Toxicity of algal-derived aldehydes to two invertebrate species: Do heavy metal pollutants have a synergistic effect?
Abstract

The recent discovery of the production of anti-proliferative aldehydes in a variety of microalgal species has lead to considerable investigation into the effects of these toxins on aquatic invertebrates. Studies have, however, rarely considered the impact pollutants may have on grazer responses to algal toxins. In this study, the acute toxicities of five aldehydes to the rotifer *Brachionus plicatilis* and nauplii of the brine shrimp *Artemia salina* are examined using immersion assays. In addition, the effect of a representative of these aldehydes in the presence of sub-lethal levels of heavy metals was examined. *B. plicatilis* generally showed greater sensitivity to the aldehydes than *A. salina*. The polyunsaturated 2-trans,4-trans-decadienal was the most toxic to both species having 24 h LD$_{50}$ values of 7 and 20 µM for *B. plicatilis* and *A. salina* respectively. The remaining aldehydes had different orders of toxicity for the two species with a stronger relationship observed between mortality and aldehyde carbon-chain length for *A. salina* whereas *B. plicatilis* mortality showed a stronger dependence on the presence of carbon-carbon double bonds in the aldehydes. The presence of 1 µM of copper sulphate in solutions of decadienal resulted in the reduction of the 24 h LD$_{50}$ of decadienal by approximately a third for both species. 1 µM of copper chloride in solutions of decadienal reduced the 24 h LD$_{50}$ of decadienal to *A. salina* nauplii by approximately 11% and 1 µM zinc sulphate caused a reduction of only 3%. Pre-exposure of the organisms to 1 µM copper sulphate had no significant impact on their subsequent mortality in decadienal. The ecological implications and the possible mechanisms for the action of copper sulphate on the response of organisms to decadienal are discussed.

**Keywords:** *Artemia salina; Brachionus plicatilis; aldehyde; acute-toxicity; heavy metals.*
Introduction

The high nutrient and light levels of coastal marine environments allows the growth of vast blooms of microalgae. In temperate and polar environments these blooms are frequently dominated by diatoms, which as a food source provide the basis for a rich variety of animal life. There have been many reports of microalgae producing harmful toxins, although until recently there have been few observations of these toxins acting directly upon grazing organisms. Many microalgal toxins are instead detrimental to organisms occupying higher trophic levels, vertebrates in particular (Anderson and White, 1992; Beltran et al., 1997). Diatoms are of high nutritional value for organisms that graze upon them and have been considered vital components in the transfer of energy within food webs. In view of this, diatom species are frequently utilised in the culture of economically important organisms (Brown et al., 1997; Renaud et al., 1999). During the last decade, however, the synonymous link between diatom primary productivity and grazer secondary production has been challenged with the discovery that several diatom species significantly reduce the reproductive success and hence population growth of invertebrates (Ianora et al., 2003 and references therein). Poulet et al. (1994, 1995) and Laabir et al. (1995) initially observed reduced egg viability and abnormal naupliar development in copepods feeding on diatoms and hypothesised that this was due to some form of chemical defence. This theory was later substantiated by the isolation of the polyunsaturated aldehydes (PUAs) 2-trans,4-trans-decadienal, 2-trans, 4-cis,7-cis-decatrienal and 2-trans,4-trans,7-cis-decatrienal from the common marine pelagic diatom Thalassiosira rotula (Miralto et al., 1999). The range of these toxic compounds produced by diatoms and other microalgae has since broadened to include octadienal, heptadienal, nonadienal and a number of other acidic aldehydes (d'Ippolito et al., 2002a, 2002b; Pohnert et al., 2002; Watson and Satchwill, 2003). The PUAs are the end-products of a lipoxygenase/hydroperoxide lyase
(LOX/HPL) cascade reaction (d'Ippolito et al., 2004). Damage to the algal cell initiates the LOX/HPL cascade by releasing phospholipase enzymes that liberate polyunsaturated fatty acids (PUFAs) from the cell membrane. These are then oxidised and cleaved to form the toxins (Pohnert, 2002). The specific type and quantity of PUFAs produced varies between species and strains, due to utilisation of different PUFAs and enzymes (d'Ippolito et al., 2003, 2004). This variability in toxin production results in a concomitant variation in toxicity (Pohnert et al., 2002; Ceballos and Ianora, 2003). Detrimental effects of a range of algal species and their toxins have been observed on the reproductive success of numerous invertebrates, most notably copepods (Laabir et al., 1995; Poulet et al., 1995; Ban et al., 1997; Ianora et al., 2004a) but also a range of other invertebrate phyla (Caldwell et al., 2002, 2003; Adolph et al., 2004; Lewis et al., 2004), algae (Castotti et al., 2005) and unicellular eukaryotes and prokaryotes (Adolph et al., 2004). The effects of PUA ingestion include the inhibition of embryogenesis (Laabir et al., 1995; Miralto et al., 1999; Starr et al., 1999), reduced egg and larval viability (Ban et al., 1997; Buttino et al., 1999; Laabir et al., 2001) and abnormal morphological development of larvae (Poulet et al., 1995, 2003; Buttino et al., 2004). Several reports have also shown reduced larval survival when feeding on specific diatom species (Carotenuto et al., 2002; Ianora et al., 2004b) as well as reduced fertilisation success (Caldwell et al., 2002; Tosti et al., 2003), sperm motility (Caldwell et al., 2004a) and larval survival (Caldwell et al., 2003; Lewis et al., 2004) following direct exposure to PUAs.

