Evidence of estrogenic mixture effects on the reproductive performance of fish

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ABSTRACT

Recent research into the effects of mixtures of estrogenic chemicals has revealed the capacity for similarly acting chemicals to act in combination, according to the principles of concentration addition. This means that, collectively, they may pose a significant environmental risk, even when each component is present at a low and individually ineffective concentration. The aim of this study was to investigate the ecological significance of mixture effects at low-effect concentrations by assessing the combined effect of estrogenic chemicals on the reproductive performance of fish. Pairs of fathead minnows were exposed to five estrogenic chemicals. Endpoints analysed included fecundity, the expression of male secondary sexual characteristics, somatic indices and vitellogenin induction. In the first phase of the study, a concentration-response analysis was performed to investigate the relative sensitivity of these endpoints. In the second phase, mixture effects at low-effect concentrations were explored by exposing fish to each of the mixture components, individually and in combination. Data from these experiments provide evidence of mixture effects on fitness and fecundity, demonstrating the capacity for chemicals to act together to affect reproductive performance, even when each component is present below the threshold of detectable effects. This has important implications for hazard assessment and contributes to our understanding of mixture effects at increasing levels of biological complexity.

KEY WORDS: mixture toxicity, estrogens, reproduction, fitness, fecundity, vitellogenin.

1. INTRODUCTION

The risk assessment of chemical mixtures remains one of the most pertinent, yet complex, issues in environmental toxicology. At present, procedures for assessing the hazard posed by chemicals in the environment focus on chemical-by-chemical assessments, despite the fact that "real world" exposures are to complex mixtures (*1*). This approach is inadequate as it fails to account for the capacity for chemicals to act in combination. Hence, the risk that exists in real exposure situations may differ from that expected on the basis of the effect assessments of the individual mixture components, due to the potential for combined effects. The majority of research on mixtures to date has centred on the effects of estrogenic chemicals, which mediate their effects on endogenous hormone function via the estrogen receptor. This group of chemicals provide an ideal model for investigating combination effects, not only because they exert their effects via the same mechanism of action, but also because of the wide range of *in vitro* and *in vivo* assays that can be used to assess their joint effects. Estrogenic mixture effects are also environmentally relevant; chemicals that interfere with sex hormone function have been associated with reduced fecundity, reproductive failure and population-level effects in a range of aquatic wildlife (*2-4*).

Recent research into the effects of estrogenic mixtures has demonstrated the capacity for these chemicals to act together in an additive manner, according to the principles of concentration addition (CA; *5*). This concept is based on the assumption that the components of the mixture act in a similar manner, such that replacing one or more chemicals either totally, or in part, with an equal fraction of an equi-effective concentration of another mixture component produces the same overall effect. The effect of the mixture can be described using a mathematical model based on the concentration and potency of the individual mixture components (*6*). The model indicates that there can be a risk of mixture effects, even when each component is present at a low and individually ineffective concentration. This has been explored *in vitro*, using assays such as the breast cancer cell proliferation assay (E-SCREEN) and the yeast estrogen screen

(YES) (*7, 8*). These studies have revealed that the principles of CA hold true, even when the individual components are combined at concentrations below the limits of detection, giving rise to dramatic mixture effects. The potential hazard posed by mixtures of chemicals at low-effect levels has become known as the "something from 'nothing'" phenomenon (*8*).

More recently, the potential for mixture effects at low doses has been demonstrated *in vivo.* Tinwell and Ashy (*9*) performed a series of experiments using an assay based on the analysis of rat uterine weight. This revealed that doses of estrogenic agents that gave a negative response when tested individually were capable of giving a positive response when tested in combination. A subsequent study using the induction of the egg yolk protein vitellogenin (VTG) in male fish as an endpoint provides further evidence of this phenomenon. Brian et al. (*10*) demonstrated that the effects of a mixture of five estrogenic chemicals were consistent with the predictions of CA. Fish were then exposed to each component of the mixture at a low-effect concentration, equivalent to one-fifth of its individual EC_{50}. While there was no evidence of VTG induction in response to the individual mixture components, the combined response was marked (50% of maximal). Furthermore, the effects of the mixture were consistent with predictions of CA, providing unequivocal evidence of the capacity for estrogenic chemicals to act together in an additive manner, at low-effect concentrations, to elicit effects on whole-organism physiology.

