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The toxicity of potentially toxic elements (Cu, Fe, Mn, Zn and Ni) to the cnidarian *Hydra attenuata* at environmentally relevant concentrations

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1. Introduction

Pollution of the aquatic environment is common near human activity and the presence of chromium (Cr), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb), tin (Sn) and zinc (Zn) is associated with antifouling paint, industry, insecticides, fertilizer use, fuel consumption and waste water treatment works (Caccia *et al.*, 2003; Deheyn and Latz, 2006; Canning-Clode *et al.*, 2011; Xu *et al.*, 2014; Rodriguez-Iruretagoiena *et al.*, 2016). Areas with intense, localised activity (e.g. harbours and ports within estuaries) are known to exhibit greater anthropogenic influence (Birch *et al.*, 2015). An example of this is the Clyde Estuary, Scotland, which has since the Industrial Revolution received pollution from ship building, dye works and petroleum installations resulting it becoming the UK's most contaminated estuarine environment (Turner, 2000; Edgar *et al.*, 2003; Vane *et al.*, 2007; Vane *et al.*, 2011).

The Water Framework Directive (2000/60/EC) and Annex II of the Environmental Quality Standards Directive (2008/105/EC) include a number of PTEs (arsenic (As), cadmium (Cd), mercury (Hg), nickel (Ni)) which are classified as 'priority substances' and 'priority hazardous substances'. Requirements of the Water Framework Directive include a target of 'good' ecological status for waterbodies and levels of pollutants within the Clyde have reduced over the last three decades and ecological recovery has been observed (Critchlow-Watton *et al.*, 2014).

Elements such as Cu, Fe, Zn and Mn, are essential in trace amounts to support and maintain functions in aquatic ecosystems (Tchounwou *et al.*, 2012), however Pb, Cu and Zn have previously found to be a 'triad' of metals associated with human influence, and at high concentrations are toxic, adversely impacting on human/animal health and the environment (ten Brink and Woudstra, 1991; McLellan *et al.*, 2013). To determine the effect of pollutants on aquatic organic substances, numerous studies exposing invertebrates to heavy metals have previously been undertaken (Lasier *et al.*, 2000; Borgmann *et al.*, 2005; Torres Guzmán *et al.*, 2010; García *et al.*, 2011; Liber *et al.*, 2011; Lopes *et al.*, 2014, Jośko *et al.*, 2016). Karntanut and Pascoe (2002) exposed four different *Hydra* species (v*ulgaris (Zurich),attenuata*, *oligactis and viridissima)* to varying concentrations of Cu, Cd and Zn. A variation in the lethal concentration (LC) between species was found with Cu being the most toxic of the three elements with an LC_{50} ranging from 0.025 to 0.084 mg/l after 96h of exposure, followed by Cd (0.16 to 0.52 mg/l), then Zn (11 to 14 mg/l).

H. attenuata (also known as *Hydra vulgaris*) is a species of cnidarian that are ubiquitously found in freshwater ecosystems and are commonly used for toxicity testing. The health status and acute toxicity of *H. attenuata* is easily observed through a series of defined morphological changes following exposure to a toxin in a relatively simple bioassay (Wilby, 1988). Other chronic endpoints used to measure toxicity include asexual reproduction (budding), feeding behaviour and attachment to a substrate (Quinn *et al.,* 2012). This species has relatively unique regenerative properties, is easy to culture and maintain in a laboratory, has a high reproductive rate and as a diploblastic organism, is sensitive to environmental pollutants and is therefore used as a bioindicator for the health of a freshwater aquatic ecosystem (Quinn *et al.,* 2012). *H. attenuata* have been widely used in cost effective bioassays to assess the toxicity of numerous contaminants including wastewater (Trottier *et al.*, 1997), industrial effluents (Blaise and Kusui, 1997), pharmaceuticals (Pascoe *et al.*, 2003; Quinn *et al.*, 2008a, 2008b, 2009) , PTEs (Holdway *et al.*, 2001; Karntanut and Pascoe, 2002; Quinn *et al.*, 2007) and more recently rare earth elements (Blaise *et al.*, 2018).

The aim of this study is to evaluate the toxicity of PTE's, both individually and as a mixture, found in the Clyde estuary in Scotland against the cnidarian *Hydra attenuata*. Water samples from various locations in the Clyde estuary (Scotland) were analysed for anthropogenic PTEs Cu, Fe, Mn, Ni and Zn. The toxicity of these metals individually and as a mixture at environmentally relevant and elevated concentrations was tested using the *H. attenuata* bioassay on the ecologically relevant endpoints of morphology, feeding, attachment and reproduction. To the best of our knowledge, this is the first toxicity study investigating the mortality of any *Hydra* species individually exposed to Fe, Mn or Ni.

