Importance of Sperm Density in Assessing the Toxicity of Metals to the Fertilisation of Broadcast Spawners

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Abstract

Many marine invertebrates reproduce through broadcast spawning, where sperm and eggs are released into the water column and are vulnerable to toxicants present in the environment. The potential impacts of toxicants on spawning success are often assessed through laboratory-based fertilisation tests. In most cases, these tests assess toxicant impacts at a single, pre-defined sperm density, based on a sperm:egg ratio that ensures high fertilisation success (≥70-80%) in a filtered seawater control. Here we show that use of a single sperm density can considerably underestimate toxicity and that assessments over a range of sperm densities can provide more ecologically relevant, conservative and informative toxicity data. Fertilisation assays were run for the polychaete *Galeolaria caespitosa* exposed to four heavy metals (Cu, Zn, Pb, Cd) across multiple sperm densities. There was a three-fold difference in the toxicity of copper and zinc when calculated at sperm densities of 10^4 and 10^6 sperm mL^{-1}, both of which result in over 80% fertilisation in filtered seawater controls. By testing across multiple sperm densities, we identified that metals impact the sperm of *G. caespitosa* during the fertilisation process. Assessing toxicity across multiple sperm densities is not always practical. This is due to the increased effort required to complete full fertilisation curves, across enough concentrations of a toxicant, to establish a concentration-response relationship. In such cases, we provide recommendations for adopting aspects of fertilisation assays that will improve on standard spermiotoxicity tests but which are still manageable for routine toxicity testing.

1.0 Introduction

Trace concentrations of metals occur naturally in the marine environment, however, metals from anthropogenic sources can lead to elevated concentrations that can be toxic to marine organisms(Hudspith, Reichelt-Brushett and Harrison, 2017). Many fish, invertebrates and algae reproduce by broadcast spawning their eggs and sperm freely into the water column where unprotected reproductive cells are directly exposed to toxicants in the water. Consequently, fertilisation success is vulnerable to metal toxicity with potential consequences for the ongoing population dynamics of affected species(Marshall, 2006).

Fertilisation assays are commonly used to investigate the effects of metals on marine invertebrates. Most studies have found concentration-response relationships with reductions in fertilisation success associated with increased concentrations of metals (see the recent review by Hudspith et al.(Hudspith, Reichelt-Brushett and Harrison, 2017)). Nearly all the studies listed by Hudspith et al.(Hudspith, Reichelt-Brushett and Harrison, 2017) use a single sperm:egg ratio that ensures at least 70-80% fertilisation in filtered seawater (FSW) controls. There are previously noted limitations to running ecotoxicological experiments at a fixed sperm:egg ratio(Marshall, 2006). First, in any natural spawning event, the density of sperm is not constant spatially or temporally and so it may be difficult to extrapolate results from laboratory experiments to field conditions(Levitan, Sewell and Fu-Shiang Chia, 1991). Second, metals can impact different aspects of the fertilisation process: for example, by reducing the number of viable gametes, decreasing sperm motility or chemotaxis(Morisawa and Mohri, 1972; Fitzpatrick *et al.*, 2008), disrupting sperm-egg binding processes(Zhang *et al.*, 2010), or affecting the efficiency of polyspermy blocks(Franchet, Goudeau and Goudeau, 1997). However, at a single sperm:egg ratio, all of these effects may present identically as a reduction in fertilisation relative to the

control(Marshall, 2006) and so while an effect of the toxicant might be detected, the mechanisms involved will likely remain unclear. Third, the effects of a toxicant can be greater at low sperm densities than at high densities, so the use of just a single sperm density may underestimate toxicity(Dinnel, Link and Stober, 1987). Finally, the use of different sperm:egg ratios for each species complicates the comparison of toxicity data among different studies and species(Marshall, 2006).

Marshall(Marshall, 2006) suggested moving away from toxicity testing at single sperm:egg ratios to using fertilisation assays that assess the impact of toxicants across a series of sperm densities. Essentially, his (theoretical) suggestion was to characterise fertilisation curves and use parameters derived from these to assess concentration-response relationships. Using a range of sperm:egg ratios can provide additional ecological information that can aid in informing environmental risk assessment and management. Schlegel et al.(Schlegel *et al.*, 2012) adopted this approach to identify the effects of ocean acidification on the fertilisation success of the Australasian sea urchin *Heliocidaris erythrogramma*. To date, however, these approaches have not yet been adopted in routine ecotoxicological testing. A good reason for this might simply be that the extra work involved in running toxicity tests across an extra dimension of sperm:egg ratios is too labour intensive.

Ecotoxicological testing of metals using fertilisation as an endpoint has been extensively researched in Cnidarians and Echinoderms (Hudspith, Reichelt-Brushett and Harrison, 2017). However, only one polychaete species (*Hydroides elegans*) has been tested for the effects of Zn, Cd and Pb to fertilisation success. With a view of expanding our knowledge on the effects of metals to fertilisation in polychaetes, the intertidal serpulid, *Galeolaria caespitosa*

presents as a model organism for ecotoxicological testing. *G. caespitosa* are easily collected and amenable to laboratory holding, have abdomens swollen with gametes throughout the year (Kupriyanova, 2006) and release large amounts of eggs or sperm when their tubes have been broken or have been disturbed mechanically (Kupriyanova and Havenhand, 2002). *G. caespitosa* is commonly used in fertilisation biology (Kupriyanova, 2006), has previously been used in ecotoxicological studies (Ross and Bidwell, 2001; Cam F Hollows, Johnston and Marshall, 2007; Lu, Lin and Aitken, 2017) and was recently incorporated into the Australian and New Zealand Environment Conservation Council (ANZECC) water quality guideline for copper (Gadd and Hickey, 2016). Like other serpulids commonly used for toxicity tests, such as *H. elegans* (Gopalakrishnan, Thilagam and Raja, 2008) and *Pomatocerous spp* (Khandeparker, Desai and Shirayama, 2005), *G. caespitosa* reproduce via broadcast spawning (Kupriyanova, 2006) and play an important role in marine communities (Gosselin and Sewell, 2013). Serpulids provide structural complexity and microhabitats that increase diversity of other marine organisms(Haines and Maurer, 1980) and are filter feeders that link the pelagic and benthic food chain (Gosselin and Sewell, 2013).

