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TOXICOLOGICAL EFFECTS OF FUNGICIDE MIXTURES ON THE AMPHIPOD *AUSTROCHILTONIA SUBTENUIS*[1]

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Abstract

Approaches to assess the toxicity of mixtures often use predictive models with acute mortality as an endpoint at relatively high concentrations. However, these approaches do not reflect realistic situations where organisms could be exposed to chemical mixtures over long periods at low concentrations at which no significant mortalities occur. The present study investigated chronic effects of 2 common fungicides, Filan® (active ingredient [a.i]) boscalid) and Systhane™ (a.i. myclobutanil), on the amphipod *Austrochiltonia subtenuis* at environmentally relevant concentrations under laboratory conditions. Sexually mature amphipods were exposed singly and in combination to Filan (1, 10, and 40 µg a.i./L) and Systhane (3µg a.i./L) over 28 d. Survival, growth, a wide range of reproduction endpoints, and glutathione-*S*-transferase (GST) activity were measured at the end of the experiment. Both fungicides had significant independent effects on male growth, sex ratio, and juvenile size. Filan mainly affected female growth and the number of embryos per gravid female while Systhane mainly affected the time for females to become gravid. The combined effects of these fungicides on numbers of gravid females and juveniles were antagonistic, causing a 61% reduction in the number of gravid females and a 77% reduction in the number of juveniles produced at the highest concentrations (40 µg a.i./L of boscalid and 3µg a.i./L of myclobutanil) compared with the controls. There were no significant effects on survival or GST activity. The present study demonstrated that the effects of mixtures were endpoint dependent and that using a variety of endpoints should be considered for a comprehensive understanding of mixture effects. Also, chronic studies are more informative than acute studies for environmentally relevant fungicide concentrations.

Keywords: Mixture toxicology, Pesticides, Aquatic invertebrates, Reproductive toxicity

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INTRODUCTION

Fungicides are a group of pesticides that are widely used in agriculture to protect plants from fungal infection. They have become an important component of plant disease management plans for agronomic crops, because fungal diseases have the potential to destroy crops, rendering them unsaleable [1]. Most modern fungicides have a single-site mode of action because this is associated with lower potential for negative impact on the environment, including nontarget organisms [2]. However, this can lead to greater fungal resistance because just a single gene mutation can alter a target site and reduce the vulnerability of fungi to the fungicide [3]. To resolve this problem, one common and effective strategy used in pesticide resistance management programs is to apply mixtures of fungicides with different modes of action [4]. As a result, fungicides with different modes of action are often detected simultaneously in agricultural areas.

The interaction effects of chemical mixtures are an area of great concern to both the public and regulatory authorities [5], leading to numerous studies of chemical mixtures over the past few decades [6]. Typical approaches to access the toxicity of mixtures often use predictive models, such as concentration addition and independent action with acute mortality as an endpoint [6,7]. These approaches are mathematical rather than biological in nature [8], and cannot explain observed interactions nor explain why mixture effects can change in time and between endpoints [7]. The concentrations used in these approaches often exceed environmentally relevant concentrations [5]. However, in reality, organisms are typically exposed to mixtures of chemicals over long periods [9] at relatively low concentrations [5]. The

main difficulties in studying chronic mixture toxicity are the complex nature of toxic mechanisms, as well as time and resource limitations [6,9]. At present, relatively few studies have observed the effects of chronic mixtures on aquatic organisms compared with acute mixture effects [9–12]. These studies have demonstrated that the toxicity of mixtures varies with duration of exposure, and the chronic mixture effects could not be predicted from acute mixture effects or chronic effects of individual chemicals.

Synergistic effects (toxicity of the mixture is greater than predicted [5]) are a specific concern in joint toxicity studies because of the potential of individual contaminants to increase the toxicity in combination [6]. In the literature, many studies were conducted to assess and predict mixture effects as well as identify the chemical groups that have high potential to cause synergistic effects [5]. The azole fungicides constitute one pesticide group over-represented in the synergistic mixtures [5]. They are known to interfere with a broad range of cytochrome P450 monooxygenases that are present in almost all living cells and are enzymes responsible for the phase I biotransformation of lipophilic compounds [5]. Hence the toxicity of lipophilic compounds is often substantially enhanced when in the presence of azole fungicides [5]. Azoles have been used extensively in agriculture not only for preventing fungal infection but also for fungal treatment [13]. One main reason for their widespread use in agriculture is their long-lasting stability [14] with half-lives in soil ranging from a month to more than a year [15,16]. Consequently, they have been frequently detected in natural environments at low nanograms to several micrograms per liter levels [17–19]. A few studies have investigated the joint toxicity of azoles fungicides with other pesticides [20–22] and have reported synergistic effects on aquatic organisms on different endpoints. Azole fungicides have been shown to enhance the effect of a pyrethroid insecticide, alpha-cypermethrin, toward *Daphnia magna* in the immobilization test up

to 12-fold (prochloraz) [20] or 13-fold (propiconazole) [22]. Zubrod et al. [21] observed a synergistic effect on the feeding of *Gammarus fossarum* (~35% deviation between predicted and observed effect) after exposure to a mixture of 5 fungicides including an azole fungicide, tebuconazole, at a total concentration of 160 µg/L for 7 d. However, these studies are based on acute experiments or non-environmentally relevant concentrations. To our knowledge, there is a lack of chronic studies that directly measure toxicity of azole mixtures at environmentally relevant concentrations.

Myclobutanil is an azole fungicide widely used in agriculture because of its broad spectrum of antifungal activity that is effective against a wide range of fungal infections in crops, seeds, fruits, and horticultural production systems [14]. Boscalid is another commonly used fungicide in agriculture because it is a systemic fungicide that is active against a broad range of fungal pathogens [23]. Both fungicides are quite stable in aquatic environments [15] and have been detected frequently in water and sediment in agricultural watersheds with very high detection rates in different areas of the world [17–19,24–27]. Boscalid and myclobutanil have also often been found to co-occur in streams [17–19]. The adverse individual effects of boscalid or mycobutanil on aquatic invertebrates have been described [23,28], but no available studies have assessed the joint effect of these fungicides.