Diatoms known to possess the chemical defence, such as *Skeletonema costatum*, frequently dominate coastal algal blooms providing abundant opportunity for numerous invertebrates to be affected by the toxins (Miralto et al., 2003; Ianora et al., 2004b). The chemical composition of coastal waters is however further complicated by a cocktail of industrial, agricultural and domestic pollutants entering the system from estuarine outflow pipes or as land based runoff (Bryan, 1971; Phillips, 1995). Heavy metals such as copper...
and zinc are common examples of these pollutants and are often highly toxic to marine organisms. Heavy metals in the marine environment frequently occur at concentrations elevated above the natural base levels due to anthropogenic pollution and can significantly impact aquatic life even at sublethal levels. Copper, for example, can greatly exceed the current environmental quality standard of 5 µg.l⁻¹ in polluted coastal waters (Environment agency, pers. comm.) and can affect larval growth, enzyme systems (Alayse-Danet et al., 1979), osmo-regulation (Macrae and Pandey, 1991; Katranitsas et al., 2003) and organogenesis (Spicer, 1995).

The majority of ecotoxicology studies are based upon single compound exposures, including several previous studies concerned with PUAs (Bisignano et al., 2001; Adolph et al., 2004), despite mixed compound toxicity studies using pollutants frequently indicating increased toxicity of certain compounds in the presence of other chemicals (Cassee et al., 1999). Copper, for example, has been utilised in several mixture toxicity studies and shows synergistic interactions with assorted other compounds (Corner and Sparrow, 1956; Moraitouapostolopoulou and Verriopoulos, 1982; Gajbhiye and Hirota, 1990). Caldwell et al. (2004b) has previously criticized methodologies involving the exposure of organisms to single PUAs. This study examined the hypothesis that the response of organisms to PUAs is influenced by the presence of pollutants within the marine environment. Two representative invertebrates are used, the rotifer Brachionus plicatilis and the nauplii of the brine shrimp Artemia salina. Both are currently important as live feed in aquaculture and are frequently utilised in toxicity studies due to easy and inexpensive culture under controlled conditions (Saliba and Krzyz, 1976; Snell and Persoone, 1989a, 1989b; Caldwell et al., 2003). Acute-toxicity immersion assays with PUAs and with mixtures of a representative aldehyde, 2-trans,4-trans-decadienal, and sublethal concentrations of selected heavy metal pollutants were performed.
Materials and Methods

Preparation of chemical standards

Chemical standards were prepared using methodology similar to that employed by Caldwell et al. (2003). All chemicals were supplied by Sigma Aldrich (UK). Aldehydes investigated included the PUAs 2-trans,4-trans-decadienal, 2-trans,4-trans-octadienal and 2-trans,4-trans-heptadienal and the saturated aldehydes decanal and undecanal. Aldehydes were initially dissolved in ethanol to facilitate the creation of standards by serial dilutions in 10 μm-filtered autoclaved seawater (FSW). Two sets of a range of concentrations were produced for each of the aldehydes. Test concentrations for *Artemia salina* toxicity assays were as follows; decadienal concentrations ranged from 0 to 38.5 μM, octadienal from 0 to 255 μM, heptadienal from 0 to 425 μM and decanal and undecanal from 0 to 330 μM. Concentrations for *Brachionus plicatilis* toxicity assays were as follows; decadienal concentrations ranged from 0 to 30 μM, octadienal from 0 to 175 μM, heptadienal from 0 to 100 μM, undecanal from 0 to 250 μM and decanal from 0 to 300 μM.

Secondly, to establish that the concentrations of the metal salts to be tested in combination with decadienal were sublethal, copper sulphate, copper chloride and zinc sulphate were dissolved in FSW to produce a range of concentrations from 0 to 200 μM. Finally, decadienal initially dissolved in ethanol to give a 20 mg.ml⁻¹ solution, was diluted in FSW creating a 100 μM standard and copper sulphate (CuSO₄•5H₂O), copper chloride (CuCl₂) and zinc sulphate (ZnSO₄•7H₂O) were dissolved in FSW producing 2 μM solutions of each. For *A. salina* assays the decadienal and copper sulphate solutions were diluted further and mixed creating ten decadienal concentrations ranging from 0 to 50 μM, with six copper sulphate concentrations from 0 to 1 μM in each decadienal concentration. This procedure was repeated using the same concentrations of copper
chloride and zinc sulphate substituted for copper sulphate and repeated for *B. plicatilis*
assays with copper sulphate at 0 to 1 µM in decadienal solutions of 0 to 20 µM.

