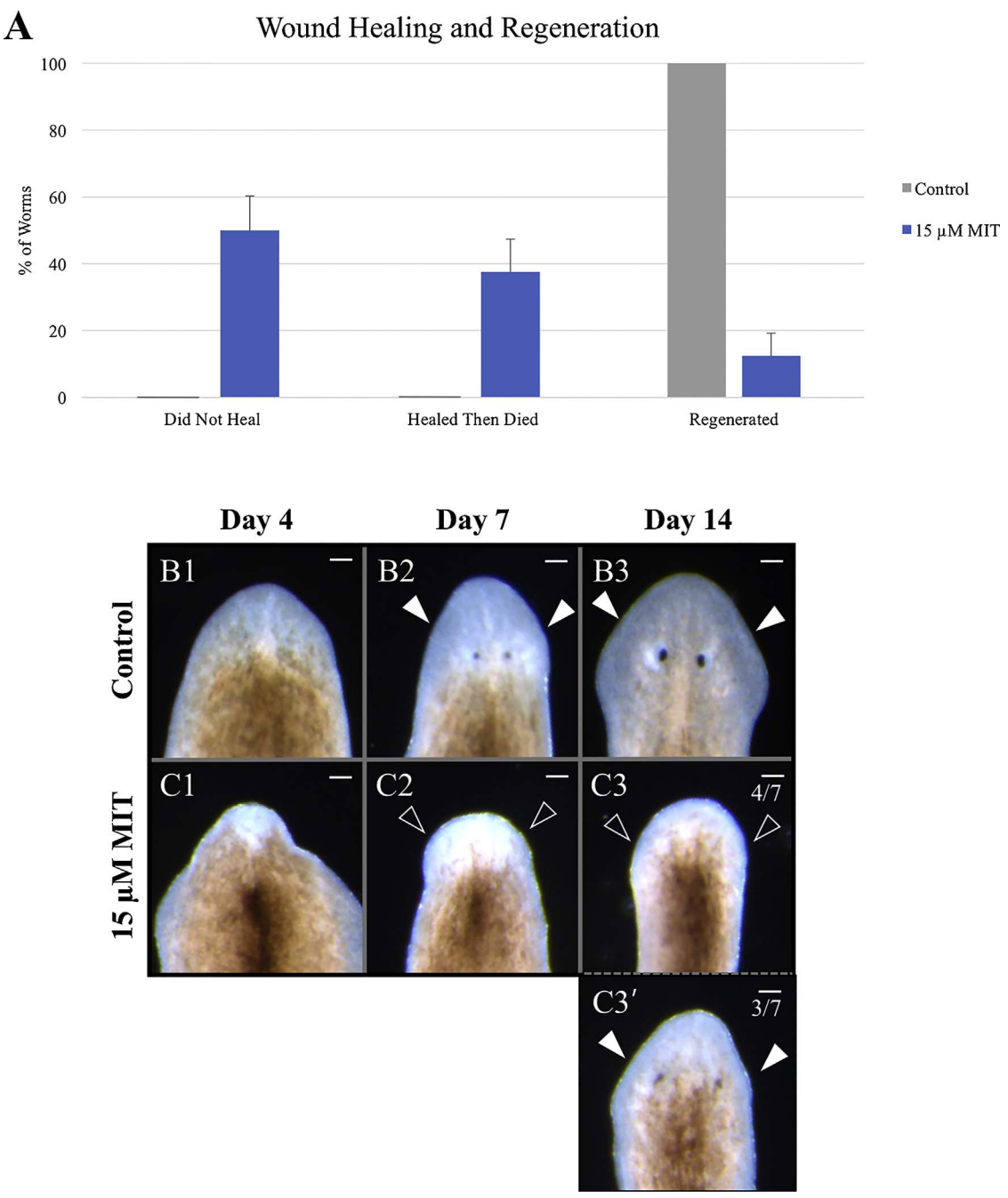


Fig. 3. MIT Exposure Causes Abnormal Regeneration in Planarians.
(A) Quantification of wound healing and regeneration over 7 days post amputation. Percentages of untreated controls and 15 µM MIT-treated *D. japonica* that: "Did Not Heal" (failed to close their wounds and died), "Healed Then Died" (died after closing their wounds), and "Regenerated" (closed their wounds and continued on to regenerate new tissues). n ≥ 21 for each condition. (B-C) Regeneration morphology over 14 days post head amputation. (B) Control (n = 5) and (C) 15 µM MIT-treated (n = 7) *D. japonica*. Note that MIT exposure blocks head regeneration (C2-3). Closed arrows = eyes present (but regenerating ectopically in preexisting tissues in C3′), open arrows = lack of eyes. Anterior is up, scale bars = 100 µm. Error bars = standard error of the proportion.