The present study investigated the chronic effects of the fungicides Filan® (active ingredient [a.i.] boscalid) and Systhane™ (a.i. myclobutanil), singly and in combination, on a freshwater amphipod *Austrochiltonia subtenuis*, at environmentally relevant concentrations. The

first objective was to investigate the long-term interaction effects of Filan and Systhane on mature *A. subtenuis* at environmentally realistic concentrations using a wide range of endpoints that span different levels of biological organization. Endpoints assessed were organism-level responses (survival), physiological responses (reproduction and growth), and suborganism level responses (using glutathione-*S*-transferase [GST]) to look at sensitivity in response to low fungicide concentrations. The second objective was to evaluate how the results of mixture studies vary between endpoints to propose suitable endpoints for mixture toxicity studies.

MATERIALS AND METHODS

Chemicals

The commercially available product Filan fungicide (Nufarm), containing 500 g a.i./kg, was used for boscalid exposures, and Systhane 400 WP fungicide (Dow AgroSciences), containing 400g a.i./kg, was used for mycobutanil exposures. Filan and Systhane were dissolved in deionized water to make a stock solution with a nominal concentration of 50 mg a.i./L. The stock solution was diluted in stream water to achieve nominal boscalid concentrations of 1, 10, and 40 µg a.i./L and mycobutanil concentration of 3 µg a.i./L. The concentrations of boscalid and myclobutanil were based on the concentrations detected in the natural environment, which ranged from 2.9 to 36 µg/L [24,26,29]. Both stock and test media were prepared immediately prior to the initiation of testing and before water changes. Water samples were collected before the experiment and at each water change. Because boscalid and myclobutanil are persistent in aquatic environments [15], their concentrations would not substantially change during the experiment. Samples from each treatment of the first week were sent to the School of Chemistry at The University of Melbourne (VIC, Australia) for analysis of boscalid and myclobutanil concentration by gas chromatrography–mass spectrometry. Measured concentrations were within

30% of nominal concentrations (Supplemental Data, Table S1). In the present study, reported concentrations are nominal concentrations.

Preconditioned leaves

Green hazel (*Pomaderris aspera*) leaves were picked from trees in the Royal Botanic Gardens Melbourne, Victoria, Australia. Leaves were cut into leaf discs (diameter 1.3 cm) by hole punch, air-dried, and stored at room temperature until use. Before the experiment, leaf discs were preconditioned for 2 wk in a 2-L beaker containing 1 L of nutrient-enriched stream water (5 mg P as K_2HPO_4, 20 mg N as $(NH_4)_2SO_4$) [30]. This process was carried out weekly to provide freshly preconditioned leaves for the amphipods throughout the duration of the present study.

Test species

Austrochiltonia subtenuis and water used in the experiment were collected from a nonpolluted stream, Deep Creek, Victoria, Australia. Amphipods were maintained in the laboratory at experimental conditions: temperature (21 ± 1 °C) and a 16:8-h light:dark photoperiod in 5-L glass aquaria with site water under constant aeration. Organisms were fed preconditioned hazel leaves ad libitum and 6 mg ground TetraMin fish food/L 3 times/wk. To obtain a known-age of amphipods for the experiment, gravid females were separated after 2 wk of acclimatization into clean 2-L glass beakers. One week later, the resulting juveniles were transferred to new 2-L glass beakers and maintained as described**<ZAQ;1>** above. The sex of less than 7-wk-old amphipods was determined under the microscope, with male amphipods distinguished from females by the second enlarged gnathopods [31]. Following this, amphipods were maintained separately for another week to recover from identification stress before being used in the experiment. The collected site water was kept at 4 °C and brought to room temperature prior to use in the experiment.

Experimental setup

The present study was a 4 (Filan fungicide: 0, 1, 10, and 40 µg a.i./L) by 2 (Systhane fungicide: 0 and 3 µg a.i./L) factorial design. Fourteen less than 8-wk-old *A. subtenuis* individuals, 7 males and 7 females, were placed randomly in 600-mL glass beakers containing 400 mL of fungicide-dosed aerated stream water. Each beaker contained 2 leaf discs (diameter 1.3 cm) as a food source and a 5 × 5-cm presoaked cotton gauze as a substrate for the amphipods. Ground TetraMin fish food was added 3 times/wk as 2.5 mg/beaker to provide additional food. Each treatment had 4 replicates. The experiment was run for 28 d using the same conditions as described in the *Test species* section. Every week, surviving amphipods were gently transferred by plastic pipette to freshly prepared test medium with fresh preconditioned leaves. Numbers of gravid females were recorded. Juveniles were counted, removed, and preserved in 70% ethanol for later size analysis based on head length (from the rostrum tip to the posterior margin of the head) [32].

At the end of the experiment, one nongravid female and one male were randomly selected from each replicate and frozen at –80 °C for GST analysis. The remaining surviving adults were preserved in 70% ethanol for further examination using a Leica MS5 microscope with an ocular micrometer. Specimens preserved in ethanol were sexed, and head length was measured to determine growth based on the final size, with the assumption that the mean size of amphipods per replicate was the same at the beginning of the experiment, because the amphipods were the same age. The number of gravid females and the number of embryos produced per gravid female were recorded. Embryo development stages were identified and followed Mann et al. [33].

GST analysis

The activity of GST was determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate as described by Habig et al. [34] and Long et al. [35] using a Synergy 2 microplate reader (Biotek Instruments). Briefly, individual amphipods (4 males and 4 females/treatment) were homogenized in 60 µL (for females) or 80 µL (for males) of 0.1 M phosphate buffer pH 6.5 (containing 1.4 mM 1,4-dithioerythritol and 1 mM ethylenediamine tetraacetic acid and 20% v/v glycerol). The homogenate was centrifuged at 4 °C and 10 000 rpm for 10 min. Activity of GST was determined following the conjugation of reduced glutathione (GSH) and CDNB at 340 nm, using a mM extinction coefficient of 6.72 (adjusted for the path length of the microplate reader), and an increase in absorbance over time was observed. The reaction buffer contained 0.1 M KH_2PO_4 (pH 6.5), 3 mM GSH, and 1 mM CDNB. The final volume in each well was 200 µL with 5 µL of supernatant. Supernatant was used to analyze protein content using a modified Lowry assay (Bio-Rad DC method) with bovine serum albumin as the standard **<ZAQ;2>**[36]. For all assays, each sample was analyzed in triplicate. Results are expressed as nmol GST activity/min/mg protein.