*Mortality assays*

*A. salina* cysts (Saunders, Great Salt Lake) were put in FSW in a conical flask with
constant light and aeration to initiate hatching. After 24 h the hatched nauplii were
concentrated into a crystallising dish with FSW. Four replicates of 2 ml of each
concentration of the aldehyde standards, metal standards or mixtures of the two were put
into 24-well multi-well plates and 15 µl of the *A. salina* nauplii suspension, containing
approximately 10-20 nauplii, placed into each. This was repeated for *B. plicatilis* using
adults filtered from a laboratory culture that was maintained at 15 ºC in a 12:12
photoperiod and fed a mixture of *Tetraselmis chui* and *Rhinomonas reticulata*. In the
mixture assays, *B. plicatilis* was only tested for the impact of copper sulphate in
combination with decadienal as this was the most toxic of the metal salts for the rotifer
and exerted the greatest impact on *A. salina* mortality in the combination assays. Finally,
decadienal solutions of 0 to 40 µM were created as above. Newly hatched *A. salina* nauplii
were pre-exposed to either a 1 µM copper sulphate solution or FSW for 24 h. These pre-
exposed nauplii were then washed thoroughly with FSW, concentrated into crystallising
dishes and 15 µl aliquots containing approximately 10-20 nauplii dispensed into multi-
well plates containing the decadienal standards. Each decadienal concentration had 12
replicates with half containing nauplii that had been pre-exposed to 1 µM copper sulphate
and half containing those pre-exposed to FSW. This was repeated for *B. plicatilis* pre-
exposed to either 1 µM copper sulphate solution or FSW for 24 h and then assayed in
decadienal at concentrations of 0 to 20 µM for a further 24 h. All multi-well plates were
sealed and kept in an incubator at 15 ºC with a 12:12 photoperiod and the mortality in
each well recorded after 24 h using a Wild Heerbrug dissecting microscope. At the end of
the assays, formalin was added to the wells and the total number of *A. salina* nauplii or *B.*
plicatilis in each well recorded. The ethanol solvent at concentrations used had no significant impact on mortality (data not shown).

**Data analysis**

Visual observations allowed the concentrations of the three metals salts sublethal to the two test organisms to be established. All remaining data were analysed using logit regressions with statistical package R. Logit regression relates the binary dependent variable (mortality) to the independent variable(s) using the basic equation $P = 1 / (1 + e^{(-a + bX)})$, where $P$ is the probability of mortality occurring, $e$ is the base of the natural logarithm and $a$ and $b$ are the coefficients generated by means of the logit regression. Odds ratios were calculated by taking the inverse natural log of the logit regression coefficient for the chemical concentration impact. Odds ratios are a summary of the relationship (effect size) between mortality and chemical concentration and effectively represent the change in probability with each unit increase in chemical concentration that mortality of an individual will occur when exposed to the chemical. The changes in the LD$_{50}$ value of decadienal over the range of metal salt concentrations were calculated by an iterative process using the logit equation. The majority of the logit regressions showed a slight amount of over-dispersion (see Table 3) suggesting that the error models utilised may be inappropriate or there were missing or inadequately defined predictors. We feel, however, that the extent of over-dispersion does not invalidate the model and can be explained in part by the variable number of organisms between the replicate wells or possibly by variation in the ages of the rotifers within the wells.

**Results**

Logit regression analyses show a highly significant relationship between aldehyde concentration and mortality for both *A. salina* and *B. plicatilis* for all aldehydes tested (Tables 1 and 2). The odds ratios and LD$_{50}$ values calculated from the logit regression
coefficients show a large range of toxicities between the aldehydes for the two species (Figure 1). Decadienal was the most toxic of the aldehydes tested, with 24 h LD$_{50}$ values of 20 $\mu$M and 7 $\mu$M for A. *salina* and *B. plicatilis* and odds ratios of 1.28 and 1.62, i.e. the odds that mortality of an individual would result from exposure to decadienal increase by 28 and 62% with each unit increase in decadienal concentration. The remaining aldehydes showed differing orders of toxicity between the two species. The lowest odds ratios for *A. salina* nauplii were obtained with heptadienal (1.01), which has an associated LD$_{50}$ of 274 $\mu$M compared to the least toxic for *B. plicatilis*, decanal (1.02), with an associated LD$_{50}$ of 176 $\mu$M. The order of toxicity for the PUAs to *A. salina* descends through decadienal ($C_{10}$), octadienal ($C_{8}$) and heptadienal ($C_{7}$). The saturated aldehydes undecanal ($C_{11}$) and decanal ($C_{10}$) were of a similar toxicity to octadienal (Figure 1). The odds ratios and LD$_{50}$ values indicate a greater sensitivity of *B. plicatilis* compared to *A. salina* to all three PUAs tested. The two saturated aldehydes were of lower toxicity for *B. plicatilis* than all three PUAs (Figure 1). The deviance of the data is however relatively high (Table 3, Figure 1), therefore suppositions about the order of toxicity of the aldehydes are approximate only.