The aim of this study was to outline and assess the value of incorporating multiple egg:sperm ratios into standardised ecotoxicological testing using fertilisation assays. The objectives of this study were; 1.) to assess the toxicity of four metals (Cu, Zn, Pb and Cd) on fertilisation in the intertidal serpulid, *G.caespitosa* across sperm densities of $10^1 – 10^6$ sperm mL^{-1} and 2.) to compare these results with traditional endpoints based on single sperm densities and 3.) to provide metal toxicity data for *G.caespitosa*.

2.0 Materials and Methods

2.1 Site

Test species and seawater (used as FSW controls and diluent in tests) were collected from the jetty pilings at Grange Beach, South Australia (-34.9026S, 138.4875E). Planned dredging activity in Port Adelaide(Minister for Planning, 2018) has the potential to increase dissolved sea water concentrations of metals and effect the water quality of local Adelaide beaches(Eggleton and Thomas, 2004) and local regulators have identified elevated metal concentrations as a key water quality issue for the area(Environment Protection Authority, 2008). Grange beach is already impacted by metals(Gaylard, 2004) and the potential impact of further metal increases on local species is unknown. The work we present may be used as part of the baseline data required to help inform risk assessments of metal increases in the area.

2.2 Study Species

Galeolaria caespitosa were held in the laboratory for no more than five days. Aggregations of *G. caespitosa* were broken apart and the individuals were carefully removed from their tubes with fine forceps. Forceps were rinsed in reverse osmosis (RO) water after any contact with an animal. Reproductively mature *G. caespitosa* immediately release gametes when their tubes have been broken or have been disturbed mechanically(Kupriyanova and Havenhand, 2002). Extracted worms were rinsed in 0.45-µm filtered seawater (FSW) and placed in individual containers with 0.5 mL of FSW to encourage sperm release. A pipette was used to collect the spawned gametes which were used for experiments within 15 minutes of collection.

2.3 Experimental Conditions

Seawater was filtered (0.45 µm) and refrigerated. All experiments were conducted at a constant room temperature of 20°C to minimise the effects of temperature on fertilisation success and gamete aging(Kupriyanova and Havenhand, 2005). Test salinity was kept at 35±2 ppt, mimicking salinity at Grange Beach. Light quality and intensity were at ambient laboratory levels. The pH of all test solutions was adjusted to 8.0±0.1 using sodium hydroxide (AR grade, Chem-Supply) and nitric acid (69%, Merck).

2.4 Experimental Design

Assessing the effects of a toxicant across a range of sperm densities and across enough concentrations of the toxicant to obtain an accurate concentration-response relationship would have required gametes from many worms at one time and, logistically, would have been almost impossible to do simultaneously (~100 sperm density x metal concentration combinations), or before gametes began to age to the point where their viability was reduced(Kupriyanova and Havenhand, 2005). Consequently, we used a paired design, measuring the response ratio between a treatment (FSW with added metal) and a matched control (FSW). We used a common batch of gametes for a pair of treatment and control fertilisation assays but tested each toxicant concentration (and paired control) with a new, independent batch of gametes. Thus, each metal treatment (and paired control) was run as a separate experiment, with a metal requiring about a week to run experiments for seven to eight treatment levels.

2.5 Treatment Preparation and Analysis

Metal stock solutions were prepared using Analar grade metal salts of $CuSO_4$, $ZnCl_2$, $CdCl_2$, and $Pb(NO_3)_2$ (99% purity, Sigma-Aldrich©) and Milli-Q water (18.2 MΩ cm^{-1}; Millipore).

Glassware was washed prior to use in 10% v/v nitric acid (69%, Merck). Test solutions for each toxicant were prepared on the day of the experiment from refrigerated stock solutions and FSW, no more than one hour prior to test commencement. An adaptive hierarchical approach was taken to determine nominal metal concentrations for each treatment, whereby the results of one paired test (control and one treatment) informed the test concentration chosen for subsequent tests. At the end of each test, sub-samples were collected from each treatment and control, filtered through acid-washed (10% HNO_3) 0.45-µm filters, and acidified to 0.2% HNO_3 (69%, Merck) for dissolved metals analysis. Metal analyses were carried out using inductively coupled plasma atomic emission spectrometry (ICP-AES; Agilent 720) by CSIRO, Lucas Heights, NSW.

2.6 Laboratory Fertilisation Assays

For each experiment, sperm was collected from five to ten males, pooled, and diluted in test solutions via a threefold serial dilution. Similarly, eggs were collected from 5 to 10 females and pooled. Gametes were pooled to minimise the effect of gamete-specific combining abilities(Kupriyanova and Havenhand, 2002). Each metal was tested for toxicity using eleven different sperm densities in fertilisation tests ranging from 10 to 5 x 10^6 sperm mL^{-1} (See Supplementary Information for sperm:egg ratios per treatment). Sperm densities were verified using a haemocytometer at 400x magnification. Sperm were exposed to test solutions for 30 minutes prior to the addition of eggs. The density of eggs was adjusted to 500 eggs mL^{-1}. Eggs were added to sperm in control or treatment solutions to allow development to occur(Cam F. Hollows, Johnston and Marshall, 2007). Development was ceased after 2.5 h, by fixing each sample with formaldehyde solution (4%, Merck). An egg control was also set up alongside each experiment to check for errant fertilisations due to accidental contamination with sperm, i.e. eggs were added to FSW without sperm under the

same exposure conditions as the FSW control. Eggs were classed as fertilised if they had begun to undergo cell division(Marshall and Evans, 2005). Each experiment consisted of a FSW control, one test concentration and one egg control. Data were not used if maximum fertilisation in the FSW control was <80%, or if the egg control showed >5% fertilisation.