Statistical analysis

Two-way analysis of variance was used to determine interaction and independent effects of Filan and Systhane. All data were checked for normality using a Shapiro–Wilk test and homogeneity of variance using Levene's test. If there were significant interaction effects, pairwise comparisons were performed to determine the simple effects of Systhane at each Filan concentration, and the synergistic effect was analyzed based on the interaction between 2 trends of the means of the mixtures and Filan treatments. If there is a synergism, these trends will diverge [37]. Statistical analysis was performed using SPSS Ver 23 (IBM).

RESULTS AND DISCUSSION

Survival

Total survival. No significant differences in mortality were observed between treatments and the control after 7 or 28 d (Figure 1). Our previous work showed that both Filan and Systhane significantly reduced the survival of *A. subtenuis* at 10 and 0.3 µg a.i./L after 7 d of exposure, respectively [28]. However, in the present study, no significant effects were observed at even higher concentrations (40 µg a.i./L of Filan and 3 µg a.i./L of Systhane), both singly and in combination. The main reason for this difference is likely differences in animal age. In previous work, we used juvenile amphipods that were <2 wk old and in the present study, we used mature amphipods that were <8 wk old. Studies in the literature have shown that the response of organisms to toxicants can depend on their life stages [36,37], and juvenile organisms are generally more sensitive to fungicides than adults [38]. Our previous work with <2-wk-old amphipods together with the present study on <8-wk-old amphipods indicates that older life stages may be less sensitive than younger life stages in terms of survival. Mortality is a typical endpoint in many mixture studies [6,9], but sublethal endpoints such as reproduction and growth should be included in mixture studies because they are more sensitive than mortality and are important for assessing the effects of toxicant mixtures on population fitness in natural environments [5].

Sex-specific survival. The sex ratio (female:male) of mature amphipods at the end of the experiment was altered by the fungicide treatments, with relatively fewer females in treatments than in the controls (Figure 2). There was no interaction effect between Filan and Systhane on sex ratio, but individually each fungicide had strong main effects on sex ratio (Table 1). The sex ratio decreased by approximately 23% at 3 µg a.i./L of Systhane compared with the control. Filan also reduced the sex ratio, but significant effects were only observed at low concentrations

of 1 and 10 µg a.i./L, at which the sex ratio was reduced by approximately 39 and 22%, respectively.

The sex ratio data in the present study showed that mature female amphipods were more sensitive to fungicides than males. Sensitivity of female amphipods was also observed in the study by Conradi and Depledge [39], who reported that overall survival of the amphipod *Corophium volutator* was unaffected but female amphipod survival was significantly decreased when mature amphipods were exposed to zinc (0, 0.2, 0.4, 0.6, and 0.8 mg/L) for 45 d. McCahon and Pascoe [40] also reported that the 48-h median lethal concentration value for cadmium for sexually mature male *Gammarus pulex* was 12.8 times greater than for sexually mature females not carrying eggs or brooding unfertilized or stage 1 eggs. Reproduction is not without cost, in terms of both post reproductive survival and future reproductive potential [39]. Furthermore, energy requirements during oogenesis and brooding in females may be higher than during spermatogenesis in males, resulting in less energy available to cope with toxic stress in females [23]. Therefore, post reproductive females are often more susceptible than post reproductive males [41]. Juvenile amphipods are commonly used in laboratory toxicity tests because they are more sensitive than at mature stages, and it is also more difficult to control the age variation when using mature amphipods. However, the sensitivity of mature female amphipods should be considered in toxicity studies because a reduction in mature females could severely impact the population structure in natural environments.

Growth

Fungicide treatments significantly reduced head lengths of male, female, and juvenile amphipods (Figure 3). However, the effects differed among males, females, and juveniles. For males, there was no interaction effect, but the main effects of both fungicides on male head

lengths were statistically significant (Table 1). The male head lengths decreased by 4% at the highest concentration of Filan alone (40 µg a.i./L) and by 6% at 3 µg a.i./L of Systhane alone compared with controls. Similarly, the individual effects of Filan and Systhane on juvenile head length were significant (Table 1). There was a 9 and 3% reduction in juvenile head length at 40 µg a.i./L concentration of Filan and 3 µg a.i./L Systhane, respectively. For females, there was only a significant effect of Filan on head lengths at the highest concentration, 40 µg a.i./L (p = 0.008), which reduced head length by 4%.

Growth has been extensively used as an endpoint in toxicity tests of individual chemicals, but it has rarely been used in mixture toxicity tests. Studies have shown that growth is a sensitive parameter in chronic mixture toxicity [10] and that results may differ from the survival endpoint [10,12]. For example, Spehar et al. [10] reported no significant effect on survival of fathead minnows after exposure to metal mixtures (As, Cd, Cr, Cu, Hg, Pb) for 32 d but observed a decrease in growth (a 30% reduction in dry wt) compared with the control. In contrast, Bao et al. [12] observed a synergistic lethal effect but only an additive effect on the developmental time of copepod larvae after exposure to mixtures of Irgarol and Cu (940 and 50 µg/L) in an 18-d life cycle test. In the present study, even though we did not observe interaction effects of the 2 tested fungicides, the independent effects still demonstrated that growth was a more sensitive endpoint than survival. To our knowledge, the present study is the first one in aquatic toxicology that assesses the effects of a mixture on growth based on sex and life stages. It was clear that the response was sex-specific, in that both fungicides had individual effects on male growth but only Filan reduced female growth. Furthermore, Filan had a significant effect on male head length at 1, 10, and 40 µg a.i./L (p = 0.01, p = 0.019, p = 0.014, respectively), while a significant effect was observed only at the highest concentration of 40 µg a.i./L for females (p = 0.008). Our

previous studies have also shown that male and female amphipods respond differently to fungicide exposure in terms of growth [28]. The present study further indicated that juveniles were more sensitive to the tested fungicides in terms of growth than mature animals. The observed effect of fungicides on juveniles may have happened during embryonic development as well as in the post hatch period, because juveniles were collected weekly in our study. Embryos and newborn juveniles are often the most sensitive stages [39,42], and therefore fungicides are likely to cause more adverse effects on these life stages than mature adults.