There was no observed impact of the three metal salts tested upon the mortality of *A. salina* at the upper limit of the concentrations tested (up to 200 $\mu$M) over 24h. The mortality of *B. plicatilis* was not affected by zinc sulphate concentrations up to 100 $\mu$M, whereas copper chloride and copper sulphate had much higher toxicities with mortality first observed at 5 $\mu$M for both compounds. These results confirm that the concentrations of up to 1 $\mu$M of copper sulphate, copper chloride and zinc sulphate that were assayed in combination with decadienal were sublethal for both *A. salina* and *B. plicatilis*. As a full range of mortalities was obtained for the *B. plicatilis* assays with the two copper salts over the concentration range tested, the LD$_{50}$ values were calculated. This showed slightly
higher toxicity of copper sulphate with an LD\(_{50}\) of 7.9 µM compared to the LD\(_{50}\) of 14.2 µM for copper chloride.

Mortality increased significantly with exposure to increasing concentrations of decadienal in all of the mixture toxicity assays. The presence of sublethal levels of the metal salts resulted in a significantly increased mortality of both \(B.\ plicatilis\) and \(A.\ salina\) exposed to decadienal and there were significant positive interaction terms for all of the metal salts in the \(A.\ salina\) mixture assay logit regression models (Table 4). The latter implies an increased mortality response of the \(A.\ salina\) to decadienal with increasing sublethal metal salt concentration, i.e. increasing the metal salt concentration results in a decreased LD\(_{50}\) value of decadienal for \(A.\ salina\) (Figure 2). There was no significant interaction term in the regression of the \(B.\ plicatilis\) data however the odds ratio for the copper sulphate concentration was considerably larger that of the \(A.\ salina\) assay (Table 4). These dissimilarities between the two species reflect a different relationship between copper sulphate concentration and mortality in decadienal, expressed in Figure 2 by the difference in shapes of the lines. Despite this, the presence of the highest concentration of copper sulphate tested (1 µM) affected the mortality of the two organisms in decadienal to a similar extent with a decrease of approximately a third in the 24 h LD\(_{50}\) value of decadienal for both species, compared to the LD\(_{50}\) value in the absence of copper sulphate (Figure 2). The odds ratios for the interaction terms of decadienal with copper chloride and zinc sulphate for the \(A.\ salina\) mixture assays show overlapping 95% confidence intervals and are both far lower than that of copper sulphate (Table 4). This implies a much less dramatic, albeit still significant interactive effect of the two salts on brine shrimp mortality in decadienal. This is demonstrated in Figure 2 with an 11% and 3% reduction in the 24 h LD\(_{50}\) of decadienal with the presence of 1 µM of copper chloride and zinc sulphate respectively compared to the 33% reduction with 1 µM copper sulphate (Figure 2). However, the mortality of the two organisms in decadienal without the
presence of copper sulphate was not affected by pre-exposure for 24 h to copper sulphate at 1 µM (Table 5).

Discussion

A wide range of toxicities were observed between the aldehydes tested for both species. Schultz and Cronin (1999) in a similar study on the toxicity of various aliphatic aldehydes to the protozoon *Tetrahymena pyriformis*, observed a strong relationship of increasing toxicity with longer carbon chains and the presence of double bonds. This relationship was observed in the current work but to a lesser extent and there were differences in this relationship between the two test organisms. There was a strong relationship of increased PUA toxicity with increasing carbon-chain length evident for *A. salina* and the saturated aldehydes showed lower toxicity relative to the PUA of similar carbon chain length (Figure 1). The increased toxicity of decadienal relative to decanal and undecanal concurs with previous work by Caldwell et al. (2003), although, in the current work there were notably smaller difference in the toxicities of the decadienal and saturated aldehydes. The acute-toxicity of the aldehydes to *A. salina* overall, however, shows a weaker dependence on the presence of carbon-carbon double bonds than reported in previous studies (Pohnert et al., 2002; Adolph et al., 2003, 2004). In contrast, the toxicity of the aldehydes to *B. plicatilis* appeared to be more dependent on the presence of carbon-carbon double bonds than carbon-chain length with both saturated aldehydes showing lower toxicity than the three PUAs (Figure 1). This concurs with the results of other studies into the impact of algal-derived aldehydes on the development of organisms (Pohnert et al., 2002; Adolph et al., 2003, 2004).

Immersion assays, whilst providing a simple and easily comparable method of assessing toxicity, may not entirely reflect the actual mechanism of aldehyde toxicity. For example,
organisms feeding on toxic algae are likely to effectively ingest a large proportion of the
Toxins rather than being directly exposed to the toxins in solution (Caldwell et al., 2004b).
This may explain some of the discrepancies observed between the current work and some
other studies into sublethal effects of aldehydes. However, many newly hatched
invertebrate larvae are non-feeding and so would not be exposed to the algal toxins via
grazing. Observations have been made of so-called “sloppy feeding” of zooplankton and
leakage from zooplankton faecal pellets resulting in the release of dissolved organic
matter into the water (Kasamatsu et al., 2004; Møller et al., 2003). This could provide one
possible mechanism for the direct exposure of newly-hatched larvae to the toxins in the
field.