2.7 Fertilisation Models

Fertilisation relationships, based on a theoretical model(Styan, Kupriyanova and Havenhand, 2008) of the distribution of sperm-egg interactions using gamete concentrations and characteristics were fitted to the measured fertilisation data for each paired treatment and control. Key model parameters, fertilisation efficiency (Fe) and polyspermy block efficiency (Be), were estimated using least squares (Styan and Butler, 2000). Average sperm swimming speed and egg diameter parameters for *G. caespitosa* were those reported by Kupriyanova(Kupriyanova, 2006).

Using the fitted fertilisation models, we calculated fertilisation success at 10^4, 10^5 and 10^6 sperm mL^{-1} for each control and treatment. The percentage fertilisation in each treatment relative to fertilisation in the respective control at these sperm densities was calculated for each concentration of each metal and used to fit concentration-response relationships.

A range of endpoints were defined to characterise different aspects of the fertilisation models. These were derived for each concentration of metal and then used to characterise concentration-response relationships (See Supplementary Information). We first estimated the maximum modelled fertilisation success (Fmax) and the sperm density that maximised fertilisation success ([Sperm]max) in each of the FSW controls. In the paired treatment fertilisation success at [Sperm]max was then calculated (F_T@[Sperm]$max_{control}$). We also estimated [Sperm]50 which was calculated as the sperm density required to achieve 50% of

the maximum fertilisation (F50) in the control and the respective fertilisation success in the paired treatment at the same sperm density (F_T@[Sperm]$50_{control}$). The density of sperm in the treatment required to obtain the same level of fertilisation as the $F50_{control}$ was also calculated ([Sperm]$50_{treatment}$) As well as the modelled values, comparisons were made between the best observed fertilisation in the control ($BestF_{control}$) assay and the observed fertilisation in the treatment ($ObservedF_{treatment}$) at the sperm density ([Sperm]$Best_{control}$) that achieved $BestF_{control}$ (see Supplementary Information for a step by step guide to calculating each value).

2.8 Statistics

The R package DRC (Ritz and Strebig, 2005) was used to model the test data for each endpoint and calculate toxicity estimates. Regression models tested included logistic, log-logistic and Weibull models with different levels of parametrization. Model comparisons were conducted using the Akaike Information Criterion (AIC) and models that best described the data were applied to determine metal concentrations that elicited a 50% (EC50) and 10% (EC10) decrease in fertilisation success (% control). The associated 95% confidence limits were estimated using the delta method. A ratio test was used to compare EC50 values through the DRC function [EDcomp()] and statistical differences were determined using the method described by Sprague and Fogel (1976)(Sprague and Fogels, 1976).

3.0 Results

Thirty experiments passed the acceptability criteria of >80% fertilisation in the FSW control and ≤5% fertilised eggs in the no sperm controls and were used to determine toxicity endpoints. There were eight unsuccessful experiments, seven of which did not exceed 80% fertilisation and one where the no sperm controls had greater than 5% fertilised eggs. These data were not used. For Zn and Cu, concentrations were tested in eight separate paired experiments, while for Pb and Cd, concentrations were tested in seven paired experiments. The concentrations of metals in the FSW controls, some of which were above Australian and New Zealand guideline values (GVs)(ANZECC, 2000), were typical of those expected for Grange Beach(Gaylard, 2004) and nearby beaches(Chakraborty and Owens, 2014). In toxicity tests with Cu, Pb, Cd and Zn, FSW controls contained 1-2 µg Cu/L, 16-66 µg Zn/L <4-23 µg Pb/L and <4-16 µg Cd/L (Table S1). Grange Beach water was considered to be of poor quality in 2004, based on exceedances of the GVs for aluminium and zinc(Gaylard, 2004). Therefore, it is likely that the community of worms sampled in this study had undergone long-term exposure to most of these contaminants over several generations, and may well yield different sensitivities to metals than worms collected from pristine environments.

3.1 Fertilisation-Sperm Density Relationships

The relationship between fertilisation success and sperm density varied considerably between tests (different batches of sperm). Therefore, each treatment was normalised to the respective control. Figure 1 shows the eight paired fertilisation assays for copper, illustrating the changes in the fertilisation-sperm density relationship with increasing concentrations of copper (relationships for the three other metals are in the Supplementary Information). Across FSW controls in all of the experiments, the observed maximum

fertilisation (80-98%) occurred at sperm densities ranging between 10^4 and 10^6 sperm mL^{-1} (60:1 to 6000:1, Sperm:Egg). At sperm densities below this, fertilisation success (%) decreased with decreasing sperm density. At higher sperm densities fertilisation success either plateaued or started to decrease slowly with increasing sperm density. However, across paired assays and experiments (i.e. among different crosses), there was considerable variability among FSW controls in the relationships between fertilisation success and sperm density and at a given sperm density fertilisation success could vary by over 50% (Figure 1). This is likely due to variability in the fertilisation success of different batches of gametes.

The presence of dissolved metals affected the relationship between fertilisation success and sperm density. There was a right shift in fertilisation curves of treatments relative to their paired control, i.e. [Sperm]50 increased (Figure 1, Supplementary Information). At the highest concentration tested for each metal there was an increase in [Sperm]50 of over four orders of magnitude, such that the slope of the curve extends beyond the sperm densities tested in this study. At low to moderate sperm densities, fertilisation was lower in treatments than in the respective FSW controls. Therefore, more sperm were required in metal treatments than in FSW controls to achieve the same level of fertilisation success. In most cases, fitted maximum fertilisation was slightly less for metal treatments than the FSW controls.

Although in most gamete crosses there were moderate decreases in fertilisation success at high densities of sperm, the presence of metals did not appear to change this much. Where there were consistent decreases in fertilisation at higher sperm densities, these appeared to occur in both the treatment and control assays (e.g. Copper 11 µg L^{-1} assay; Figure 1).