Reproduction

Maturation. In the present study, maturation was investigated by 2 endpoints: time to become gravid and the number of gravid females.

Only Systhane had a significant effect on the time for females to become gravid (Table 1). It took approximately 1 wk longer than controls for females to become gravid at 3 µg a.i./L of Systhane (Supplemental Data, Figure S1).

The number of gravid females was significantly reduced in all fungicide treatments (Figure 4). There was a significant interaction effect between Filan and Systhane (Table 1) and the effect was antagonistic, because there was no significant difference between the number of gravid females in the mixture and in the Filan treatment with increasing Filan concentrations (Figure 4). It seems that Systhane was the major factor contributing to the reduction in number of gravid females, because the effect of Systhane was observed at 0 µg a.i./L of Filan ($F_{(1,24)} = 28.68$, $p < 0.001$), at which the reduction was approximately 61% compared with the control. In fact, the presence of Filan did not affect the cumulative number of gravid females in the fungicide mixtures.

The results in terms of time to become gravid and the number of gravid females

consistently showed that Systhane delayed amphipod maturation. Carrying a brood is likely to cost energy. If the energy status of a female is reduced (e.g., by stress) to the extent that by incubating a brood she jeopardizes her own survival, then her overall fitness may be increased by sacrificing the broodings and reproducing at a later date [39]. Current reproduction versus survival is the most prominent life-history trade-off for the animal, and current reproduction versus parental growth is also another possible trade-off [43] that has been observed in amphipods [44]. Thus, a possible explanation for the effects of Systhane on amphipod maturation could be the allocation of energy resources from reproduction to body maintenance or development, thereby increasing the likelihood of survival and growth by postponing the reproduction of brood. Our results in terms of female growth and survival strongly support this hypothesis because Systhane had no effects on female head length (Table 1) and female survival (data not shown). These findings are also in agreement with our previous study showing that juvenile amphipods exposed to Systhane (0.3, 3, and 30 µg a.i./L) reached maturation later than control amphipods [28].

Fecundity. Only Filan affected amphipod fecundity (number of embryos/gravid female; Table 1). The number of embryos/gravid female tended to decrease in all Filan treatments compared with the control (Figure 5), but a significant effect was observed only at 10 µg a.i./L. There was no evidence of an interaction between the fungicides.

Animal size has been shown to be important in reproductive success in a variety of species including amphipods, because the animals have to reach a certain size before reproduction can occur [39,45]. Amphipod reproductive success is closely linked to female [39,44] and male body sizes [44]. The present data on amphipod growth is consistent with the result for female fecundity. Both fungicides had individual effects on male size, but only Filan

had a significant effect on female size. As a result, only Filan had a significant effect on the number of embryos/gravid female. For single toxicants, the relationship between growth and reproduction has been extensively documented and discussed in the literature [23,39,44]; in mixture studies, growth and reproduction endpoints are rarely measured, but the relationship between them has been reported [11,46]. Growth has been considered a more useful parameter than reproduction, because determinations of the latter are generally subject to greater variations [46]. Moreover, if a chemical affects growth of the exposed organism, it thereby automatically alters the toxicokinetics of all mixture components and their effects on reproduction [7,46]. However, the relationship between growth and reproduction has been shown to exhibit a number of forms, each dependent on the manner in which energy is allocated between somatic (growth) and gametic (reproduction) tissue development. A proportional (linear) relationship between growth and reproduction could be seen if the energy allocations to growth and reproduction are affected similarly by toxicants [47]. For example, a proportional relationship between growth and reproduction was observed in an experiment in which *D. magna* was exposed to mixtures of 10 organic compounds with diverse modes of actions [46]. Conversely, the proportional relationship may not exist if growth and reproduction respond with differential sensitivity [39]. Cleuvers et al. [11] reported that mixture effects of 3 nonsteroidal anti-inflammatory drugs on *D. magna* growth were associated with reproduction except for one treatment where the *D. magna* body length increased with increasing mixture concentrations but reproduction clearly decreased at the same time. Further work needs to address the complicated relationship between growth and reproduction in mixture toxicity.

Fertility. In the present study, fertility was defined as cumulative number of viable juveniles per replicate, because the juveniles were collected on a weekly basis and the number of

females was only determined at the end of the experiment. There was a significant interaction effect between Filan and Systhane on the cumulative number of juveniles (Table 1), resulting in a 54 to 77% reduction of juveniles. Exposure to Systhane resulted in a decrease in the number of juveniles with increasing Filan concentrations (Figure 6). However, significant effects of Systhane on amphipod fertility were observed at 0, 1, and 10 µg a.i./L of Filan ($F_{(1,24)} = 83.74$, $p < 0.001$; $F_{(1,24)} = 23.13$, $p < 0.001$; $F_{(1,24)} = 4.71$, $p = 0.04$, respectively) but not at 40 µg a.i./L ($F_{(1,24)} = 2.22$, $p = 0.15$). The effect of the fungicide mixture on cumulative number of juveniles was antagonistic, because the difference between cumulative number of juveniles in the mixture and in the Filan treatment decreased with increasing Filan concentrations (Figure 6).