2. Materials and Methods

2.1. Test organism

Hydra were maintained in glass bowls containing 0.5 L of *Hydra* media (147 mg/l $CaCl_2H_2O$, 110 mg/l TES [N-Tris(hydroxymethyl) methyl 1-2-aminoethanesulfonic acid], pH adjusted to 7 using 0.5 M NaOH), maintained at 18± 2°C with a 12 h light 12 h dark photoperiod. *Hydra* were fed 3 times per week with newly hatched *Artemia* *salina* nauplii and were fasted 48 h prior to exposure. To avoid algal contamination, *Hydra* media was regularly changed after each feeding.

2.2. PTE determination

Water samples were collected from two estuarine (Kelburn Park and Erskine Harbour) and one freshwater (Gourock Burn) sites along the Clyde estuary (Fig. 1). Samples were collected in polypropylene sample bottles with pH and temperature recorded immediately (Mettler Toledo). Samples were then acidified with conc. HNO_3 (Fisher Trace Grade, UK) on site for preservation, transported to the laboratory and refrigerated at 4°C until analysis. Prior to analysis samples were filtered to <45μm (Filtermate, Environmental Express, USA).

Potentially toxic elements were determined by ICP-OES (Thermo Fisher, iCAP); a calibration series (0 mg/l, 2 mg/l and 10 mg/l of multi-element standard, ME/1001/05; Fisher Scientific, UK) was determined. Samples were analysed in triplicate. ICP-OES conditions were as follows: rf generator: 1.15 kW; Plasma: 1.4 l/min; Auxillary: 0.5 l/min; Nebuliser: 0.8 l/min; sample flow rate 1.5 ml/min. Averages of the sample concentrations were calculated; Limits of Detection were calculated using standard practice (e.g. (McLellan *et al.*, 2013)) (Table 1). It can be seen that levels of Fe within the Gourock Burn were very high therefore it was decided not to put this forward at the reported concentrations. Ni was taken at 0.5 mg/l to reflect potential toxicity levels within the selected biota. These are the 'environmentally relevant' concentrations.

2.3. Test solutions

Environmental relevant PTE solutions (1x) of metals in hydra media (HM) were prepared for the individual elements and for the mix solution. These stock solutions were then diluted with HM to give concentrations: 0.0001x, 0.001x, 0.01x, 0.1x. 10x, 100x and 1000x concentrations were made from a 1000x stock solution (Table 2).

2.4. *Hydra* toxicity tests

All PTE exposures to Hydra *attenuata* were undertaken in quadruplicate (4 repetitions of each concentration) and the whole experiment was undertaken in triplicate for the PTE mixture and duplicate for each individual metal exposure. A 4 ml sample of the relevant solution was added to 4 wells in a 24 multiwell plate, containing a single *Hydra*, and the wells wrapped in parafilm to prevent evaporation and kept at 18± 2°C for 96 h. Healthy *Hydra* with a morphology score of 10 on the Wilby table (Table 3) and having one bud (2 hydranths) were used in each exposure. Selection of healthy *Hydra* was undertaken using a binocular microscope. Morphology, hydranth number and attachment were observed at 24, 48, 72 and 96 h. The *Hydra*'s ability to ingest prey (feeding endpoint) was tested on all *Hydra* that scored > 5 on the Wilby score table after 96 h as per Quinn et al., (2007). These *Hydra* were placed individually into a well of a clean 24 well multi-well plate containing 4 ml of *Hydra* media. Freshly hatched *Artemia* were rinsed three times with HM with 5 individuals added to each well at time 0, taking care not to add them directly to the tentacles of the *Hydra*. The number of ingested prey were observed every 20 min for 120 min.

2.5. Statistical Data Analysis

The 96 h LC_{50} values for the mortality exposure were calculated using the Probit analysis program. The mortality exposure the sub-lethal LOEC (Lowest Observable Effect Concentration) was reported for ≥2 *Hydra* with score 8 or below and the NOEC (No Observable Effect Concentration) was based on *Hydra* with a score >8 (Quinn et al., 2009). A toxicity threshold (TT) was determined from the LOEC and NOEC using the following equation: TT=(NOECxLOEC)/2 (US EPA, 1989). Variability in all endpoints (morphology, attachment, hydranth number, feeding behaviour) between the exposed and control *Hydra* were tested by one-way analysis of variance (ANOVA). Significance was set at $p \leq 0.05$. The Pearson correlation coefficient was used to measure the strength of the association between the concentration of the pollutant and the endpoints.

3 Results

3.1. Toxicity of individual metals to *Hydra attenuata*

Complete (100%) population mortality (indicated by a score ≤5 on the Wilby scale) was found at 0.1x (0.05 mg/l) for Cu (Fig. 2 A), 0.1x (0.3 mg/l) for Fe (Fig. 2 C), 10x (5 mg/l) for Ni (Fig. 2 D). For Mn, 100% mortality of all *Hydra* exposed was found at 100x (200 mg/l). Although some mortality was observed at 1x, mortality numbers were low (Fig. 2 B). Highly significant ($p = < 0.005$) and negative correlations were found with hydranth number and feeding behaviour (Table 5). For Zn, 100% mortality was found at 1000x (100 mg/l). Although mortality was detected at 100x (10 mg/l), mortality numbers were low (Fig. 2 E). An extremely significant ($p = < 0.001$) and negative correlation was found with hydranth number, and a very significant ($p = < 0.005$) negative correlation was found for feeding behaviour (Table 5). The 96 h LC_{50} values were determined as follows: Cu 0.0225 mg/l, Mn 20 mg/l, Fe 0.135 mg/l, Ni 2.25 mg/l, and Zn 31.622 mg/l. The Toxicity thresholds were calculated at: Cu 0.000125 mg/l, Mn 0.2 mg/l, Fe 0.000045 mg/l, Ni 0.0125 mg/l, Zn 5 mg/l (Table 4).