900 µM (Bester et al., 2014; Carbajo et al., 2015; Rafoth et al., 2007; Baranowska and Wojciechowska, 2013; Bollmann et al., 2014). Interestingly, the toxicity of whole animals and injured planarians to MIT was roughly similar, even though injured worms might be predicted to have higher mortality levels. However, the sensitivity of injured planarians to MIT was predictably much lower for sub-lethal effects, with concentrations as low as 5 µM significantly inhibiting wound closure (Fig. 2D).

In this study, different concentrations of MIT resulted in a range of phenotypic responses, including epithelial integrity and neuromuscular defects (Fig. 1). The broadest range of phenotypes was observed at 6 h of exposure, as by 24 h many phenotypic animals had died. Past 24 h, little change in phenotypic distributions was seen (data not shown). These data suggest that MIT affects planaria quickly following exposure. The lysing phenotypes we observed (lesions and head regression) suggest that at a minimum MIT exposure affects epithelial cells. However, head regression is known to occur over several days following stem cell loss (Oviedo et al., 2008; 2004), suggesting a possible explanation for animal deaths at later time points. The neuromuscular effects observed strongly suggest that neuronal populations are affected by MIT; inhibition of neurotransmitter levels (e.g. cholinergic or dopaminergic activity) is known to result in both c-shape and screw-like

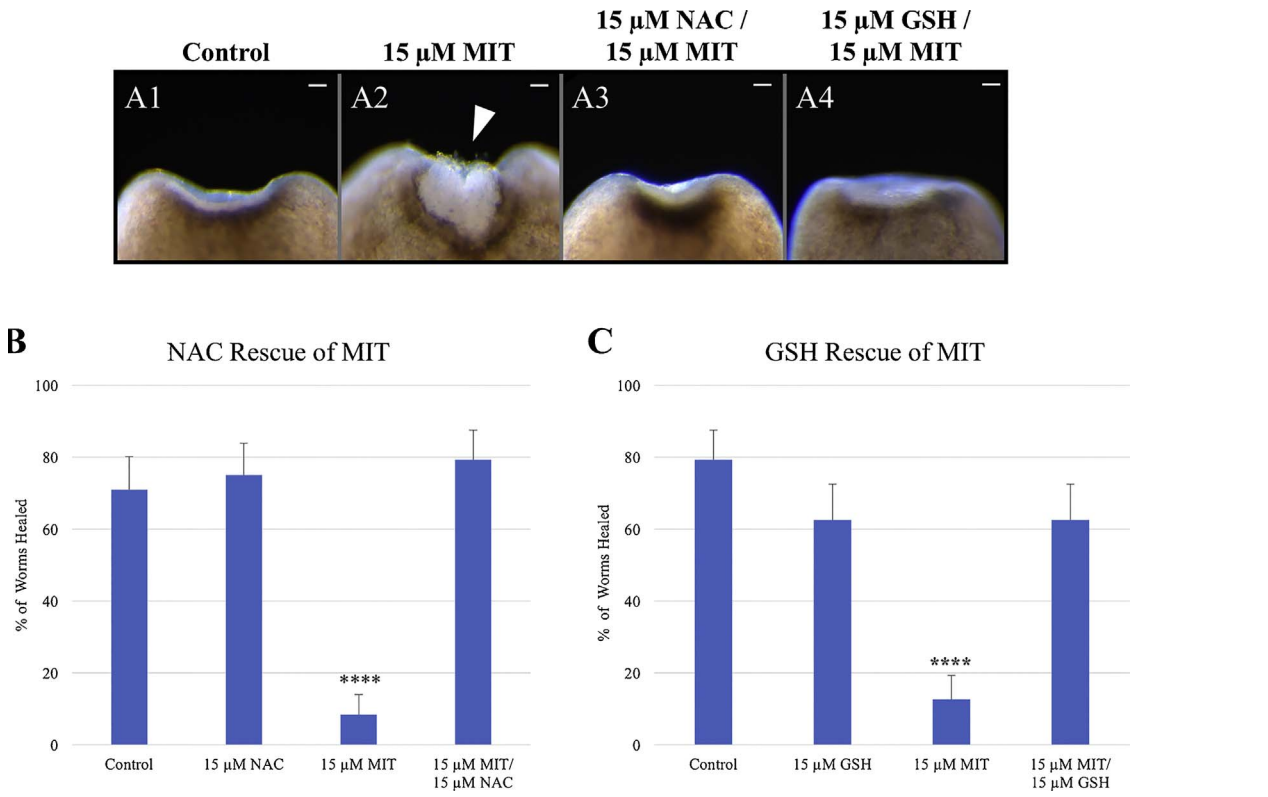


Fig. 4. Antioxidants Rescue the Effects of MIT Exposure.
Wound healing at 1 h post head amputation in (A1) untreated control (A2) 15 µM MIT-treated, (A3) 15 µM MIT plus 15 µM *n*-acetyl cysteine (NAC) treated and (A4) 15 µM MIT plus 15 µM glutathione (GSH) treated *D. japonica*. Arrows = unclosed wound, anterior is up, scale bar = 50 µm. (B-C) Quantification of antioxidant rescue. Both (B) NAC and (C) GSH rescue wound healing at a 1:1 molar ratio with MIT. Error bars = standard error of the proportion, n = 24 for each condition, **** $p \leq 0.0001$.