Amphipod fertility is affected by all the previously mentioned reproduction measurements. The decrease in the number of juveniles is closely related to the reduction in number of gravid females and the increasing time to become gravid. Even though there were no interaction effects of Filan and Systhane and only Filan had a main effect on amphipod fecundity (Table 1), the mean brood size between early and late stages of development could be reduced if female amphipods are exposed to contaminants while carrying eggs, because embryos developing in the maternal brood pouch could be a very sensitive life stage [42]. In the present study, the embryo development stage in fungicide treatments was mostly at early (1 and 2) and middle (3 and 4) stages (~77%); it is likely that the survivorship of embryos at the last stages (5 and 6) before hatching could be affected, resulting in a significant decline in the number of juveniles produced. Another plausible explanation is mortality of newborn juveniles. In the present study, juveniles were collected on a weekly basis so it is possible that mortality of newborn juveniles occurred in between collections.

Overall findings on reproduction showed that reproduction was the most sensitive and the

only endpoint at which interactions were observed. Even though Systhane is an azole fungicide that potentially causes synergistic effects, interaction effects observed in the present study were antagonistic. The present study used environmentally relevant concentrations, whereas the synergistic effects of azole fungicides observed in other studies occurred at lethal concentrations in acute tests [20–22]. It has been reported that true synergistic interactions are rare and often occur at high concentrations [5]. For example, Charles et al. [48] investigated the acute toxic effects of Cu and Ni on the amphipod *G. pulex*, reporting that toxicity was synergistic when both metals were exposed at equal lethal concentrations (LC1–90), but was antagonistic when Ni was used at sublethal concentrations. Furthermore, it has been reported that when one is dealing with compounds that act through different mechanisms in mixture studies, an increase in the sensitivity of the parameter studied will lead to a decrease in additivity of the joint action of the mixture [46]. Although the modes of action of Filan and Systhane on aquatic invertebrates are unclear, modes of fungicide action could predict analogous mechanisms of toxicity, target sites, and toxic effects for nonfungal species, because many biochemical pathways and processes are conserved across species [49]. Filan belongs to the succinate dehydrogenase inhibitor group of fungicides, which disrupt fungal respiration, while Systhane belongs to the demethylation inhibitor group, which inhibits sterol biosynthesis in fungal membranes [15]. This difference in modes of fungicide action could result in a decrease in the additivity of the interaction effect on reproduction. Nevertheless, the present reproductive results suggest that fungicides act selectively on reproduction variables; reproduction was the most sensitive parameter and could provide insight for a better understanding and evaluation of mixture effects in amphipods.

We have demonstrated that mixture toxicity of Filan and Systhane could be underestimated if only total mortality (both acute and chronic) is used, because no significant

effects were observed on survival of the amphipods, but significant effects were observed on growth, sex-specific survival, and reproduction.

GST activity

Activity of GST was altered in all fungicide treatments (Supplemental Data, Figure S2), but no significant effects were observed (Table 1). Such activity was 60% higher in males than in females in the control (Supplemental Data, Figure S2). For males, GST activity increased by approximately 60% in Systhane treatments compared with the treatments without Systhane, but no significant main effect was observed.

The GSTs play a significant role in detoxification across multiple kingdoms and phyla [50] and have been widely used as biomarkers to assess pesticide contamination [51], especially organochlorine compounds [52]. However, GSTs generally have not shown predictable behavior in invertebrates [51]. In crustaceans, GST activity was found to be elevated [52], inhibited [53], or not modified [54] after pesticide exposure. In addition to the natural seasonal and spatial variation, factors inherent to the endogenous characteristic of test species, such as size, gender, reproduction status, and sexual maturity can also influence biomarker response in many invertebrates [51,55]. In the present study we observed higher levels of GST in males than in females, in both the control and fungicide treatments. This is in agreement with the study conducted by Correia et al. [56], who found that glutathione-related enzyme activity in the amphipod *Gammarus locusta* was 50% lower in females than males. The difference in GST activity between genders may be related to size because the male amphipods are generally bigger than the females. In the present study, the head length of control amphipods was 33% longer in males than females. Depending on species, the size of the test organism may have different effects on level of GST activity. For example, Robillard at al. [57] found that GST activity

increased with the length of the mussel *Anodonta cygnea*. In contrast, Jemec et al. [55] reported that GST activity in *D. magna* decreased with size (directly related to age). The observed high variations in GST activity in both genders in the present study could be related to reproductive status. The present study used sexually mature amphipods, and during the experiment the amphipods may have experienced one or more reproductive cycles [58]. The amphipods that were randomly chosen for GST analysis may have been at different phases of the reproductive cycle, which may impact GST activity [59]. Our GST results imply that biomarkers may not always be more sensitive than whole-organism responses, and more endogenous characteristics of *A. subtenuis* need to be known before biochemical biomarkers are used in this species as diagnostic tools for environmental contamination assessments.

The present study and our 2 previous studies [23,28] report on the wide range of effects that the fungicides Filan and Systhane™ have on 2 Australian amphipod species, *Allorchestes compressa* and *A. subtenuis* at environmentally relevant concentrations under laboratory conditions. In summary, reproduction was the most sensitive endpoint for both single and mixture exposures. Growth was also significantly affected following fungicide exposure but varied depending on sex, fungicide concentration, type of fungicide, animal age, and test species. As expected, survival was less sensitive than reproduction or growth. Fungicide exposure caused no significant effects on survival of amphipods when they were exposed at the mature stage but caused significant effects when they were exposed as juveniles. Biochemical biomarkers were altered after fungicide exposure but varied depending on test species, type of fungicides, and sex.

CONCLUSIONS

In conclusion, both tested fungicides (Filan and Systhane) caused adverse effects on the growth and reproduction of the amphipod *A. subtenuis* singly and in combination at

environmentally relevant concentrations. Reproduction was the most sensitive endpoint and the only one where interaction effects of 2 fungicides were observed. However, these joint effects were antagonistic, and Systhane seems to be a major factor in these interaction effects. Our results demonstrated that the effects of fungicide mixtures were endpoint dependent and that a variety of endpoints should be considered for a comprehensive understanding of mixture effects, because different single endpoints could lead to a completely different interpretation. Measuring total mortality only (both acute and chronic) may be insufficient to assess the toxicity effects of mixtures at environmentally realistic concentrations. Growth and reproduction are important endpoints in chronic mixture studies, but toxicants may act selectively on reproduction variables, and growth alone is not a good indicator for overall population fitness. Biochemical biomarker endpoints may not always be more sensitive than whole-body endpoints, and their use should be evaluated based on the species and toxicants studied. The present study emphasizes the importance of chronic mixture studies and suggests that reproduction-related endpoints could provide better insights for understanding and evaluating mixture toxicity.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3809.