_A. salina_ and _B. plicatilis_ were exposed directly to the aldehydes in solution; therefore
dissimilarities in the permeability of integument as well as in detoxification abilities and
general tolerance to toxic compounds could explain the different order of toxicities for the
aldehydes between the two test organisms. For example _A. salina_ nauplii possess a highly
impermeable chitinous integument and are relatively tolerant to toxins whereas _B._
plicatilis has a much thinner chitinous outer layer (Yu and Cui, 1997). This is reflected in
the generally lower tolerance of _B. plicatilis_ to the aldehydes in comparison with _A._
salina. Unlike the between species variation in aldehyde toxicity, differences within the	wo species in the toxicity of the five aldehydes in relation to their chemical structure may
be explained by the behaviour of the aldehydes in solution. The basis of aldehyde acute-
toxicity is the interaction of the aldehyde functional groups with biological molecules,
such as amine, sulphydryl or hydroxyl containing molecules, within an organism/cell
resulting in a disruption of their function. For example, aldehydes react with the amine
groups of amino acids, proteins and enzymes to form imines or Schiff bases (Bender and
Brubacher, 1973; Bisignano et al., 2001). When in solution a reversible reaction can occur
between aldehydes and water resulting in the formation of hydrates that do not undergo
reactions with the biological molecules. The proportion of aldehyde to hydrate in solution is affected by both the carbon chain length and the degree of saturation, with a greater proportion of hydrates formed with saturated aldehydes of shorter chain length (Brown, 1976). The Michael acceptor properties of the PUAs tested, i.e. conjugation of a carbon-carbon double bond to the aldehyde functional group, also confers increased reactivity with nucleophillic groups such as amines (Lipnick, 1991).

When in combination with decadienal, sublethal levels of copper sulphate caused an increase in the mortality of both *A. salina* and *B. plicatilis* (Table 4). Figure 2 demonstrates the impact of the copper sulphate on the toxicity of decadienal to *A. salina* showing the characteristic curved line of a synergistic interaction. In contrast, the lack of a significant interaction term in the *B. plicatilis* mixture assay, evident in the straight line in Figure 2 implies a more simple additive effect of the copper sulphate. However, the copper sulphate concentrations utilised were sublethal when tested on both species without the presence of decadienal. If the actual impact of copper sulphate upon the mortality of the *B. plicatilis* was simply additive, then there should be no significant impact of copper sulphate on mortality. It is possible that the high significance of copper sulphate as a single variable could be masking an interaction between the copper salt and decadienal in the *B. plicatilis* mixture assays. Despite this difference, the presence of 1 µM copper sulphate had an impact of a similar magnitude on the mortality of both organisms in decadienal, reducing the LD$_{50}$ of decadienal by approximately one third.

It has been assumed that the copper is the substance causing the observed effect on mortality in decadienal and the lower impact of zinc sulphate seems to confirm that the sulphate component of copper sulphate is having little or no effect. However, following this reasoning, the reduced impact of copper chloride compared to copper sulphate for *A. salina* also implies that the copper is not responsible for the observed effect. The addition of 1 µM sulphate to a litre of seawater represents an increase in the water sulphate content
of only 0.004% whereas 1 µM copper is proportionally a far greater increase of
approximately 7000% due to the lower basal concentrations of copper in natural seawater.
This along with the greater toxicity of copper to marine organisms suggests that it is more
probable that the copper is responsible for the observed synergistic effects. A possible
reason for the differing impact of the copper salts is discussed below.

The sublethal effects of copper upon marine organisms have been well documented, for
example it affects organogenesis and ion-exchange abilities of developing brine shrimp
nauplii (Spicer, 1995) and modifies the swimming behaviour of the freshwater rotifer,
Brachionus calyciflorus (Charoy and Janssen, 1999). It is possible that the sublethal
physiological effects of copper could indirectly reduce the ability of both species to
tolerate decadienal. However, pre-exposure of both organisms to 1 µM copper sulphate
for 24 h had no significant impact on their subsequent mortality when exposed to
decadienal without the presence of copper sulphate (Table 5). As well as this there were
no differences observed in the upper limits of the sublethal concentrations of copper
sulphate and copper chloride for B. plicatilis and Saliba and Krzyz (1976) reported that
copper sulphate was actually of lower acute-toxicity than copper chloride for A. salina.

Following this, if the direct toxic effects of the copper salts upon A. salina were an
important factor copper chloride should have shown a greater impact upon the mortality
of A. salina in decadienal. These two facts suggest that there is a more direct interaction
between the aldehyde and metal salts in the mixture assays causing at least part of the
observed effect. It is, however, unlikely that there is any direct chemical reaction between
the aldehydes and copper as such a reaction only occurs in highly acidic conditions and
the products of the reaction, carboxylic acids are generally of lower toxicity than
aldehydes (Brown, 1976). In solution, copper sulphate forms separate ions of copper (II)
and sulphate. Copper (II) is a well-documented catalyst for many chemical reactions. It is
possible that the copper may have acted as a catalyst in the reaction between decadienal
and the biological molecules of the test organisms, increasing the rate of reaction and thus
effectively acting synergistically on the toxicity of the decadienal. Following this, as
chloride ions are more effective complexing agents than sulphate ions they would bind
more of the copper (II) in complexes in solution reducing the bioavailability of the
catalytic form (Gardner and Comber, 2003). The addition of such low concentrations of
the salts in seawater relative to the natural levels already present, however, are unlikely to
have an extensive effect upon the chemistry of the copper (Lofts, pers comm.).