3.2 Toxicity of PTE mixture

For the PTE mixture, 100% mortality was found at 0.1x (Fig. 3). The 96h LC_{50} value was calculated as 0.045x. The LOEC was 0.01x and NOEC was 0.001x. The toxicity threshold was calculated at 0.000005x (Table 4). The high toxicity of Cu was not entirely responsible for the very high toxicity of the mixture. The toxicity threshold for the mixture (0.000005x) showed that the mixture was more toxic than Cu individually, which had a toxicity threshold of 0.000125 mg/l (0.00025x).

3.3 Toxicity of heavy metals at environmental concentration

Both Cu and Fe when exposed individually to the concentration of their respective metals found in the environment resulted in 100% mortality of all *Hydra* exposed (Fig. 2 A & C). A significant ($p = < 0.001$) toxic effect occurred when *Hydra* were exposed to Mn and Ni at the environmentally relevant concentration. Zn remained at a perfect morphology score of 10 when exposed to the Zn concentration found in the environment (Fig. 2 E). The concentration of Zn found in the environment also had no significant toxic effect on hydranth number, feeding behaviour or attachment of *Hydra* to a substrate. When exposed to the concentration of Mn found in the environment, a significant ($p = < 0.01$) toxic effect occurred in the attachment of *Hydra* to a substrate (Fig. 2 B). The concentration of Mn found in the environment had no significant toxic effect on hydranth number or feeding behaviour. The concentration of Ni found in the environment resulted in a significant ($p = < 0.05$) toxic effect on the feeding behaviour of *Hydra* (Fig. 2 D). The concentration of Ni found in the environment had no significant toxic effect on hydranth number or attachment of *Hydra* to a substrate. *Hydra* morphology was monitored at 24 h, 48 h, 72 h and 96 h of exposure to the concentration found in the environment with any toxic effect occurring within the first 24 h of exposure (Fig. 4).

4 Discussion

Since the 18th Century and the beginning of the Industrial Revolution, the Clyde has had a diverse heritage and there is a well-documented legacy of pollutants e.g. (Hursthouse *et al.*, 1994; Edgar *et al.*, 2003; Vane *et al.*, 2007; Vane *et al.*, 2011). The sample locations chosen for this site are near former landfill sites (Gourock Burn and Kelburn Park) or wastewater treatment works (Erskine Harbour) and there is potential for continued contamination from these sources. This is in addition to former industrial activity e.g. metal plating near Kelburn Park (Miller, 1986). Despite the improving physical and ecological status of the outer Clyde estuary (Critchlow-Watton *et al.*, 2014), it is concerning that this study has found that PTE levels are above legislative requirements (Table 6) which may be caused by the proximity of potential point sources of pollutants. In that light, the Clyde is similar to other estuaries where point sources can be attributed to elevated PTE levels (Larrose *et al.*, 2010; Birch *et al.*, 2015; Petit Jérôme *et al.*, 2015; Rodriguez-Iruretagoiena *et al.*, 2016). Levels of all heavy metals tested were higher than levels in the Thames river in London, Canada (Environment and Engineering Services, 2018) and the Ganga river in India (Central Water Commission, 2018) (Table 6). The maximum acceptable limits for copper (0.00376 mg/l) and iron (1 mg/l) based on EU / UK legislative requirements are higher than the *H. attenuata* LC_{50}'s for copper (0.0225 mg/l) and iron (0.135 mg/l).

To the best of our knowledge, this is the first toxicity study investigating the toxicity of any *Hydra* species exposed to Fe, Mn or Ni. This study calculated the LC_{50} values, LOEC, NOEC and Toxicity Thresholds for Cu, Fe, Mn, Zn and Ni (Table 4). The 96 h LC_{50} results for Cu (0.0225mg/l) are similar to those reported by Karntanut and Pascoe (2000) (0.032 mg/l) for *H. vulgaris* (also known as *H. attenuata*) and for 4 different species of Hydra; *H. vulgaris* Zurich (0.042 mg/l), *H. vulgaris* (0.056 mg/l), *H. oligactis* (0.084 mg/l), *H. viridissima* (0.025 mg/l) (Karntanut and Pascoe, 2002). The Cu LC_{50} value in the current study were higher than the LOEC value which is unusual but is due to the dilution range used for the serial dilution.