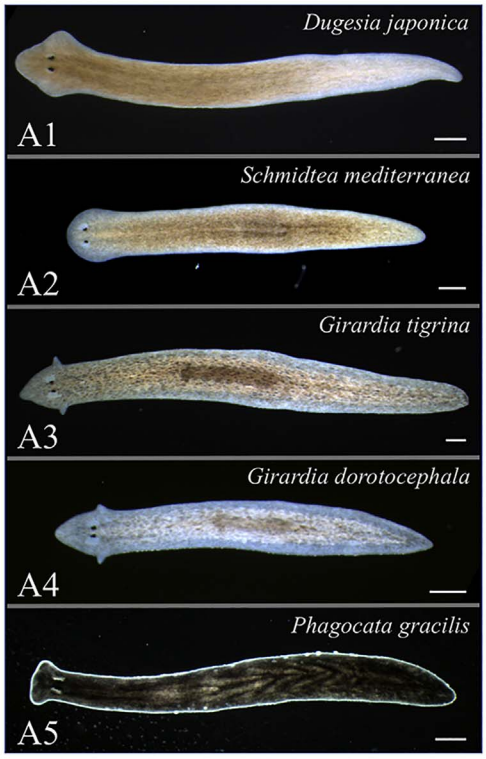
phenotypes (Buttarelli et al., 2000; Passarelli et al., 1999; Venturini et al., 1989; Wu et al., 2015).

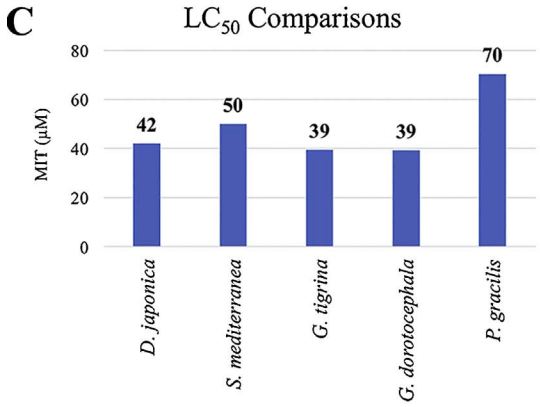
Planaria normally close their wounds within 1 h post amputation (hpa) (Reddien and Sanchez Alvarado, 2004). Our results revealed that the majority of planarians exposed to 15 µM MIT failed to close their wounds even several days post amputation, as compared to untreated controls where 100% of animals completed wound healing within 24 h (Fig. 2B-C). In our previous work, we found that wound healing was also inhibited in *Xenopus laevis* (the African clawed frog) by 50 µM MIT (Delos Santos et al., 2016). Together, these data suggest that aquatic organisms are highly sensitive to low concentrations of MIT, and that MIT exposure may interfere with wound healing processes in general. Without proper wound healing, the first step prior to the initiation of regeneration (Wenemoser and Reddien, 2010), planarians subsequently died and did not undergo regeneration (Fig. 3A). However, a small proportion of animals were able to close their wounds after a significant delay, and these animals were examined for regeneration-specific defects (Fig. 3B). Planarian tail fragments that survived exposure to 15 µM MIT had inhibited (total lack of head structures) or abnormal (stunted eyes found only in preexisting tissues) anterior regeneration (Fig. 3C). These data suggest that the lack of proper wound healing (either a failure of proper closure or delayed timing) inhibits subsequent regeneration, similar to what has been found in previous studies of *Xenopus* (Delos Santos et al., 2016; Ho and Whitman, 2008).