Despite the differences in toxicities of the PUAs tested, it could be assumed that they
have a toxic mechanism based on similar principles. The addition of the heavy metals
would therefore be likely to exert a similar impact on the mortality of organisms exposed
to aldehydes other than decadienal. Copper concentrations in polluted coastal regions can
frequently exceed the environmental quality standard of approximately 5 µg.l⁻¹ (80 nM)
reaching concentrations of twenty times this (1.6 µM), for example, in coastal regions of
Tyne and Wear (UK) (Environmental Agency, pers. comm.). These concentrations are
above those tested in the current work in combination with decadienal and therefore the
potential exists for harmful synergistic effects of copper upon organisms exposed to algal
aldehydes in the marine environment. There have been no reports into the aldehyde
concentration in the seawater occupied by a toxic algal bloom, however, Wichard et al.
(2004) have reported the production of decatrienal, a ten carbon PUA of similar toxicity
to decadienal, at approximately 25 to 30 fmol.cell⁻¹ in a natural phytoplankton sample.
The base LD₅₀ values of decadienal for A. salina and B. plicatilis are 20 and 7 µM
respectively, assuming an effective aldehyde production of 30 fmol.cell⁻¹, cell densities of
6.7x10⁵ and 2.3x10⁵ cells.ml⁻¹ could, upon damage, produce enough toxins to cause 50%
mortality of A. salina and B. plicatilis. The addition of copper sulphate would reduce
these values to 4.5x10⁵ and 1.6x10⁵ cells.ml⁻¹ respectively. These are high cell counts
even in bloom conditions and less direct mechanisms of exposure of non-feeding
invertebrate larvae to the toxins as discussed earlier would mean higher cell concentrations would be required to produce the same effective aldehyde concentration. However this work measured acute-toxic effects, whereas sublethal effects of the aldehydes upon reproduction would occur at much lower levels and with reduced exposure time (Pohnert et al., 2002; Romano et al., 2003; Caldwell et al., 2004a; Ianora et al., 2004b). In the field, ingestion and potential accumulation of a large proportion of the aldehyde toxins by zooplankton grazing on toxin producing microalgae could increase the effectiveness of the toxins by removing the barrier of the organism’s integument. Again, this may mean that lower concentrations of toxin producing algal cells would be required to exert a negative impact on actively grazing invertebrates. Finally, study has only examined the possible impact of one pollutant. Schröder et al. (1998) for example have reported a similarly detrimental effect showing that caulerpin, a toxin of the green algae Caulerpa taxifolia, inhibits the ability of the marine sponge, Geodia cydonium, to resist tributyltin by affecting its detoxification abilities. With the cocktail of pollutants present in coastal regions and the possibility of numerous interactions between compounds in the aquatic environment, pollutants could potentially have an important impact on interactions between toxin producing algae and their grazers and this should be taken into consideration when studying organism interactions in the field.

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Table 1

Logit regression coefficients and odds ratios for 24 h acute-toxicity assays of aldehydes with *Artemia salina* nauplii (** = significant to <0.001).

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>Predictors</th>
<th>Estimate ± SE</th>
<th>Z</th>
<th>Odds ratio and 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decadienal</td>
<td>Intercept</td>
<td>-4.81 ± 0.32</td>
<td>-15.17**</td>
<td>1.278 (1.241, 1.316)</td>
</tr>
<tr>
<td></td>
<td>Concentration (µM)</td>
<td>0.245 ± 0.015</td>
<td>16.09**</td>
<td></td>
</tr>
<tr>
<td>Octadienal</td>
<td>Intercept</td>
<td>-2.46 ± 0.19</td>
<td>-13.18**</td>
<td>1.022 (1.020, 1.024)</td>
</tr>
<tr>
<td></td>
<td>Concentration (µM)</td>
<td>0.022 ± 0.001</td>
<td>16.40**</td>
<td></td>
</tr>
<tr>
<td>Heptadienal</td>
<td>Intercept</td>
<td>-3.33 ± 0.21</td>
<td>-15.48**</td>
<td>1.012 (1.010, 1.014)</td>
</tr>
<tr>
<td></td>
<td>Concentration (µM)</td>
<td>0.012 ± 0.001</td>
<td>14.70**</td>
<td></td>
</tr>
<tr>
<td>Decanal</td>
<td>Intercept</td>
<td>-3.49 ± 0.32</td>
<td>-11.01**</td>
<td>1.044 (1.038, 1.050)</td>
</tr>
<tr>
<td></td>
<td>Concentration (µM)</td>
<td>0.043 ± 0.003</td>
<td>12.67**</td>
<td></td>
</tr>
<tr>
<td>Undecanal</td>
<td>Intercept</td>
<td>-2.46 ± 0.24</td>
<td>-10.19**</td>
<td>1.033 (1.028, 1.037)</td>
</tr>
<tr>
<td></td>
<td>Concentration (µM)</td>
<td>0.032 ± 0.002</td>
<td>13.05**</td>
<td></td>
</tr>
</tbody>
</table>
### Table 2

Logit regression coefficients and odds ratio for 24 h acute-toxicity assays of aldehydes with *Brachionus plicatilis* (** = significant to <0.001, * = significant to <0.05).