The 96 h LC_{50} value calculated for Zn in the present study (31.6 mg/l) is higher than those reported for *H. vulgaris* (7.4 mg/l) (Karntanut & Pascoe, 2000) *H. vulgaris Zurich* (14 mg/l), *H. vulgaris* (13 mg/l), *H. oligactis* (14 mg/l), *H. viridissima* (11 mg/l) (Karntanut & Pascoe, (2002). In the current study Zn was tested at a concentration of 10 mg/l and a mortality percentage of 12.5% was found. The large divisions used in the serial dilutions resulted the high LC_{50} value of 30 mg/l that was calculated, as the next concentration tested after 10 mg/l was 100 mg/l.

The same could be true of Mn (with an LC_{50} value of 20 mg/l) but as this is the first time this metal has been used in a toxicity test to study mortality of *Hydra*, there is no literature for comparison. Harford *et al.*, (2015) however, exposed *Hydra viridissima* to varying levels of Mn to test population growth. The highest concentration tested by Harford *et al.,* (2015) was 10 mg/l at which the population of

H. viridissima was still growing but had dropped to 10% growth compared to the control. A very significant (p = <0.005) negative correlation was found for hydranth number and feeding behavior when exposed to Mn.

An extremely significant (p = <0.001) negative correlation for hydranth number and a very significant (p = <0.005) negative correlation was found when exposed to Zn. However, there was no significant correlation for attachment when exposed to any of the tested metals and no significant correlation for attachment, hydranth number or feeding behavior when exposed to Cu, Fe or Ni.

In this study, a significant toxic effect occurred when *Hydra* were exposed to the Cu, Fe, Mn and Ni at concentrations found in the Clyde estuary (Fig. 2A-D). *Hydra* morphology was unaffected and remained at a score of 10 when exposed to the concentration of Zn found in the environment (Fig. 2E). Mortality levels of 100% were measured when *Hydra* were exposed to the heavy metal mixture (Fig. 3) and to Cu and Fe (individually) (Fig. 2A, C) at concentrations found in the Clyde. These results indicate that *Hydra attenuata* are unable to survive in aquatic environments with the metal concentrations found in the Clyde estuary, which may also have an impact on *Hydra* predators and prey.

The results also indicate that the PTE mixture (including the individual concentrations of Cu, Fe, Mn and Ni) could potentially prove significantly toxic to other invertebrates. The concentration of Cu found in the Clyde estuary was measured at 0.5 mg/l, this was 22 times higher than the LC_{50} found for *Hydra attenuata*. When compared with other studies (Table 7), the levels of Cu found in the Clyde would also be toxic to aquatic vertebrates such as *Rasbora sumatrna,* the guppy (*Poecilia reticulata)* and the zebrafish (*Danio rerio).* The concentration of Fe found in the Clyde estuary was measured at 3 mg/l, this was also 22 times higher than the LC_{50} found for *Hydra attenuata* and would be toxic to other aquatic invertebrates such as *Daphnia magna*, and aquatic vertebrates, such as the brown trout (*Salmo trutta*) (Table 7).

For the PTE mixture, a significant ($p \leq 0.05$) toxic effect was seen at the lowest concentration studied (0.0001x) (Fig. 3). The LC_{50} was calculated as 0.045x for the mixture and the toxicity threshold was calculated as 0.000005x. The toxicity threshold (TT) was lower than any of the corresponding values of the individual metals contained within the mixture (Table 4). This result indicates that the metals have a cumulative effect, with each metal behaving cumulatively, contributing to the total effect of the mixture and further increasing the toxicity.

Morphology was found to be the most significant endpoint in studying the toxic effects of metals. Using the additional endpoints of hydranth number, attachment and feeding behavior, Quinn *et al.,* (2007) found a significant decrease in hydranth number, attachment and feeding behavior as the concentration of the toxin increased. In the present study, a similar significant negative correlation was observed for hydranth number and feeding behavior following exposure to Mn and Zn. There was no significant correlation found with attachment in any of the exposures undertaken.

Most toxicity tests involving *Hydra* spp expose the organism to a toxin for 96 h. In this study, it was observed that any significant toxic effect of a pollutant occurred within the first 24 h of exposure. A review of other toxicity studies using *Hydra* as a test organism shows that the toxic effect of a contaminant occurs within the first 24 h of exposure (Blaise and Kusui, 1997; Karntanut and Pascoe, 2000, 2002). It may therefore be necessary to only expose *Hydra* to a toxin for 24 h to test a compounds toxicity. However, more research is needed to confirm this. The potential replacement of a 96 h exposure with a 24 h one would greatly reduce the time needed for toxicity testing, helping to reduce the cost and potentially increasing the number of toxins that can be tested within a given time period.