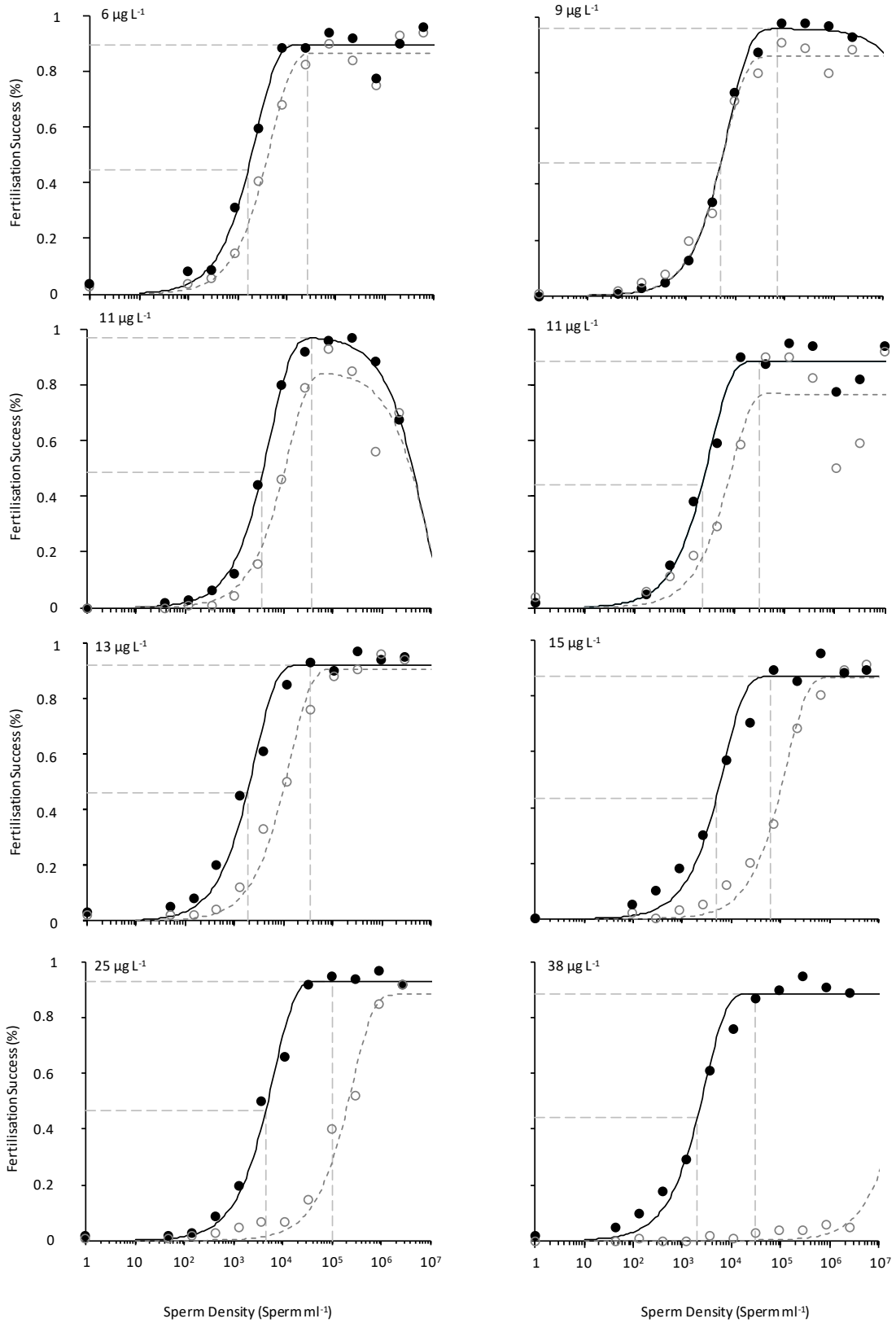

Figure 1*:* Fertilisation curves for *Galeolaria caespitosa* sperm exposed to copper prior to fertilisation. FSW controls are represented by the solid line and treatments by the dashed line. Measured dissolved (<0.45 µm) copper concentration is reported for each treatment in the upper left of the graph.

3.2 Fertilisation success at specific sperm densities

Based on the fitted fertilisation models to calculate fertilisation success at a given sperm density (10^4, 10^5 or 10^6 sperm mL^{-1}), the concentration-response relationships all showed a decrease in fertilisation with increasing metal concentration (Figure 2). Resulting EC50 values for each metal, measured between 10^4 and 10^6 sperm mL^{-1}, ranged from 12 to 33 µg Cu L^{-1}, from 160 to 550 µg ZnL^{-1}, from 560 to 1500 µg Pb L^{-1} and from 4900 to 6100 µg Cd L^{-1} (Table 1). Resulting EC10 values for each metal, measured between 10^4 and 10^6 sperm mL^{-1}, ranged from 8.2 to 27 µg Cu L^{-1}, from 68 to 200 µg Zn L^{-1}, from 65 to 910 µg Pb L^{-1} and from 3900 to 4200 µg Cd L^{-1} (Table 1).