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Data Availability—Data, associated metadata, and calculation tools are available from the corresponding author (htvu@student.unimelb.edu.au).

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Figure 1. Percentage of survival (mean ± standard error) of *Austrochiltonia subtenuis* in control and fungicide treatments after 7 (**A**) and 28 (**B**) d exposure, $n = 4$. Blue bars are Filan-only treatments, and red bars are mixtures of Filan and Systhane.

Figure 2. Sex ratio (mean ± standard error) of *Austrochiltonia subtenuis* in control and fungicide treatments after 28 d of exposure, $n = 4$. Blue bars are Filan-only treatments, and red bars are mixtures of Filan and Systhane.

Figure 3. Head length of males (**A**), females (**B**), and juveniles (**C**), (mean ± standard error) of *Austrochiltonia subtenuis* in control and fungicide treatments after 28 d of exposure, $n = 4$. Blue bars are Filan-only treatments, and red bars are mixtures of Filan and Systhane.

Figure 4. Cumulative number of gravid females (mean ± standard error) of *Austrochiltonia subtenuis* in control and fungicide treatments after 28 d of exposure, $n = 4$. Blue bars are Filan-only treatments, and red bars are mixtures of Filan and Systhane.

Figure 5. Number of embryos per gravid female (mean ± standard error) of *Austrochiltonia subtenuis* in control and fungicide treatments after 28 d of exposure, $n = 4$. Blue bars are Filan-only treatments, and red bars are mixtures of Filan and Systhane.

Figure 6. Cumulative number of juveniles (mean ± standard error) of *Austrochiltonia subtenuis* in control and fungicide treatments after 28 d of exposure, $n = 4$. Blue bars are Filan-only treatments, and red bars are mixtures of Filan and Systhane.

<<ENOTE>>AQ1: Please delete the word "above" and add the section heading (if different than *Test species*) to avoid confusion.

<<ENOTE>>AQ2: Ref. 38 is cited out of order. References 36–38 have been renumbered, please check for accuracy.

<<ENOTE>>AQ3: Reference 1: please give date accessed prior to present study's acceptance.

Table 1. Individual and interactive effects of Filan (F) and Systhane (S) on the amphipod *Austrochiltonia subtenuis* after 28 d of exposure tested with a two-way analysis of variance[a]

Dependent variable	Factor	*df*	*F*	*p*
28-d survival percentage	F	3	1.514	0.236
	S	1	0.086	0.772
	F × S	24	0.257	0.855
Sex ratio	F	3	5.075	0.007*
	S	1	8.333	0.008*
	F × S	24	1.382	0.272
Male head length	F	3	3.521	0.030*
	S	1	33.338	<0.001*
	F × S	24	2.216	0.112

Female head length	F	3	6.321	0.003*
	S	1	2.278	0.145
	F × S	21	2.261	0.108
Juvenile head length	F	3	14.262	<0.001*
	S	1	8.663	0.007*
	F × S	24	2.196	0.115
Time to become gravid	F	3	2.867	0.058
	S	1	16.200	<0.001*
	F × S	24	0.733	0.542
No. of gravid female	F	3	7.707	0.001*
	S	1	9.366	0.005*
	F × S	24	7.707	0.001*
Embryos/female	F	3	3.048	0.048*
	S	1	0.962	0.336
	F × S	24	0.635	0.600
No. of juveniles	F	3	47.813	<0.001*
	S	1	77.625	<0.001*
	F × S	24	12.060	<0.001*
GST activity in male	F	3	0.748	0.539
	S	1	3.848	0.067
	F × S	16	1.012	0.413
GST activity in female	F	3	0.539	0.667
	S	1	0.002	0.964

	F × S	14	0.250	0.860

[a] The degrees of freedom, *F* values, and *p* values are shown.

* Significant at $p < 0.05$.

GST = glutathione-*S*-transferase.