Evidence of estrogenic mixture effects highlights the limitations of the chemical-by-chemical approach to environmental risk assessment by demonstrating that similarly-acting chemicals can act together to produce a significant combined response, even at concentrations that are deemed to be environmentally acceptable from the analysis of their individual effects. Uncertainty factors of 10-1000 are currently employed to account for factors such as interactive effects and variations in sensitivity (between sexes, species and stages of development) (*11*). However, these margins are essentially arbitrary and may not be sufficient to account for mixture effects

due to the sheer number of chemicals present in the environment. This may lead to the underestimation of potential hazards, and hence, erroneous conclusions regarding the risk they pose. As a result, further evidence is required to establish the ecological significance of combination effects for wildlife exposed to mixtures of chemicals in the low-effect concentration range.

The aim of this study was to establish whether estrogenic chemicals have the capacity to act in combination to affect the fitness and reproductive performance of fish. This was addressed by exposing pairs of breeding fathead minnow (FHM) to five estrogenic chemicals at low-effect concentrations, both individually and in combination. The data were used to determine whether the combined effects of the mixture exceeded the response to the individual mixture components, thereby providing evidence that estrogenic chemicals act in a combination to affect reproductive performance. The pair-breeding system of the FHM provides an ideal model for investigating estrogenic mixture effects on population-level processes because it forms the basis of an assay that was developed for investigating endocrine disrupting effects on reproductive performance (*12*, *13*). Endpoints include fecundity and the expression of secondary sexual characteristics. The analysis of combined estrogenic effects on FHM builds on existing evidence of additive effects on VTG induction (*10*) and contributes to the current understanding of estrogenic mixture effects at increasing levels of biological complexity.

2. METHODS

2.1 Experimental Set-up

An established reproductive performance test was employed (*12*). In this test, male and female FHM are isolated in pairs and provided with a spawning substrate. Spawning frequency and egg production is then recorded daily for six weeks: 3 weeks prior to chemical exposure and for a further 3 weeks, during which the fish are exposed to the chemical, or chemicals, of interest.

Treatment-related effects can then be assessed by pre- and post-exposure comparison. At the end of the assay, the effects of treatment on somatic indices and biochemical endpoints, in addition to the expression of secondary sexual characters, can also be assessed.

At the beginning of each experiment, adult fish (>6 months old) were removed from communal holding tanks and placed in 30L glass aquaria (0.6m x 0.3m x 0.3m), supplied with a continuous flow of dechlorinated tap water heated to 25˚C. Two pairs were placed in each aquarium: each pair was separated by a perforated stainless steel barrier. The fish were maintained under a 16h light: 8h dark light regime with 20 min dawn and dusk transition periods. They were fed four times daily; twice with frozen brine shrimp and twice with flaked fish food.

2.2 Chemical Treatment

The chemicals tested in this study were based on the mixture analysed by Brian et al. (*10*). These were the natural steroid estrogen, 17β-estradiol (E2), the synthetic steroid estrogen, 17α–ethynylestradiol (EE2), and the estrogen mimicking compounds, 4-*tert*-nonylphenol (NP), 4-*tert*-octylphenol (OP) and bisphenol-A (BPA). All of these chemicals have been detected in the aquatic environment (*14*). Stock solutions were made by dissolving the chemicals in HPLC grade dimethylformamide (DMF). Stock solutions were diluted 1:15000 with dechlorinated tap water before entering the tanks. The dosing system is described in Brian et al. (*10*).

The chemical concentrations in the tanks were determined at three time points during each pair-breeding study. The first set of water samples was collected after one week of dosing. The second set was taken after two weeks and the third set was taken after the third and final week, on the day the experiment was terminated. The analytical methods are described in Brian et al. (*10*).

2.3 Reproductive Analysis

The reproductive output of each pair of fish was assessed each day at 12:00 ± 1 hour. The spawning substrate was removed from each tank and the presence or absence of eggs was recorded to determine the mean number of spawning events performed by each pair of fish. The number of eggs attached to the spawning substrate was counted using a dissecting microscope. Any eggs that had failed to adhere to the surface of the spawning substrate were collected in a glass Petri dish and added to the egg count to determine the number of eggs produced during each spawning event. Cumulative egg production was recorded pre- and post-exposure.