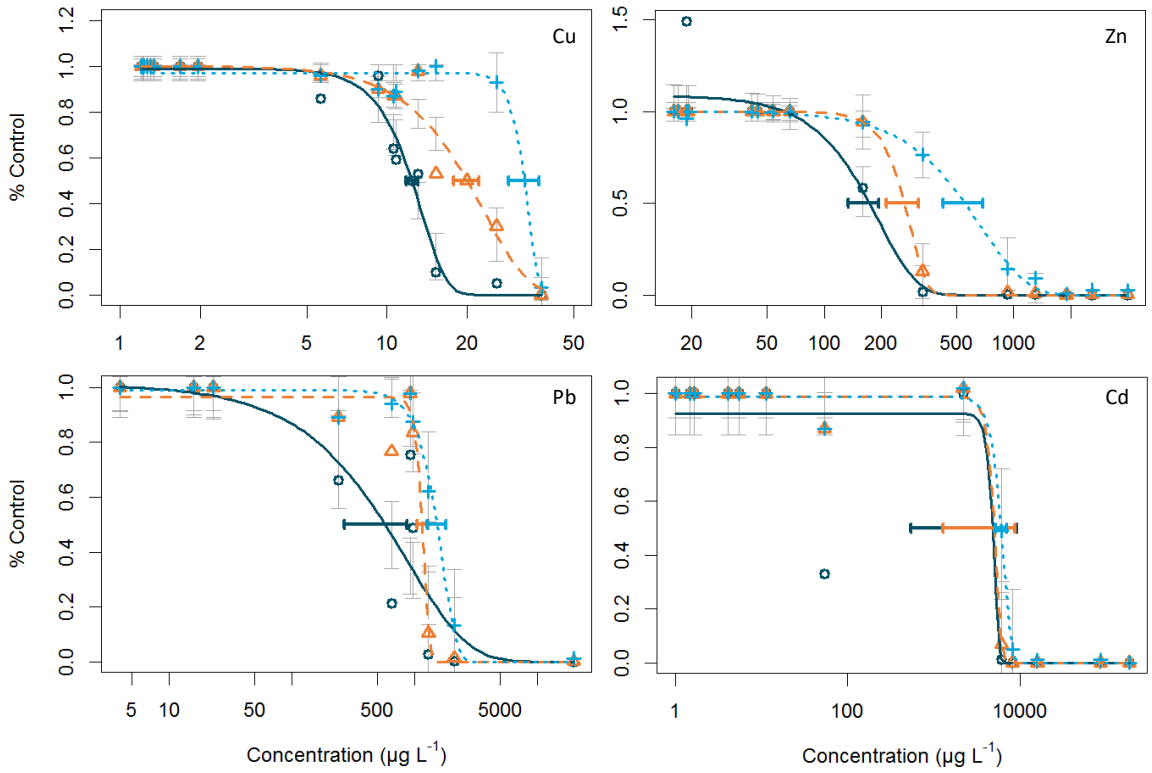

Figure 2: Concentration-response relationships for exposing *Galeolaria caespitosa* sperm to; a.)Cu, b.)Zn, C.)Pb and d.)Cd prior to and during fertilisation. Relationships were fitted at three sperm densities: 10^4 (black, solid), 10^5 (orange, dashed) and 10^6 (blue, dotted) sperm

mL^{-1}. EC50 values were calculated for each sperm density (solid horizontal lines) with associated confidence intervals. Error bars in the vertical direction represent confidence in the model.

Table 1: EC10, EC50 values and associated 95% confidence limits calculated based on fertilisation success at three standard sperm concentrations for *Galeolaria caespitosa* (2SF)[a]

	Sperm Density (Sperm ml^{-1})	**EC10 ($\mu g\ L^{-1}$)**	**EC50 ($\mu g\ L^{-1}$)**
Cu	10^4	8.2 (7.0-9.4)	12 (12-13)
	10^5	9.6 (7.4-11)	20 (18-22)
	10^6	27 (21-34)	33 (29-37)
Zn	10^4	68 (31-110)	160 (130-190)
	10^5	180 (94-270)	260 (210-320)
	10^6	200 (85-310)	550 (420-680)
Pb	10^4	65 (0-180)	560 (270-860)
	10^5	980 (770-1,200)	1,200 (1,000-1,300)
	10^6	910 (620-1,200)	1,500 (1,300-1,800)
Cd	10^4	3900 (0-10,000)	4900 (540-9,300)
	10^5	3900 (3,000-11,000)	5100 (1,300-8,900)
	10^6	4200 (2100-6300)	6100 (5,300-7,000)

[a] Measured dissolved (<0.45 µm) values.

Table 2: Statistical comparison of EC50 values at different sperm densities for *Galeolaria caespitosa*

	Sperm density	EC50 ratio	Magnitude	Significance
Cu	$10^4 : 10^6$	0.38	2.6	$p < 0.05$*
	$10^5 : 10^6$	0.61	1.6	$p < 0.05$*
Zn	$10^4 : 10^6$	0.30	3.4	$p < 0.05$*
	$10^5 : 10^6$	0.48	2.1	$p < 0.05$*
Pb	$10^4 : 10^6$	0.37	2.7	$p < 0.05$*
	$10^5 : 10^6$	0.76	1.3	$p < 0.05$*
Cd	$10^4 : 10^6$	0.80	1.3	$p > 0.05$
	$10^5 : 10^6$	0.83	1.2	$p > 0.05$

*Significant difference.

For Cu, Zn and Pb, there were significant decreases in toxicity with increase in sperm density evidenced by greater EC50 and EC10 values at higher sperm densities (Table 1,Table 2). For Cd, however, there was no significant difference between the toxicity values derived at each sperm density. Zinc toxicity was most impacted by sperm density with a three-fold increase in EC50 between 10^4 and 10^6 sperm mL^{-1} (Table 2). Of the four metals, copper was most toxic to *G. caespitosa* fertilisation with the lowest EC50 (12-33 µg/L) and EC10 values (8.2-27 µg/L) when tested with sperm densities of 10^4 to 10^6 sperm mL^{-1}. The final ranking of metal toxicity for all three sperm densities tested was Cu>Zn>Pb>Cd.

3.3 Fertilisation model endpoints

To identify which of the fertilisation model endpoints would be most useful to assess toxicity, the fitted fertilisation model parameters Fmax, F50, [Sperm]50 and Observedmax were used to construct concentration-response relationships for all metals (Figure 3). For Cu, Zn and Pb, F50 and Sperm[50] were the most sensitive endpoints, resulting in the lowest EC10 and EC50 values (Table 3). For Cd, there was no significant difference between endpoints and a wide range in confidence intervals within the slope of the response curves (Figure 3d).

Effect concentrations (10 and 50%) calculated using [Sperm]50 and F50 were consistently lower than those calculated for standard fertilisation success at 10^6 sperm mL^{-1}.