5. Conclusion

This paper shows that a significant toxic effect was observed on *Hydra* exposed to the PTE mixture at the concentration found in the environment (1x) after a short-term exposure period (24 h). The high toxicity of Cu was not entirely responsible for the very high toxicity of the mixture. The toxicity threshold for the mixture (0.000005x) showed that the mixture was more toxic than Cu individually, which had a toxicity threshold of 0.000125 mg/l (0.00025x). The toxicity threshold (TT) for the PTE mixture was lower than that for the same metals when exposed individually to *Hydra*, indicating that metals may act cumulatively in a mixture. However, a significant toxic effect occurred when *Hydra* were exposed individually to Cu, Fe, Mn and Ni at concentrations found in the environment, with 100% mortality when exposed individually to the environmental concentrations of Cu and Fe. These high environmental concentrations of PTE would impact, not only on the predator and prey interactions within the *Hydra* community but also could potentially prove significantly toxic to other aquatic organisms.

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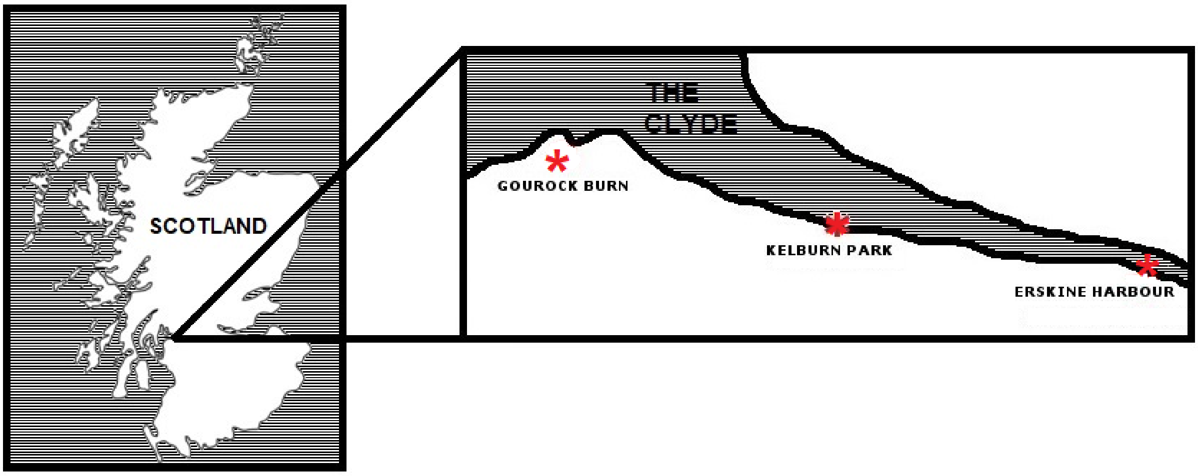

Fig. 1: Overview of heavy metal sampling locations along the Clyde estuary, Scotland. * indicates sample location.