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th>Predictors</th>
<th>Estimate ± SE</th>
<th>Z</th>
<th>Odds ratio and 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decadienal</td>
<td>Intercept Concentration (µM)</td>
<td>-3.41 ± 0.29</td>
<td>-11.79**</td>
<td>1.619 (1.506, 1.741)</td>
</tr>
<tr>
<td></td>
<td>0.482 ± 0.037</td>
<td></td>
<td>13.00**</td>
<td></td>
</tr>
<tr>
<td>Octadienal</td>
<td>Intercept Concentration (µM)</td>
<td>-2.47 ± 0.19</td>
<td>-12.80**</td>
<td>1.068 (1.058, 1.079)</td>
</tr>
<tr>
<td></td>
<td>0.066 ± 0.005</td>
<td></td>
<td>12.00**</td>
<td></td>
</tr>
<tr>
<td>Heptadienal</td>
<td>Intercept Concentration (µM)</td>
<td>-2.97 ± 0.33</td>
<td>-9.07**</td>
<td>1.100 (1.078, 1.121)</td>
</tr>
<tr>
<td></td>
<td>0.095 ± 0.010</td>
<td></td>
<td>9.88**</td>
<td></td>
</tr>
<tr>
<td>Decanal</td>
<td>Intercept Concentration (µM)</td>
<td>-3.27 ± 0.23</td>
<td>-14.29**</td>
<td>1.019 (1.017, 1.021)</td>
</tr>
<tr>
<td></td>
<td>0.019 ± 0.001</td>
<td></td>
<td>13.27**</td>
<td></td>
</tr>
<tr>
<td>Undecanal</td>
<td>Intercept Concentration (µM)</td>
<td>-3.05 ± 0.19</td>
<td>-15.83**</td>
<td>1.044 (1.038, 1.050)</td>
</tr>
<tr>
<td></td>
<td>0.043 ± 0.003</td>
<td></td>
<td>15.59**</td>
<td></td>
</tr>
</tbody>
</table>
Table 3

Deviance and dispersion values for the logit regressions for two test species assayed in aldehydes and aldehyde-metal salt combinations. A dispersion of approximately 1 indicates assumptions of binomial data are acceptable.

<table>
<thead>
<tr>
<th>Test species</th>
<th>Assay solution</th>
<th>Deviance (degrees of freedom)</th>
<th>Dispersion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Null</td>
<td>Residual</td>
</tr>
<tr>
<td><strong>Artemia salina</strong></td>
<td>Decadienal</td>
<td>758.265 (47)</td>
<td>68.911 (46)</td>
</tr>
<tr>
<td></td>
<td>Octadienal</td>
<td>530.21 (65)</td>
<td>110.56 (64)</td>
</tr>
<tr>
<td></td>
<td>Heptadienal</td>
<td>434.02 (67)</td>
<td>107.8 (66)</td>
</tr>
<tr>
<td></td>
<td>Decanal</td>
<td>655.549 (47)</td>
<td>32.201 (46)</td>
</tr>
<tr>
<td></td>
<td>Undecanal</td>
<td>587.465 (45)</td>
<td>67.986 (44)</td>
</tr>
<tr>
<td></td>
<td>Decadienal and CuSO$_4$</td>
<td>5589.02 (231)</td>
<td>365.23 (228)</td>
</tr>
<tr>
<td></td>
<td>Decadienal and CuCl$_2$</td>
<td>4965.69 (231)</td>
<td>417.68 (228)</td>
</tr>
<tr>
<td></td>
<td>Decadienal and ZnSO$_4$</td>
<td>4031.69 (235)</td>
<td>375.09 (232)</td>
</tr>
<tr>
<td><strong>Brachionus plicatilis</strong></td>
<td>Decadienal</td>
<td>664.912 (55)</td>
<td>64.206 (54)</td>
</tr>
<tr>
<td></td>
<td>Octadienal</td>
<td>553.337 (53)</td>
<td>78.928 (52)</td>
</tr>
<tr>
<td></td>
<td>Heptadienal</td>
<td>501.976 (48)</td>
<td>53.668 (47)</td>
</tr>
<tr>
<td></td>
<td>Decanal</td>
<td>368.642 (66)</td>
<td>83.952 (65)</td>
</tr>
<tr>
<td></td>
<td>Undecanal</td>
<td>893.356 (66)</td>
<td>82.691 (65)</td>
</tr>
<tr>
<td></td>
<td>Decadienal and CuSO$_4$</td>
<td>2960.72 (239)</td>
<td>223.25 (236)</td>
</tr>
</tbody>
</table>
Table 4
Logit regression coefficients and odds ratio for 24 h acute-toxicity assays of decadienal in combination with different metal salts (\(^*\) = significant to <0.001, \(^*\) = significant to <0.05, \(\text{ns}\) = not significant). Odds ratios for non-significant coefficients are excluded.