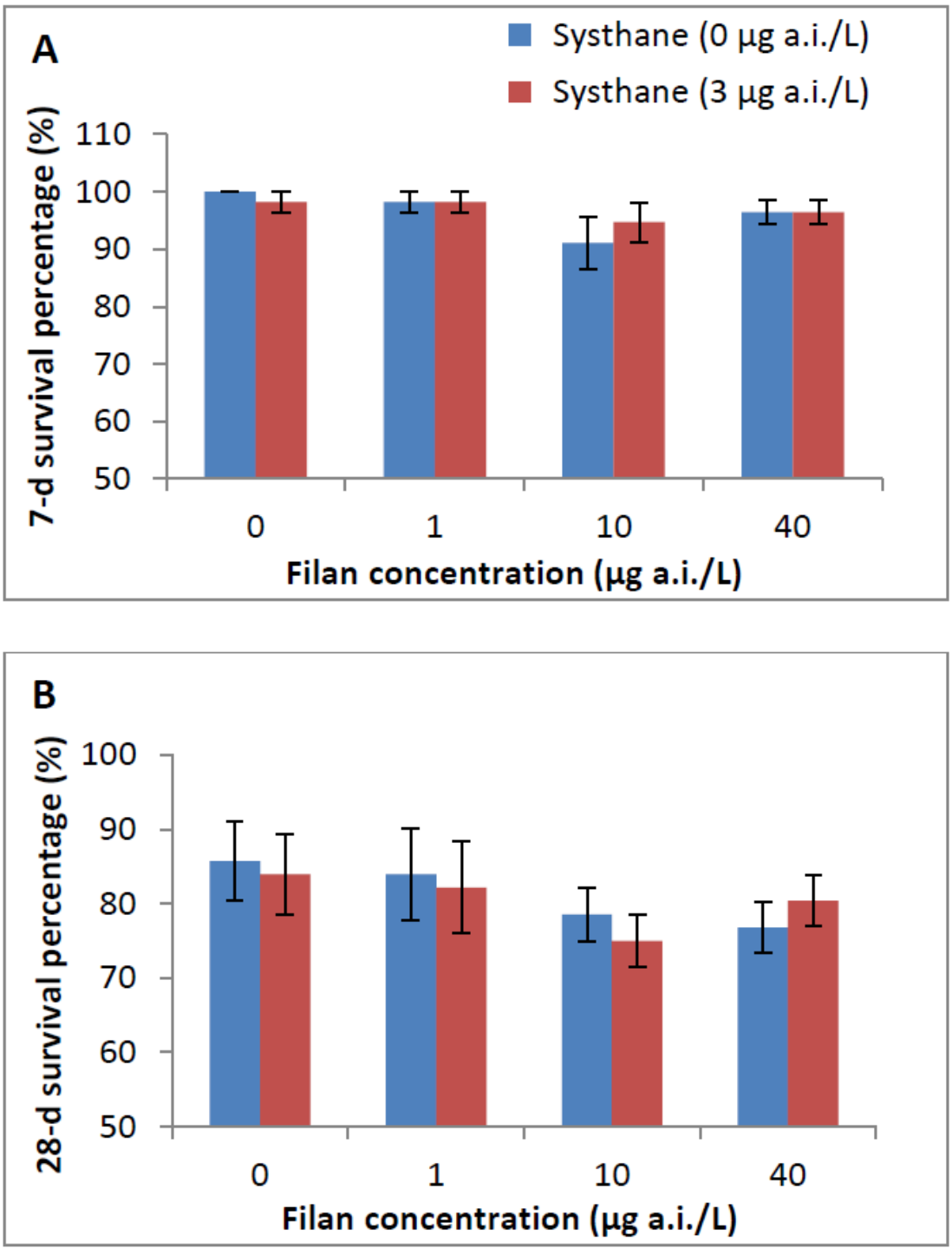
A
Systhane (0 µg a.i./L)
Systhane (3 µg a.i./L)
7-d survival percentage (%)
110
100
90
80
70
60
50
0
1
10
40
Filan concentration (µg a.i./L)
B
28-d survival percentage (%)
100
90
80
70
60
50
0
1
10
40
Filan concentration (µg a.i./L)