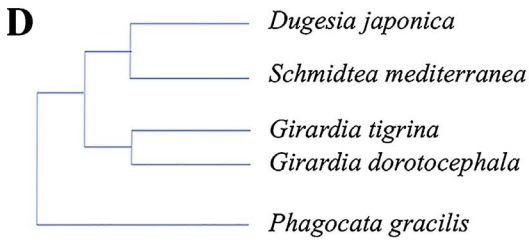
Previous LC_{50} data from the Environmental Protection Agency (EPA) reports that MIT is toxic to a variety of aquatic organisms, including *Lepomis macrochirus* (Bluegill) and *Oncorhynchus mykiss* (Rainbow Trout) (National Library of Medicine, 2015). Our data show that after just 6 h of exposure low concentrations of MIT are lethal to planaria (Fig. 5). We investigated MIT toxicity in five different planarian species: the two species most commonly used for research (*D. japonica and S. mediterranea)*, and three species that are commercially available and thus easily obtained. Interestingly, we found that toxicity levels grouped by the known phylogenetic relationships of the species tested (Fig. 5C and D) (Alvarez-Presas et al., 2008). *G. tigrina* and *G. dorotocephala* (both native to North America) are the most closely related species and had the same LC_{50} values (39 µM); *D. japonica* (an East Asian species) and *S. mediterranea* (found in Mediterranean countries) also clade together and had similar LC_{50} values (42 µM and 50 µM, respectively); while the phylogenetic outlier *P. gracilis* (which is also native to North America) had a much higher tolerance to MIT (LC_{50} at 70 µM). These data suggest that evolutionary relationships may be a good predictor of toxicity.

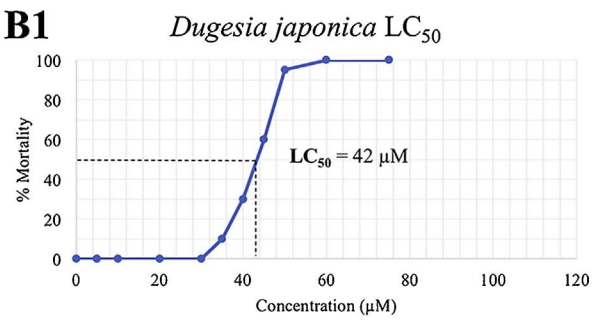
The mechanism of action for MIT is still under investigation. However, previous research suggests MIT contains a sulfur moiety that reacts with available cysteines (Collier et al., 1990). Studies also suggest MIT thiol interactions have neurotoxic effects such as interruption of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) signaling in neurons, and that activation of ERK signaling can lead to higher levels of intracellular reactive oxygen species (ROS) resulting in cell death (He et al., 2006; Du et al., 2002). MIT effects can be prevented by the addition of thiol-containing antioxidants like glutathione (GSH) and *n*-acetyl cysteine (NAC) that occupy MIT and prevent it from interacting with available cysteines (Bruchhausen et al., 2003). The rescue of wound healing and regeneration in *Xenopus laevis* was demonstrated previously when GSH or NAC were added along with MIT (Delos Santos et al., 2016). We hypothesized that GSH and NAC would also rescue MIT-inhibited wound healing in planaria, which our results support (Fig. 4). Together, these data suggest MIT does inhibit wound healing via thiol interactions.

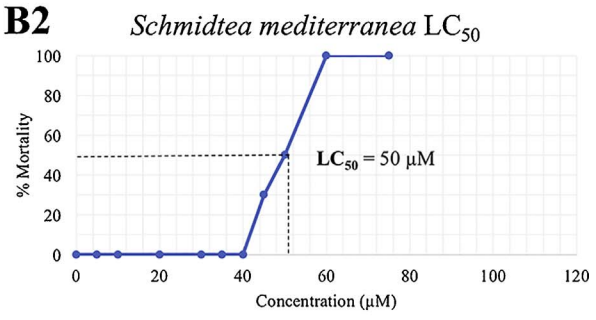
The molecular pathways that regulate this process are not well understood. We are not aware of any reported gene inhibition studies that have resulted in a loss of planarian wound closure. However, several genes have been identified that are activated by wounding.