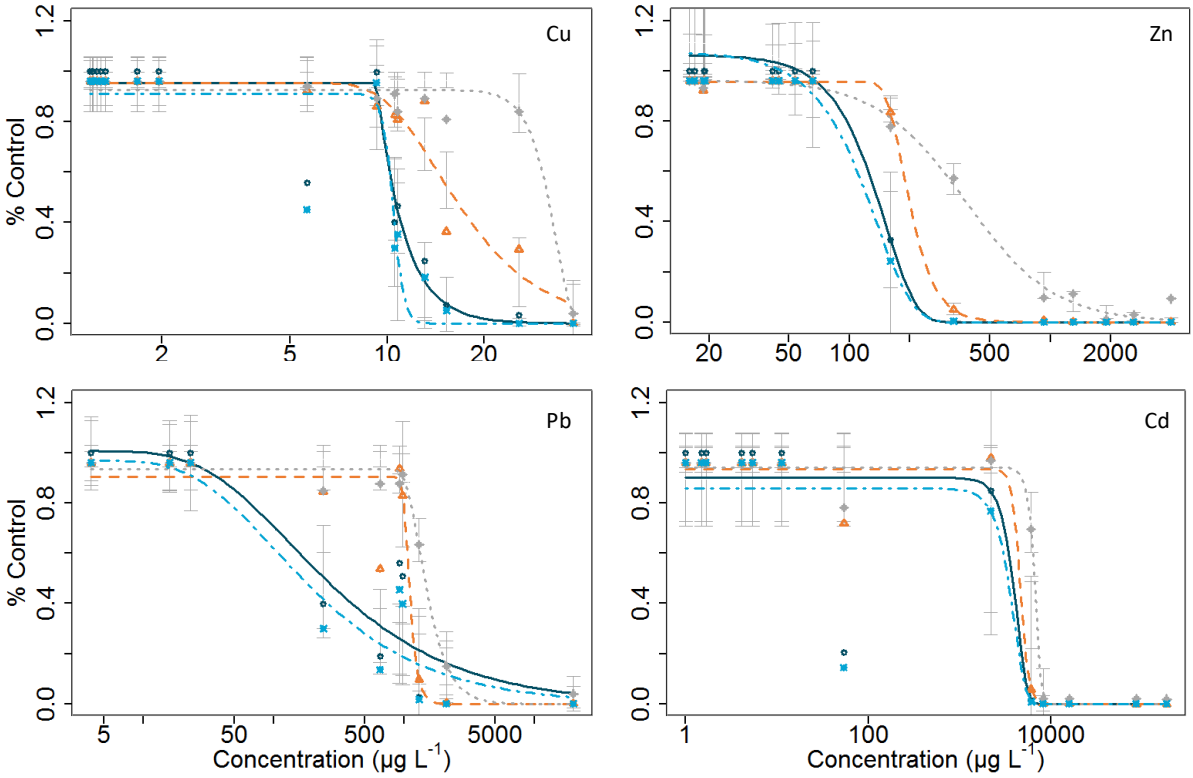

Figure 3: Concentration-response relationships for: a) Cu, b) Zn, c), Pb, and d) Cd. Curves were calculated using four different endpoints; F50 (navy, circles, solid), Fmax (orange, triangles, dashed), [Sperm]50 (Blue, "X", dash-dot) and Observedmax (grey, plus, dotted). Shaded regions show 95% confidence intervals of the model.

Table 3: EC10, EC50 values and associated 95% confidence limits calculated using fertilisation model endpoints for *Galeolaria caespitosa* (2SF).

	Endpoint	EC10 (µg L^{-1})	EC50 (µg L^{-1})
Cu	[Sperm]50	4.5 (0-13)	9.8 (5.5-14)
	F50	9.5 (8.9-10)	11 (10-11)
	Fmax	10 (8.4-12)	17 (14-19)
	Observedmax	26 (22-30)	32 (29-35)
Zn	[Sperm]50	54 (0-110)	120 (70-160)
	F50	68 (18-120)	130 (103-160)
	Fmax	160 (150-160)	200 (190-200)
	Observedmax	120 (90-160)	380 (330-430)
Pb	[Sperm]50	30 (0-75)	180 (22-330)
	F50	36 (0-100)	240 (0-490)
	Fmax	980 (850-1,100)	1,100 (940-1,300)
	Observedmax	1070 (950-1,200)	1,500 (1,400-1,600)
Cd	[Sperm]50	2200 (1,600-6,000)	3,700 (0-9,900)
	F50	2500 (4,000-7,800)	4,000 (0-9800)
	Fmax	3700 (4,600-9,000)	4,600 (880-8,400)
	Observedmax	5400 (4,800-6,100)	6,800 (6,300-7,300)

4.0 Discussion

4.1 Single sperm densities

The toxicity of Cu, Zn and Pb to fertilisation success in *G. caespitosa* was dependent on sperm density. We found significant differences in EC50 values calculated at fixed sperm densities of 10^4, 10^5 and 10^6 sperm mL^{-1}, with up to threefold differences in toxicity between estimates at 10^4 and 10^6 sperm mL^{-1}. Although similar trends were observed for cadmium, there was no statistical difference in EC values with sperm density. These findings are consistent with those of Hollows et al. (Cam F Hollows, Johnston and Marshall, 2007) who found that the effects of copper to *G. caespitosa* sperm were sperm-density dependent, with stronger effects at low sperm densities, than at high sperm densities (Cam F. Hollows, Johnston and Marshall, 2007). Work on other species, has also found that the sensitivity of sperm bioassays decreases as sperm:egg ratios increase (Dinnel, Link and Stober, 1987).

Fertilisation tests using single sperm densities may underestimate toxicity. While some test protocols expose invertebrate sperm at low sperm densities(Simon and Laginestra, 1997; Williams, Bentley and Hardege, 1997), the majority involve exposing sperm at densities as high as 10^6 sperm mL^{-1} (USEPA, 1995). Most of the studies listed in the review by Hudspith et al.(Hudspith, Reichelt-Brushett and Harrison, 2017) have used single sperm densities of between ~10^5 and ~10^6 sperm mL^{-1}. These sperm densities are used as they would likely generate greater than 70-80% fertilisation success in FSW controls for a given species. Had we only conducted our experiments at 10^6 sperm mL^{-1}, we would have underestimated the potency of Cu, Zn and Pb to fertilisation success relative to experiments run at 10^5sperm ml^{-1}. Both experiments would have generated >70% fertilisation in FSW controls, but the

measured toxicity (EC50 or EC10) of metals (Cu, Zn, Pb) would have been significantly lower at 10^5 sperm mL^{-1} ($p < 0.05$; Table 2). Interestingly, this would not have been the case for Cd as it has a much steeper slope in concentration-response than the other metals. This suggests that the sperm can tolerate Cd until a threshold concentration, after which the potency of Cd is great and has an almost absolute toxic effect to sperm. The data (figure 2) suggest that this effect is irrespective of sperm density. The mechanisms underlying this phenomenon are unclear and require further investigation.