<table>
<thead>
<tr>
<th>Test species</th>
<th>Metal salt</th>
<th>Predictors</th>
<th>Estimate ± SE</th>
<th>Z</th>
<th>Odds ratio and 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Intercept</td>
<td>-6.25 ± 0.37</td>
<td>-16.92(^*)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decadienal</td>
<td>0.237 ± 0.015</td>
<td>15.68(^*)</td>
<td>1.267 (1.231, 1.305)</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>Metal salt</td>
<td>-2.17 ± 0.70</td>
<td>-3.12(^*)</td>
<td>0.114 (0.029, 0.450)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Interaction</td>
<td>0.239 ± 0.033</td>
<td>7.21(^*)</td>
<td>1.270 (1.190, 1.355)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intercept</td>
<td>-4.26 ± 0.24</td>
<td>-17.45(^*)</td>
<td>NA</td>
</tr>
<tr>
<td>Artemia salina</td>
<td>Copper chloride</td>
<td>Decadienal</td>
<td>0.291 ± 0.017</td>
<td>17.07(^*)</td>
<td>1.338 (1.315, 1.361)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metal salt</td>
<td>-0.53 ± 0.43</td>
<td>-1.23(\text{ns})</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Interaction</td>
<td>0.076 ± 0.031</td>
<td>2.42(^*)</td>
<td>1.079 (1.046, 1.113)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intercept</td>
<td>-4.06 ± 0.24</td>
<td>-16.91(^*)</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decadienal</td>
<td>0.205 ± 0.012</td>
<td>17.56(^*)</td>
<td>1.228 (1.199, 1.257)</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>Metal salt</td>
<td>-1.25 ± 0.44</td>
<td>-2.88(\text{ns})</td>
<td>0.287 (0.121, 0.679)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Interaction</td>
<td>0.072 ± 0.022</td>
<td>3.31(^*)</td>
<td>1.075 (1.029, 1.122)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intercept</td>
<td>-3.98 ± 0.25</td>
<td>-15.86(^*)</td>
<td>NA</td>
</tr>
<tr>
<td>Brachionus plicatilis</td>
<td>Copper sulphate</td>
<td>Decadienal</td>
<td>0.775 ± 0.052</td>
<td>14.83(^*)</td>
<td>2.171 (2.061, 2.286)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metal salt</td>
<td>1.46 ± 0.36</td>
<td>4.05(^*)</td>
<td>4.306 (3.004, 6.172)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Interaction</td>
<td>-0.084 ± 0.082</td>
<td>-1.02(\text{ns})</td>
<td>--</td>
</tr>
</tbody>
</table>
**Table 5**

The logit regression output of 24 h acute-toxicity of decadienal using animals pre-
exposed to 1µM copper sulphate or filtered seawater for 24 h (** = significant to <0.001,  
\( ns \) = not significant)

<table>
<thead>
<tr>
<th>Test species</th>
<th>Predictors</th>
<th>Estimate ± SE</th>
<th>Z</th>
<th>Deviance (degrees of freedom)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Null</td>
</tr>
<tr>
<td>Artemia salina</td>
<td>Intercept</td>
<td>-5.69 ± 0.43</td>
<td>-5.30**</td>
<td>1909 (103)</td>
</tr>
<tr>
<td></td>
<td>Decadienal</td>
<td>0.325 ± 0.023</td>
<td>4.92**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pre-exposure</td>
<td>0.595 ± 0.544</td>
<td>-1.69 ns</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Interaction</td>
<td>-0.055 ± 0.029</td>
<td>1.56 ns</td>
<td></td>
</tr>
<tr>
<td>Brachionus plicatilis</td>
<td>Intercept</td>
<td>-2.67 ± 0.50</td>
<td>-13.15**</td>
<td>1026 (106)</td>
</tr>
<tr>
<td></td>
<td>Decadienal</td>
<td>0.283 ± 0.058</td>
<td>13.89**</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pre-exposure</td>
<td>-0.585 ± 0.347</td>
<td>1.09 ns</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Interaction</td>
<td>0.061 ± 0.039</td>
<td>-1.92 ns</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

![Graph showing the relationship between metal salt concentration (µM) and Decadienal LD\textsubscript{50} (% change).]
**Figure Legends**

**Figure 1:** 24 h LD$_{50}$ values and standard error bars for *Artemia salina* nauplii (solid squares) and *Brachionus plicatilis* (empty diamonds) exposed to five algal-derived aldehydes.

**Figure 2:** Percentage change in the 24 h LD$_{50}$ value of decadienal to *Artemia salina* nauplii with the addition of copper sulphate (dot-dashed line), copper chloride (dashed line) and zinc sulphate (dot line) and to *Brachionus plicatilis* in with the addition of copper sulphate (solid line).