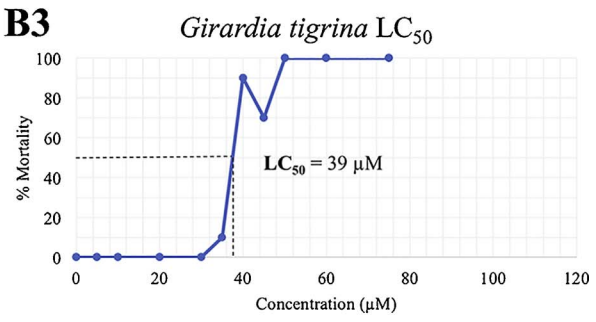


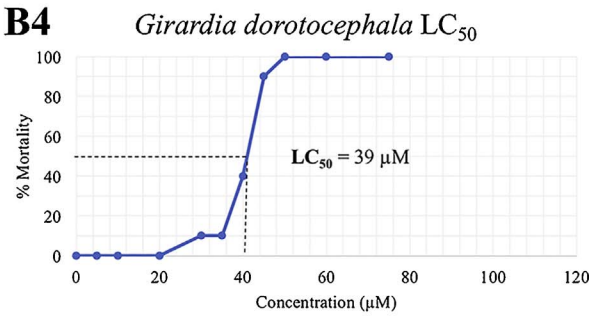












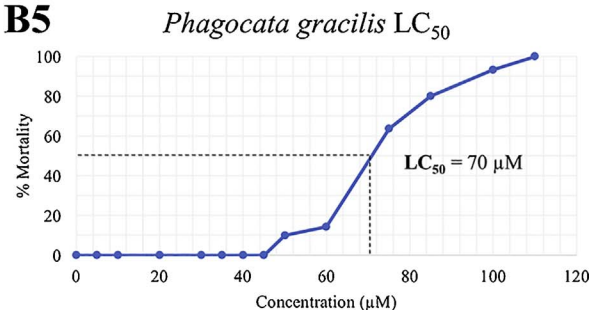


Fig. 5. MIT Toxicity Across Planarian Species.
(A and B) Images of the planarian species tested and corresponding LC_{50} graphs of MIT exposure at 6 h of exposure in intact animals. (A1, B1) *Dugesia japonica*, (A2, B2) *Schmidtea mediterranea*, (A3, B3) *Girardia tigrina*, (A4, B4) *Girardia dorotocephala* and (A5, B5) *Phagocata gracilis*. Anterior to the left, scale bars = 500 µm, n ≥ 10 for each concentration for all species. (C) Comparison of LC_{50} values at 6 h of exposure across species. (D) Phylogenetic scheme of species relationships (based on Alvarez-Presas et al., 2008).

Early planarian wound healing responses (~30 min) include the expression of *jun-1* and *fos-1* (Wenemoser et al., 2012), and ERK has been linked to activation of c-Fos (Monje et al., 2005). While it is possible that interrupted ERK signaling by MIT (as occurs in other systems) prevents c-Fos activation in planarians to inhibit early wound healing responses, inhibition of planarian ERK signaling alone does not result in a wound healing phenotype (Agata et al., 2014; Tasaki et al., 2011; Umesono et al., 2013). Similarly, apoptosis is upregulated at the injury site in response to wounding and is thought to play a role in planarian wound healing (Pellettieri et al., 2010). While MIT could possibly affect rates of apoptosis, inhibition of apoptosis alone also does not interfere with planarian wound closure (Beane et al., 2013). Interestingly, ROS (such as hydrogen peroxide) are known to oxidize thiols, and ROS signaling is linked to both normal wound healing as well as chronic wounds (Dunnill et al., 2017; Novo and Parola, 2008). However, while ROS have been shown to accumulate near the wound site after injury in planarians, again loss of ROS signaling does not lead to a loss of wound closure in planarians (Pirotte et al., 2015).

In this study, we showed MIT is toxic to a broad range of planarian species, causing neuromuscular impairments at sub-lethal concentrations and inhibiting normal wound healing and regeneration by interacting with available thiols. Many wound healing mechanisms are conserved across taxa (Bielefeld et al., 2013), and further studies should be done to establish potential risks to other aquatic species. Because the data suggest that even at low concentrations MIT acts quickly, the possible consequences of its detrimental effects on aquatic organisms should be considered in future investigations.

5. Conclusions

Consistent with previous studies in *Xenopus laevis*, we demonstrated that MIT inhibits planarian wound healing and regeneration. Furthermore, we showed that MIT is toxic to planaria at low concentrations and some of the sub-lethal effects include epithelial and neuromuscular impairments. We used thiol-containing antioxidants to verify the MIT mechanism of action is through free thiol interactions. Lastly, we determined by LC_{50} that planarian MIT toxicity levels correlate with the phylogenetic relationships of the species tested. These data suggest that MIT exposure may be a greater risk to aquatic organisms than previously thought, affecting both normal developmental and regenerative processes as well as injury responses. Future studies should investigate the extent to which other organisms may be impacted by MIT.

Author contributions

Conceived and designed the experiments: WSB, AT. Performed the experiments: AV, WSB. Analyzed the data: AV, WSB, AT. Contributed to writing the manuscript: AV, WSB, AT.