Natural spawning sperm densities for most marine invertebrates, including *G. caespitosa* are unknown, and are likely to vary due to a range of individual, demographic, species-specific and environmental factors (Levitan, 1998; Havenhand and Styan, 2010; Crimaldi and Zimmer, 2014) and, as such, the use of a single standardised sperm density for toxicity testing may not be suitable. Thus, if spermiotoxicity tests are to be run at a single sperm density, as most are(Hudspith, Reichelt-Brushett and Harrison, 2017), then care is needed in determining what that density should be, as the toxicity results might be conditional on this. Consequently, metrics that are independent of sperm density should also be considered for routine toxicity testing.

4.2 Multiple sperm densities

Fertilisation assays across multiple sperm densities can provide information about the mechanism of toxicant impacts on fertilisation across a range of toxicant concentrations. When we examined the toxicity of metals across a range of sperm densities, we observed a shift in the fertilisation relationships to the right relative to FSW controls (see Figure 1 and Supplementary Information). This suggests that metals impact the fertilisation process mainly through effects on sperm. This effect was consistent across the range of

cocnentrations of all metals. Figure 4 represents how the relationship between fertilisation success and sperm density should change when a toxicant impacts different aspects of the fertilisation process. An impact to sperm viability will reduce the sperm-egg encounter rate, with the effect resulting in a horizontal (right) shift in the fertilisation relationship. In contrast, an impact on eggs or early developing zygotes would result in a decrease in the maximum number of eggs that are able to be fertilised (Figure 4e). When toxicants disrupt polyspermy blocks, there will not be much of a difference between treatment and control at low sperm densities but an increasing difference at higher sperm densities. In such instances, maximum fertilisation in the treatment would be lower and would occur at a lower sperm density than the control (Figure 4f). Whilst there were slight decreases in modelled maximum fertilisation success in some metal treatments, no clear effects on eggs or polyspermy were detected.

Had we chosen to test only one sperm density, while we may have been able to detect an impact on fertilisation (at that sperm density; Figure 4a, b ,c), we would not have been unable to determine which part of the fertilisation process was affected. Thus, although the fertilisation assays here involved more effort, we believe our work is a good example of the potential value of this added complexity in ecotoxicology assessments where fertilisation is measured as an endpoint.

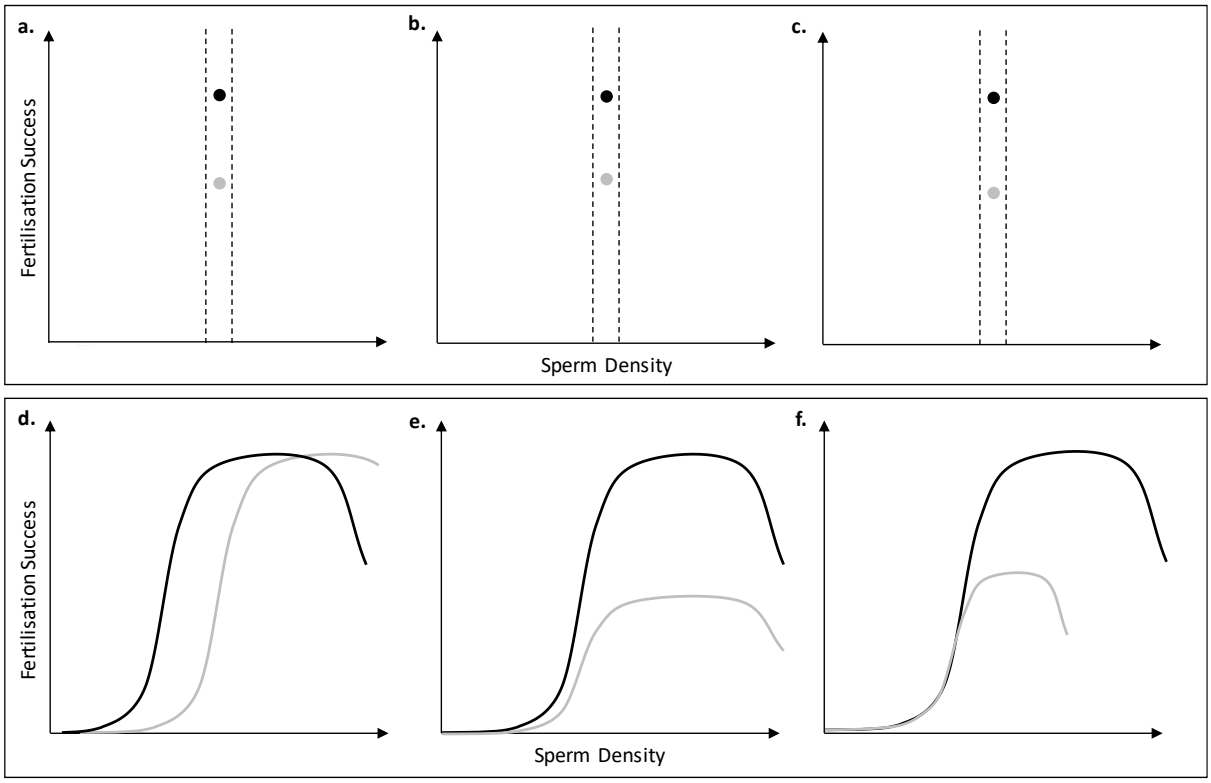

Figure 4: The potential effects of a toxicant on fertilisation success: a,b,c) show the impact of a toxicant to sperm, eggs and the efficiency of polyspermy blocks (respectively) when only testing at a fixed sperm density; d,e,f) show the same effects but across a range of sperm densities. Adapted from Marshall(Marshall, 2006).