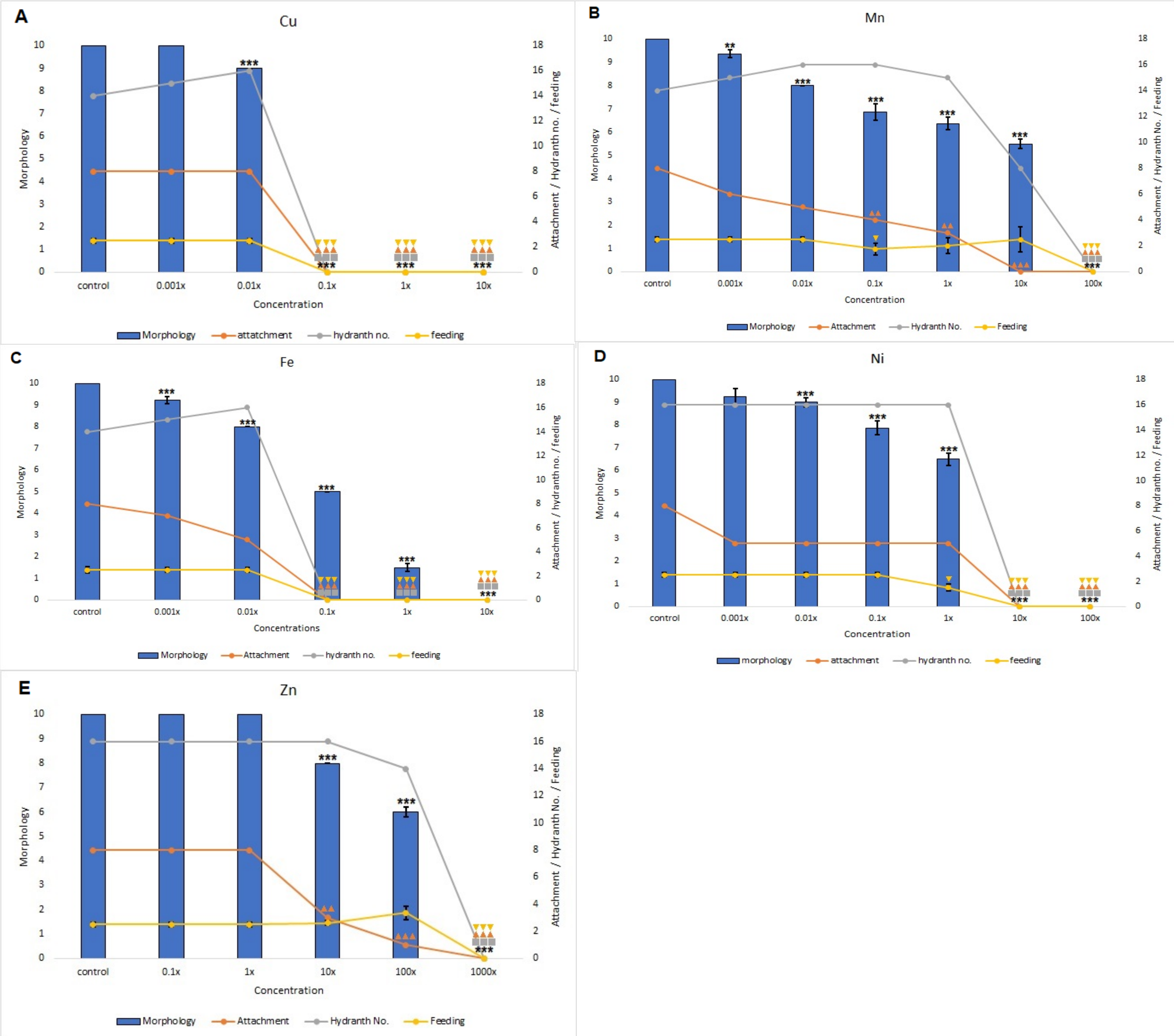

Fig. 2: – Lethal and sub-lethal effects of individual metals at varying concentrations on *Hydra* morphology, hydranth number, attachment and feeding after 96hr exposure. Points at Morphology and feeding represent the mean score (n=8)±standard error. Points at attachment and hydranth number represent sum(n=8). Significance for morphology at *=p≤0.05; **=p≤0.01; ***=p≤0.001. Significance for hydranth number at ■=p≤0.05; ■■=p≤0.01; ■■■=p≤0.001. Significance for attachment at ▲=p≤0.05; ▲▲=p≤0.01; ▲▲▲=p≤0.001. Significance for feeding at ▼=p≤0.05; ▼▼=p≤0.01; ▼▼▼=p≤0.001.

Note: Error bars do not show at points where results had no variability.

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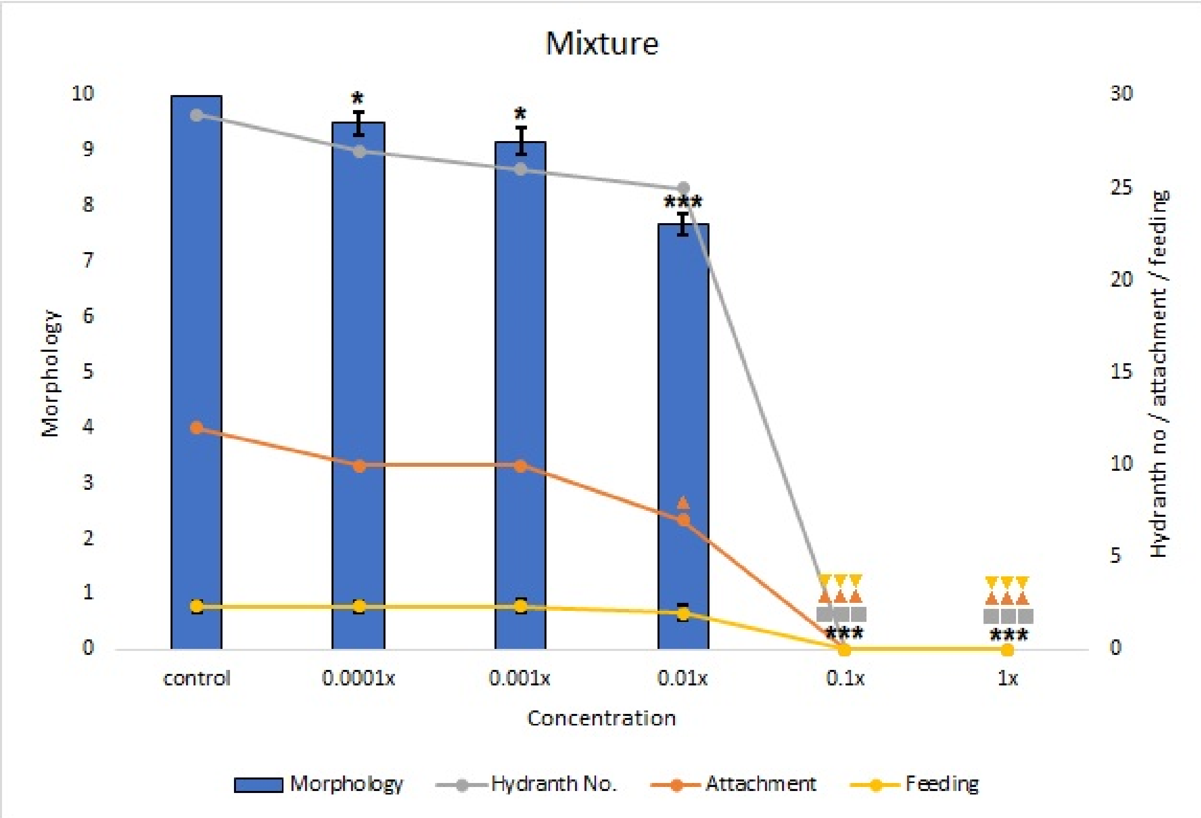