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References

Aerts, O., Goossens, A., Giordano-Labadie, F., 2015. Contact allergy caused by methylisothiazolinone: the Belgian-French experience. Eur. J. Dermatol. 25, 228–233.
Agata, K., Tasaki, J., Nakajima, E., Umesono, Y., 2014. Recent identification of an ERK signal gradient governing planarian regeneration. Zoology (Jena) 117, 161–162.
Alvarez-Presas, M., Baguna, J., Riutort, M., 2008. Molecular phylogeny of land and freshwater planarians (Tricladida: Platyhelminthes): from freshwater to land and back. Mol. Phylogenet. Evol. 47, 555–568.
Böttcher, I., Bellinghausen, I., König, B., Knop, J., Saloga, J., 2008. Different regulation of T helper 1- and T helper 2-promoting cytokine signalling factors in human dendritic cells after exposure to protein versus contact allergens. Immunology 123, 139–144.
Balestrini, L., Di Donfrancesco, A., Rossi, L., Marracci, S., Isolani, M.E., Bianucci, A.M., Batistoni, R., 2017. The natural compound sanguinarine perturbs the regenerative capabilities of planarians. Int. J. Dev. Biol. 61, 43–52.
Baranowska, I., Wojciechowska, I., 2013. The determination of preservatives in cosmetics and environmental waters by HPLC. Pol. J. Environ. Stud. 22, 1609.
Beane, W.S., Tseng, A.-S., Morokuma, J., Lemire, J.M., Levin, M., 2012. Inhibition of planar cell polarity extends neural growth during regeneration, homeostasis, and development. Stem Cells Dev. 21, 2085–2094.
Beane, W.S., Morokuma, J., Lemire, J.M., Levin, M., 2013. Bioelectric signaling regulates head and organ size during planarian regeneration. Development 140, 313–322.
Becker, D., Valk, E., Zahn, S., Brand, P., Knop, J., 2003. Coupling of contact sensitizers to thiol groups is a key event for the activation of monocytes and monocyte-derived dendritic cells. J. Invest. Dermatol. 120, 233–238.
Bester, K., Vollertsen, J., Bollmann, U.E., 2014. Water driven leaching of biocides from paints and renders. The Danish Environmental Protection Agency, Pesticide Research No. 156.
Bielefeld, K.A., Amini-Nik, S., Alman, B.A., 2013. Cutaneous wound healing: recruiting developmental pathways for regeneration. Cell. Mol. Life Sci. 2059–2081.
Bjorkner, B., Bruze, M., Dahlquist, I., Fregert, S., Gruvberger, B., Persson, K., 1986. Contact allergy to the preservative Kathon CG. Contact Dermat. 14, 85–90.
Bollmann, U.E., Tang, C., Eriksson, E., Jonsson, K., Vollertsen, J., Bester, K., 2014. Biocides in urban wastewater treatment plant influent at dry and wet weather: concentrations: mass flows and possible sources. Water Res. 60, 64–74.
Bruchhausen, S., Zahn, S., Valk, E., Knop, J., Becker, D., 2003. Thiol antioxidants block the activation of antigen-presenting cells by contact sensitizers. J. Invest. Dermatol. 121, 1039–1044.
Bruze, M., Dahlquist, I., Fregert, S., Gruvberger, B., Persson, K., 1987a. Contact allergy to the active ingredients of Kathon CG. Contact Dermat. 16, 183–188.
Bruze, M., Fregert, S., Gruvberger, B., Persson, K., 1987b. Contact allergy to the active ingredients of Kathon CG in the guinea pig. Acta Derm. Venereol. 67, 315–320.
Buttarelli, F.R., Pontieri, F.E., Margotta, V., Palladini, G., 2000. Acetylcholine/dopamine interaction in planaria. Comp. Biochem. Physiol. C: Pharmacol. Toxicol. Endocrinol. 125, 225–231.
Buttarelli, F.R., Pellicano, C., Pontieri, F.E., 2008. Neuropharmacology and behavior in planarians: translations to mammals. Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 147, 399–408.