4.3 Fertilisation endpoints

Fertilisation endpoints that are independent of sperm density provided sensitive measures of toxicity. There was a consistent ordering of how sensitive each endpoint appeared across the four metals; [Sperm]50, F50 and then Fmax, followed by Observedmax. The first three endpoints were all significantly lower ($p < 0.05$) than EC50 values calculated at 10^6 sperm mL^{-1}. The most sensitive were [Sperm]50 and F50, with no significant difference between EC50s using these endpoints. The mode of toxicity of metals on fertilisation (affecting sperm viability) is likely the key factor in determining the relative order of endpoints here. Had metals impacted egg viability we would expect that Fmax (and possibly Observedmax) might

then be more sensitive measures of toxicity (see Figure 4). Thus, information regarding the mode of toxicity is required to pick an appropriate endpoint.

4.4 Practicalities for routine toxicity testing

Our work illustrates that obtaining toxicity data using fertilisation assays can give a more complete and ecologically relevant assessment of toxicity than standard spermiotoxicity tests. However, we recognise there may be situations in which this approach may be impractical in routine ecotoxicological testing, because of the time or costs involved, or because regulatory standards refer to simpler, standardised methodologies (Dinnel, Link and Stober, 1987; USEPA, 1995; Simon and Laginestra, 1997). Nonetheless, we believe there are two modifications to standard spermiotoxicity testing that could be made based on our work.

First, we recommend that during a pre-test to determine an appropriate sperm density (sperm:egg ratio), one moderate level of the toxicant should be tested simultaneously, to determine something about the aspects of fertilisation that are likely to be affected. Similar to Lewis et al.(Lewis, Pook and Galloway, 2008) who exposed *Neries virens* sperm to water-accommodated fractions of crude oil across sperm densities ranging from 10^3 to 10^8 sperm mL^{-1} during a pre-test when determining their optimum sperm concentration for subsequent toxicity tests. Provided the concentration of the toxicant chosen provides some discernible impact on fertilisation (but not so much as to prevent it altogether), this should help to identify the mode of toxicity, which in turn might inform the most sensitive sperm density to establish concentration-response data from.

Our second recommendation then is to use information about the likely mode of impact of the toxicant on fertilisation, either from a pre-test, as above, or from previous work, to help

set what the target fertilisation rate should be in FSW controls and thus the sperm density that will be used during the main spermiotoxicity test. Where there is an indication that the toxicant affects sperm viability, as illustrated in our results, a more sensitive test would be obtained by reducing the sperm density to one that gives 50% (or lower) in FSW controls. Alternatively, if the main impact appears to be on egg/larval viability, then a (greater) sperm density more likely to achieve near 100% fertilisation in FSW controls would maximise the precision with which decreases in fertilisation can be measured; and if toxicants appear to affect polyspermy blocks, then a greater sperm density again will be needed to measure effects. Finally, if more than one of these effects are expected then a fertilisation assay approach will be needed, like the approach we have outlined in this paper.

4.5 *Galeolaria caespitosa*

As an ecotoxicological test species, *G. caespitosa* are almost ideal to work with and possess a range of attributes that enabled us to attempt more complex experimental assessments than might usually be done in assessments of the impacts of metals on fertilisation. Importantly, the worms are easily collected and amenable to laboratory holding and most adults within *G. caespitosa* aggregations have abdomens swollen with gametes throughout the year(Kupriyanova, 2006), from which they immediately release large amounts of eggs or sperm when their tubes have been broken or have been disturbed mechanically(Kupriyanova and Havenhand, 2002). Thus, being able to collect and use ripe animals over a prolonged period enabled us to run the thirty separate crosses we needed to assess the effects of metals using fertilisation assays.

The species also appears to be relatively sensitive to metals which led to clear concentration response relationships. Fertilisation in *G. caespitosa* was sensitive to metals, with effect

concentrations (10%) for Cu, Zn, Pb, and Cd estimated at between 4.5-27, 54-200, 30-910 and 2200-4200 µg L^{-1} respectively, depending on the sperm density used in fertilisation assays and on the metric used to assess toxicity. The toxicity values for Cu and Zn are the lowest so far reported for a polychaete, while those for Pb and Cd are within the range of those reported for marine invertebrates by Hudspith et al.(Hudspith, Reichelt-Brushett and Harrison, 2017). This suggests that this test species is a credible candidate for use in risk assessments. The polychaete community we studied is currently persisting in waters where the metal concentrations already periodically exceed the EC10 values we derived for fertilization success(Gaylard, 2004). Therefore, any further increases in metals in this area will likely reduce fertilization success of gametes more frequently, which could lead to population loss and potentially cause community collapse.

It is also possible that *G. caespitosa* collected from more pristine environments would yield different (greater) sensitivities to the metals we tested. For example, the sperm of killfish (*Fundulus heteroclitus*) from a contaminated site showed greater tolerance to metal toxicity (methylmercury) than the sperm of those from pristine environments(Khan and Weis, 1987). Research has indicated that populations living in metal polluted environments can become tolerant to metals(Klerks and Weis, 1987; Weis and Weis, 1989; Durou, Mouneyrac and Amlard-Triquet, 2005; Wang and Rainbow, 2005; Bankar *et al.*, 2018). Therefore, although the data presented here are highly relevant for use in assessing potential impact of increased metals at the study site, they may underestimate the toxicity of dissolved metals to fertilisation in *G. caespitosa* from a pristine environment.

5.0 Conclusion

Use of single sperm densities to determine the effects of metals to fertilisation can considerably underestimate toxicity. Toxicity tests that evaluate the impact of a toxicant to fertilisation using multiple sperm densities can provide ecologically important information that can be masked in tests using single sperm densities. Therefore, we recommend that future ecotoxicological testing based on fertilisation should use assays across a range of sperm densities to determine toxicity. Where this is impractical, we recommend that prior information about the likely nature of the impact on fertilisation should be used to determine the appropriate level of fertilisation in FSW controls and to set the sperm density used in experiments accordingly.

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