Fig. 3 – Lethal and sub-lethal effects of several concentrations (0.0001×–1×) of the heavy metal mixture found in the environment on *Hydra* morphology, hydranth number, attachment and feeding after 96hr exposure. Points at Morphology and feeding represent the mean score (n=12) ±standard error. Points at attachment and hydranth number represent sum (n=12). Significance for morphology at *=p≤0.05; **=p≤0.01; ***=p≤0.001. Significance for hydranth number at ■=p≤0.05; ■■=p≤0.01; ■■■=p≤0.001. Significance for attachment at ▲=p≤0.05; ▲▲=p≤0.01; ▲▲▲=p≤0.001. Significance for feeding at ▼=p≤0.05; ▼▼=p≤0.01; ▼▼▼=p≤0.001.

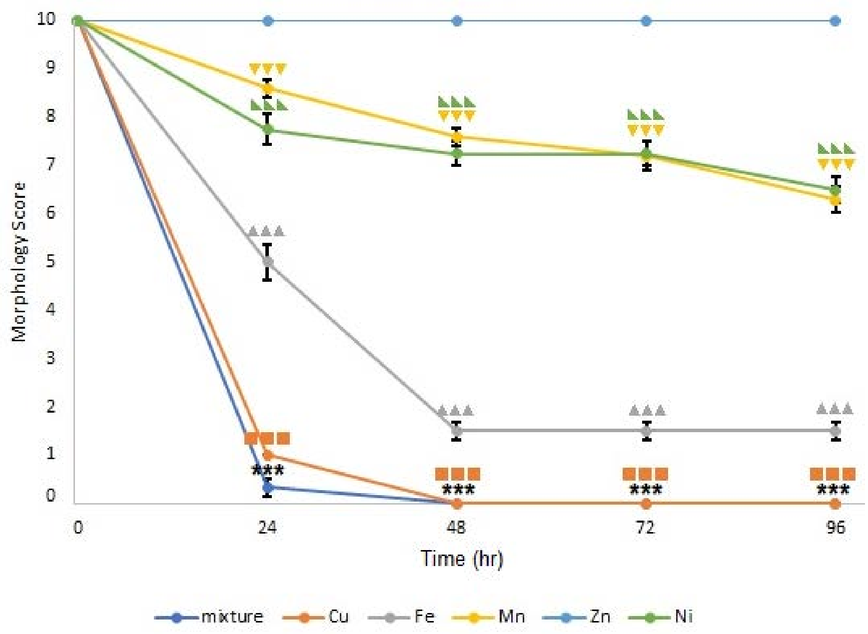

Fig. 4 – Effect of heavy metal concentrations found in the environment on *Hydra* morphology at 24, 48, 72 and 96 h exposure. Mean scores are represented the individual metals (n=8) and metal mixture (n=12)±standard error. Significance for morphology at *=p≤0.05; **=p≤0.01; ***=p≤0.001. Significance for Cu at ■=p≤0.05; ■■=p≤0.01; ■■■=p≤0.001. Significance for Fe at ▲=p≤0.05; ▲▲=p≤0.01; ▲▲▲=p≤0.001. Significance for Mn at ▼=p≤0.05; ▼▼=p≤0.01; ▼▼▼=p≤0.001. Significance for Ni at ◣=p≤0.05; ◣◣=p≤0.01; ◣◣◣=p≤0.001.

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Table 1: Estuarine and freshwater concentrations of heavy metals found in the environment.

Element	Gourock Burn (mg/l)	Kelburn Park (mg/l)	Erskine Harbour (mg/l)	Average (mg/l)
Cu	<LOD	0.98	0.67	0.82
Fe	33.78	<LOD	9.87	21.82
Mn	1.72	<LOD	<LOD	1.72
Ni	<LOD	1.74	1.42	1.58
Zn	<LOD	0.20	0.23	0.21

Table 2: The concentrations of PTEs used in the exposure tests (mg/l). Based on the concentrations found in the environment.

Metal	0.001x (mg/l)	0.01x (mg/l)	0.1x (mg/l)	1x Environmental concentration (mg/l)	10x (mg/l)	100x (mg/l)	1000x (mg/l)
Copper	0.0005	0.005	0.05	0.5	5	-	-
Iron	0.003	0.03	0.3	3	30	-	-
Manganese	0.002	0.02	0.2	2	20	200	-
Zinc	-	-	0.01	0.1	1	10	100
Nickel	0.0005	0.005	0.05	0.5	5	50	-

Table 3: Hydra morphology score table used to assess acute toxicity, based on the Wilby morphology score (Wilby, 1988).