Carbajo, J.B., Perdigon-Melon, J.A., Petre, A.L., Rosal, R., Leton, P., Garcia-Calvo, E., 2015. Personal care product preservatives: risk assessment and mixture toxicities with an industrial wastewater. Water Res. 72, 174–185.
Castanedo-Tardana, M.P., Zug, K.A., 2013. Methylisothiazolinone. Dermatitis 24, 2–6.
Collier, P.J., Ramsey, A., Waigh, R.D., Douglas, K.T., Austin, P., Gilbert, P., 1990. Chemical reactivity of some isothiazolone biocides. J. Appl. Bacteriol. 578–584.
De Groot, A.C., Liem, D.H., Weyland, J.W., 1985. Kathon CG: cosmetic allergy and patch test sensitization. Contact Dermat. 12, 76–80.
Delos Santos, N., Azmat, S., Cuenca, Y., Drenth, J., Lauper, J., Tseng, A.S., 2016. Effects of the biocide methylisothiazolinone on Xenopus laevis wound healing and tail regeneration. Aquat. Toxicol. 181, 37–45.
Du, S., Mclaughlin, B., Pal, S., Aizenman, E., 2002. In vitro neurotoxicity of methylisothiazolinone, a commonly used industrial and household biocide, proceeds via a zinc and extracellular signal-regulated kinase mitogen activated protein kinase-dependent pathway. J. Neurosci. 22, 7408–7416.
Dunnill, C., Patton, T., Brennan, J., Barrett, J., Dryden, M., Cooke, J., Leaper, D., Georgopoulos, N.T., 2017. Reactive oxygen species (ROS) and wound healing: the functional role of ROS and emerging ROS-modulating technologies for augmentation of the healing process. Int. Wound J. 14, 89–96.
Finney, D.J., 1952. Probit analysis (2nd ed). J. Inst. Actuaries 78 (3), 388–390.
Grebe, E., Schaeffer, D.J., 1991. Planarians in toxicology: standardization of a rapid neurobehavioral toxicity test using phenol in a crossover study. Bull. Environ. Contam. Toxicol. 46, 866–870.
Hagstrom, D., Cochet-Escartin, O., Zhang, S., Khuu, C., Collins, E.M., 2015. Freshwater planarians as an alternative animal model for neurotoxicology. Toxicol. Sci. 147, 270–285.
Hagstrom, D., Cochet-Escartin, O., Collins, E.M., 2016. Planarian brain regeneration as a model system for developmental neurotoxicology. Regeneration (Oxf.) 3, 65–77.
He, K., Huang, J., Lagenaur, C.F., Aizenman, E., 2006. Methylisothiazolinone, a neurotoxic biocide, disrupts the association of SRC family tyrosine kinases with focal adhesion kinase in developing cortical neurons. J. Pharmacol. Exp. Ther. 317, 1320–1329.
Ho, D.M., Whitman, M., 2008. TGF-β signaling is required for multiple processes during Xenopus tail regeneration. Dev. Biol. 315, 203–216.
Horvat, T., Kalafatic, M., Kopjar Kovacevic, N.G., 2005. Toxicity testing of herbicide norflurazon on an aquatic bioindicator species—the planarian Polycelis felina (Daly.). Aquat. Toxicol. 73, 342–352.
Leiva-Salinas, M., Francés, L., Silvestre, J., 2014. Actualización en La dermatitis de contacto Alérgica por metilcloroisotiazolinonametilisotiazolinona Y metilisotiazolinona. Actas Dermosifiliogr. 105, 840–846.
Lobo, D., Beane, W.S., Levin, M., 2012. Modeling planarian regeneration: a primer for reverse-engineering the worm. PLoS Comput. Biol. 8, 1002481.
Lundov, M.D., Krongaard, T., Menne, T.L., Johansen, J.D., 2011a. Methylisothiazolinone contact allergy: a review. Br. J. Dermatol. 165, 1178–1182.
Lundov, M.D., Zachariae, C., Johansen, J.D., 2011b. Methylisothiazolinone contact allergy and dose-response relationships. Contact Dermat. 64, 330–336.
Monje, P., Hernandez-Losa, J., Lyons, R.J., Castellone, M.D., Gutkind, J.S., 2005. Regulation of the transcriptional activity of c-Fos by ERK. A novel role for the prolyl isomerase PIN1. J. Biol. Chem. 280, 35081–35084.
National Library of Medicine, 2015. Methylisothiazolinone [Online]. Toxicology Data