Figure 1

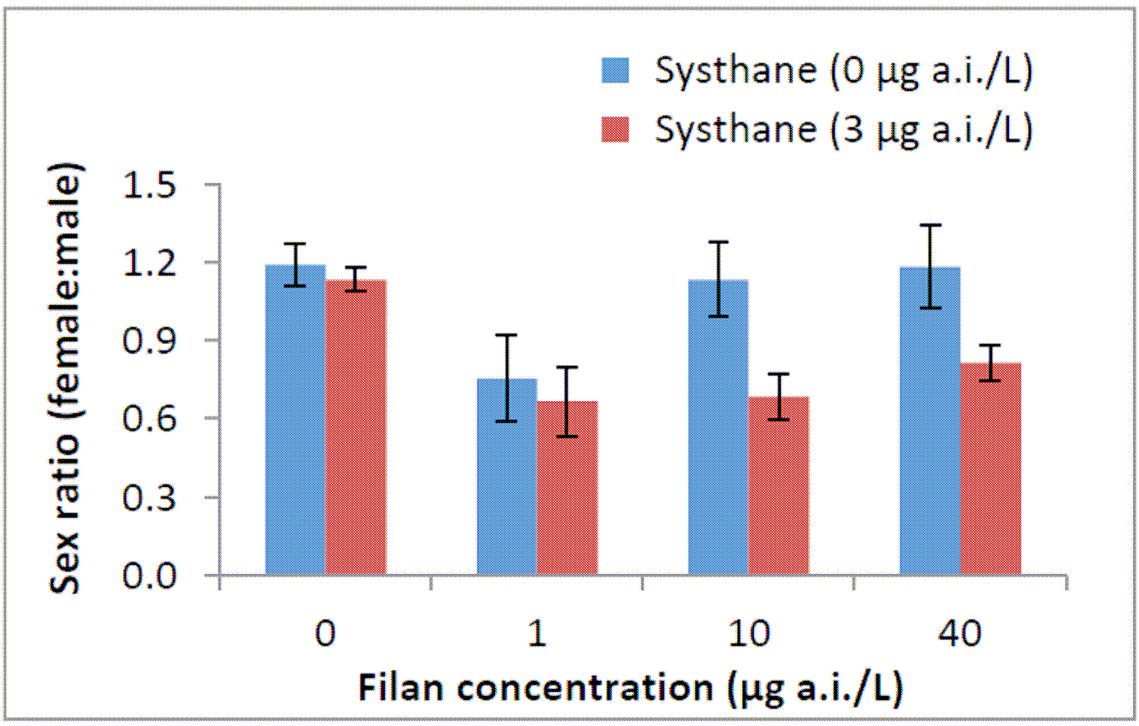

Figure 2

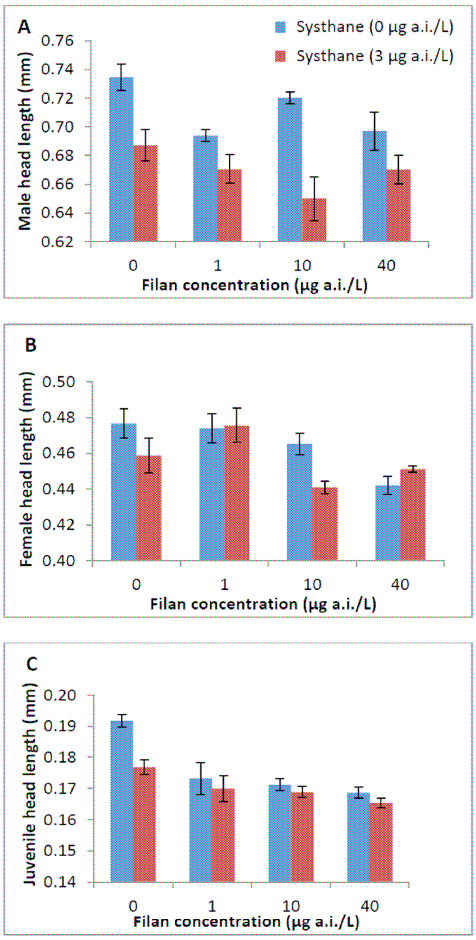

Figure 3

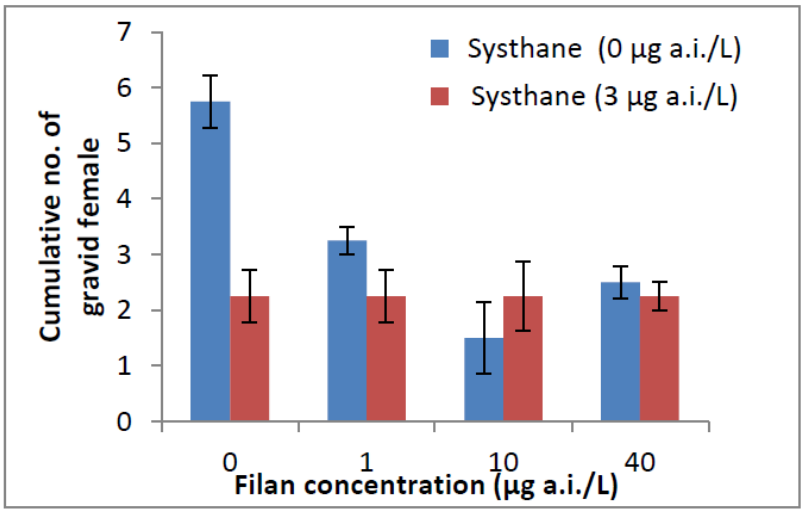

Figure 4

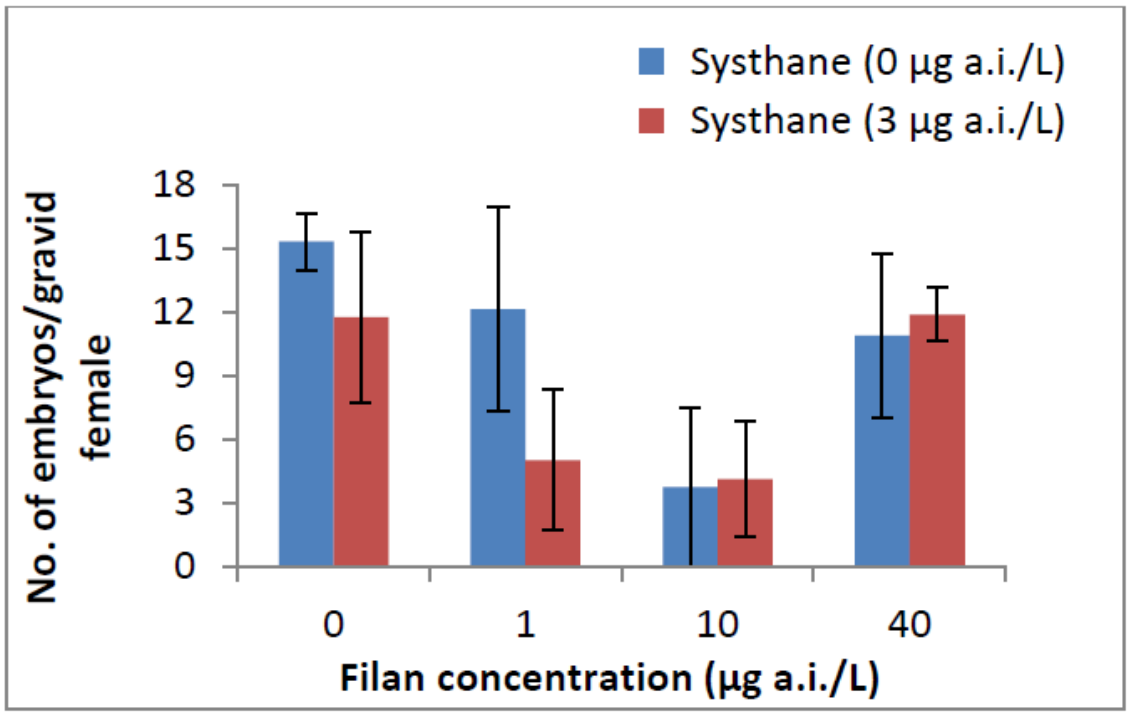

Figure 5

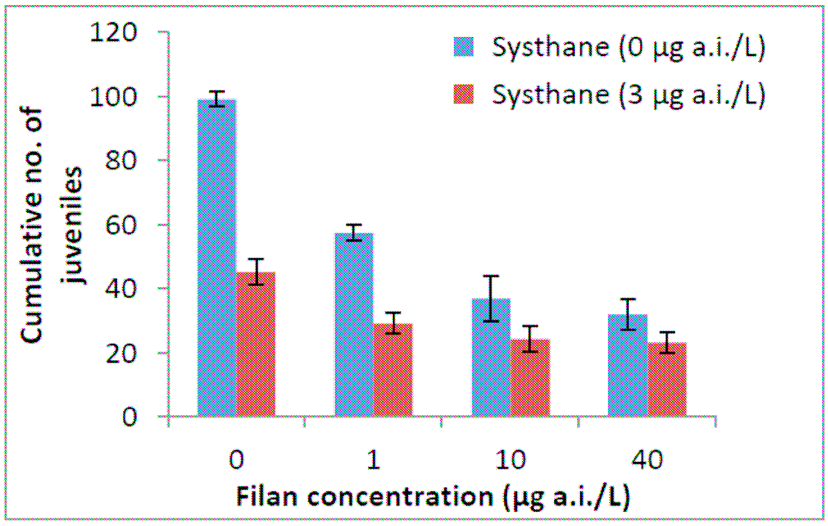

Figure 6

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