10	Healthy, long tentacles and body reactive	
9	Partially contracted, slow reactions	
8	Clubbed tentacles, body slightly contracted	Alive
7	Shortened tentacles, body slightly contracted	
6	Tentacles and body shortened	
5	Totally contracted, tentacles visible	
4	Totally contracted, no visible tentacles	
3	Expanded, tentacles visible	Dead
2	Expanded, no visible tentacles	
1	Dead but intact	
0	Disintegrated	

Table 4: LC_{50}, LOEC and NOEC values based on morphology for *Hydra attenuata* exposed to heavy metals individually and as a mixture. Toxicity Threshold (TT=(NOECxLOEC)/2). Actual concentrations measured in the environment are also presented.

Metal	**Concentration in environment (mg/l)**	**LC_{50} (mg/l)**	**LOEC (mg/l)**	**NOEC (mg/l)**	**TT (mg/l)**
Mixture	1x	0.045x	0.01x	0.001x	0.000005x
Copper	0.5	0.0225	0.05	0.005	0.000125
Iron	3	0.135	0.03	0.003	0.000045
Manganese	2	20	2	0.2	0.2
Zinc	0.1	31.622	10	1	5
Nickel	0.5	2.25	0.5	0.05	0.0125

Table 5: Pearson correlation coefficient of heavy metal pollutants and attachment, hydranth number and feeding behaviour endpoints.

	Mixture	**Cu**	**Fe**	**Mn**	**Ni**	**Zn**
Attachment	-0.6742	-0.5048	-0.4882	-0.6132	-0.6666	-0.6662
Hydranth no.	-0.702	-0.5032	-0.5032	**-0.9257****	-0.717	**-0.9996*****
Feeding	-0.7013	-0.5048	-0.5048	**-0.9308****	-0.6848	**-0.922****

Significant results indicated by bold with significance set at *p <0.05, **p <0.005, ***p <0.001

Table 6: A comparison of the heavy metal concentrations found from the Clyde, Thames and Ganga rivers with the maximum acceptable limits based on EU / UK legislative requirements.

Metal	EU / UK[a] (mg/l)	Average measured Concentration (mg/l)	River
Copper	0.00376	0.5	Clyde, Glasgow, Scotland[b]
		0.001	Thames, London, Canada[c]
		0.022	Ganga, Kachlabridge, India[d]
Iron	1	3	Clyde, Glasgow, Scotland[b]
		0.044	Thames, London, Canada[c]
		0.0004	Ganga, Kachlabridge, India[d]
Manganese	-	2	Clyde, Glasgow, Scotland[b]
		0.011	Thames, London, Canada[c]
		-	Ganga, Kachlabridge, India[d]
Zinc	0.0079	0.1	Clyde, Glasgow, Scotland[b]
		0.002	Thames, London, Canada[c]
		0.00009	Ganga, Kachlabridge, India[d]
Nickel	0.0086	0.5	Clyde, Glasgow, Scotland[b]
		0.004	Thames, London, Canada[c]
		0.006	Ganga, Kachlabridge, India[d]

[a] SEPA (2018)
[b] Present study
[c] Environment and Engineering Services (2018)
[d] Central Water Commission (2018)

Table 7: Comparison of LC_{50} for *H. attenuata* with those from other species for selected heavy metals.

Metal	Organism	LC_{50} (mg/l)	Source
Copper	*Hydra attenuata*	0.0225	Present study
	Danio rerio	0.01166	Alsop & Wood (2011)
	Rasbora sumatrna	0.0056	Shuhaimi-Othman *et al.*, (2010)
	Capoeta fusca	1.1	Ebrahimpour *et al.,* (2010)
	Poecilia reticulata	0.0379	Shuhaimi-Othman *et al.,* (2010)
Iron	*Hydra attenuata*	0.135	Present study
	Daphnia magna	0.23	García *et al.,* (2011)
	Salmo trutta	0.05	Dalzell and MacFarlane (1999)
	Hyalella azteca	>1	Borgmann *et al.,* (2005)
Manganese	*Hydra attenuata*	20	Present study
	Rutilus rutilus caspicus	300	Hoseini *et al.,* (2014)
	Mogurnda mogurnda	240	Harford *et al.,* (2015)
	Ceriodaphnia dubia	6.2	Lasier *et al.,* (2000)
	Garra gotyla gotyla	3.2	Sharma & Langer (2014)
Zinc	*Hydra attenuata*	31.622	Present study
	Danio rerio	2.535	Alsop & Wood (2011)
	Daphnia magna	0.76	Lopes *et al.,* (2014)
	Capoeta fusca	13.7	Ebrahimpour *et al.,* (2010)
	Lecane quadridentata	0.12	Torres Guzman *et al.,* (2010)
Nickel	*Hydra attenuata*	2.25	Present study
	Clarias gariepinus	8.87	Ololade & Oginni (2010)
	Hyalella azteca	2	Liber *et al.,* (2011)
	Danio rerio	0.5898	Alsop & Wood (2011)
	Chironomus dilutus	119.5	Liber *et al.,* (2011)