Network. Available: https://toxnet.nlm.nih.gov/cgi-bin/sis/search/a?dbs+hsdb:@term+@DOCNO+8200.
Novo, E., Parola, M., 2008. Redox mechanisms in hepatic chronic wound healing and fibrogenesis. Fibro. Tissue Repair 1, 5.
Oviedo, N.J., Nicolas, C.L., Adams, D.S., Levin, M., 2008. Planarians: a versatile and powerful model system for molecular studies of regeneration, adult stem cell regulation, aging, and behavior. Cold Spring Harb. Protoc. 2008 pdb.emo101.
Pagán, O.R., Coudron, T., Kaneria, T., 2009. The flatworm planaria as a toxicology and behavioral pharmacology animal model in undergraduate research experiences. J. Undergrad. Neurosci. Educ. 7, 48–52.
Passarelli, F., Merante, A., Pontieri, F.E., Margotta, V., Venturini, G., Palladini, G., 1999. Opioid-dopamine interaction in planaria: a behavioral study. Comp. Biochem. Physiol. Part C: Pharmacol. Toxicol. Endocrinol. 124, 51–55.
Pellettieri, J., Fitzgerald, P., Watanabe, S., Mancuso, J., Green, D.R., Sanchez Alvarado, A., 2010. Cell death and tissue remodeling in planarian regeneration. Dev. Biol. 338 (1), 76–85.
Pirotte, N., Stevens, A.S., Fraguas, S., Plusquin, M., Van Roten, A., Van Belleghem, F., Paesen, R., Ameloot, M., Cebrià, F., Artois, T., Smeets, K., 2015. Reactive oxygen species in planarian regeneration: an upstream necessity for correct patterning and brain formation. Oxid. Med. Cell. Longevity 2015, 392476.
Prá, D., Lau, A.H., Knakievicz, T., Carneiro, F.R., Erdtmann, B., 2005. Environmental genotoxicity assessment of an urban stream using freshwater planarians. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 585, 79–85.
Raffa, R.B., Rawls, S.M., 2009. Planaria: a model for drug action and abuse. Landes Biosci.
Raffa, R.B., Valdez, J.M., 2001. Cocaine withdrawal in planaria. Eur. J. Pharmacol. 430, 143–145.
Rafoth, A., Gabriel, S., Sacher, F., Brauch, H.J., 2007. Analysis of isothiazolinones in environmental waters by gas chromatography–mass spectrometry. J. Chromatogr. A 1164, 74–81.
Rawls, S.M., Patil, T., Tallarida, C.S., Baron, S., Kim, M., Song, K., Ward, S., Raffa, R.B., 2011. Nicotine behavioral pharmacology: clues from planarians. Drug Alcohol Depend. 118, 274–279.
Reddien, P.W., Sanchez Alvarado, A., 2004. Fundamentals of planarian regeneration. Annu. Rev. Cell Dev. Biol. 20, 725–757.
Rink, J.C., 2013. Stem cell systems and regeneration in planaria. Dev. Genes Evol. 223, 67–84.
Sakaguchi, H., Miyazawa, M., Yoshida, Y., Ito, Y., Suzuki, H., 2007. Prediction of preservative sensitization potential using surface marker CD86 and/or CD54 expression on human cell line, THP-1. Arch. Dermatol. Res. 298, 427–437.
Sanchez Alvarado, A., Newmark, P.A., 1998. The use of planarians to dissect the molecular basis of metazoan regeneration. Wound Repair Regen. 6, 413–420.
Tasaki, J., Shibata, N., Nishimura, O., Itomi, K., Tabata, Y., Son, F., Suzuki, N., Araki, R., Abe, M., Agata, K., Umesono, Y., 2011. ERK signaling controls blastema cell differentiation during planarian regeneration. Development 138, 2417–2427.
Thume, I.S., Frizzo, M.E., 2017. Sertraline induces toxicity and behavioral alterations in planarians. Biomed. Res. Int. 2017, 5792621.
Thyssen, J.P., Sederberg-Olsen, N., Thomsen, J.F., Menné, T., 2006. Contact dermatitis from methylisothiazolinone in a paint factory. Contact Dermat. 54, 322–324.
Umesono, Y., Tasaki, J., Nishimura, Y., Hrouda, M., Kawaguchi, E., Yazawa, S., Nishimura, O., Hosoda, K., Inoue, T., Agata, K., 2013. The molecular logic for planarian regeneration along the anterior-posterior axis. Nature 500, 73–76.
Uter, W., Geier, J., Bauer, A., Schnuch, A., 2013. Risk factors associated with methylisothiazolinone contact sensitization. Contact Dermat. 69, 231–238.
Venturini, G., Stocchi, F., Margotta, V., Ruggieri, S., Bravi, D., Bellantuono, P., Palladini, G., 1989. A pharmacological study of dopaminergic receptors in planaria. Neuropharmacology 28, 1377–1382.
Wenemoser, D., Reddien, P.W., 2010. Planarian regeneration involves distinct stem cell responses to wounds and tissue absence. Dev. Biol. 344, 979–991.
Wenemoser, D., Lapan, S.W., Wilkinson, A.W., Bell, G.W., Reddien, P.W., 2012. A molecular wound response program associated with regeneration initiation in planarians. Genes Dev. 26, 988–1002.
Wilford, J.E., De Gannes, G.C., 2017. Methylisothiazolinone contact allergy prevalence in western Canada: increased detection with 2000 ppm patch test allergen. J. Cutan. Med. Surg. 21, 207–210.
Williams, T.M., 2007. The mechanism of action of isothiazolinone biocides. Power Plant Chem. 9, 14–22.
Wu, J.P., Li, M.H., Chen, J.S., Chung, S.Y., Lee, H.L., 2015. Disturbances to neurotransmitter levels and their metabolic enzyme activity in a freshwater planarian exposed to cadmium. Neurotoxicology 47, 72–81.
Yazar, K., Lundov, M.D., Faurschou, A., Matura, M., Boman, A., Johansen, J.D., Lidén, C., 2015. Methylisothiazolinone in rinse-off products causes allergic contact dermatitis: a repeated open-application study. Br. J. Dermatol. 173, 115–122.