At the end of each study, fish were sacrificed by overdose with anaesthetic (MS222). After recording length and weight, treatment-related effects on male secondary sexual characteristics were investigated by analysing the relative fatpad weight and the expression of nuptial tubercles. The fatpad is a mucus-secreting dorsal pad, which was carefully removed using a scalpel. Tubercle number was recorded and their prominence scored using the methods described by Smith (*15*). Blood samples were collected for the determination of plasma VTG (*16*). Gonad and liver weights were recorded for the calculation of gonado- and hepatosomatic indices (GSI and HSI).

2.4 Experimental Design

Pairs of fish that had spawned successfully in the week before each experiment were randomly allocated to treatment groups. Each treatment consisted of two or, in some cases, three tanks. Each tank contained two pairs of fish (i.e. four or six pairs per treatment group). Water and solvent controls were run alongside the tanks containing the test chemicals in order to provide baseline data throughout the exposure period.

In this type of experiment, low-effect levels are usually identified using concentration-response data for the individual mixture components (e.g. *4*, *7*, *8*). However, due to practical constraints associated with the pair breeding assay (i.e. time, labour and resource limitations), such data

were not available. As a result, it was necessary to proceed using a two-phase experimental design:

In the first phase, fish were exposed to a dilution series of the mixture in order to characterise the concentration-response curve. A fixed-ratio design was employed: the relative concentration of each component was based on its EC50 for VTG induction (*10*). These values were multiplied by a factor of five (the 5-fold rule) to account for the fact that the reproductive endpoints were expected to be less sensitive than the induction of VTG (KL Thorpe, pers. comm.). A master stock was prepared and dispensed into the highest treatment to give nominal concentrations of 3ng/l EE2, 125ng/l E2, 35µg/l NP, 225µg/l OP and 750µg/l BPA. This 100% stock was then diluted to give 40, 20 and 10% mixture concentrations. The concentration-response was then analysed to establish the relative sensitivity of each endpoint and, hence, to identify the lowest concentration of the mixture that adversely affected the majority of the reproductive endpoints. This information was used to design the experiments in Phase 2.

The second phase aimed to establish the potential for mixture effects when each component was present at a low-effect concentration. This "low dose" study involved the parallel testing of the mixture components both individually, and in combination, using the approach described by Silva et al. (*8*). A single concentration of each chemical was tested: this corresponded with the concentrations present in the lowest dilution of the mixture that adversely affected the majority of the reproductive endpoints, as determined in Phase 1. The responses in the individual and combined exposure treatments were compared to determine whether there was evidence of mixture effects.

2.5 Data Analysis

Continuous data were examined for normal distribution and homogeneity of variance (Shapiro-Wilk's and Bartlett's tests), and if relevant, data were $\log_{10}$ transformed (VTG, fatpad) or Box-Cox transformed (egg production, with maximum likelihood estimated parameter 0.94). Dunnett's one-tailed test (α=5%) was then employed to determine whether any of the treatment groups differed in relation to the solvent controls. Treatment-related effects were assessed by comparison of the pre- and post-exposure data (paired t-tests, α=5%).

All remaining quantal endpoints were analysed using generalized linear modelling approaches (GLIM), assuming the numbers of spawnings to be Poisson-distributed and the tubercle number and prominence to be binomial-distributed. Mean effects were estimated by Maximum Likelihood and 95% confidence intervals adjusted for multiplicity by the Hochberg method. These intervals were used to assess treatment-related effects during the pre- and post-exposure periods and differences between treatment groups in relation to the solvent controls. All analyses were done using the SAS procedure PROC GENMOD and PROC MULTTEST (*17*).

3. RESULTS

3.1 Exposure Concentrations

The measured concentrations of each chemical after one week of dosing are presented in the supporting information. In general, these values were close to the nominal concentrations, although the levels of BPA were slightly lower than expected. Analysis of the measured concentrations throughout the exposure revealed little deviation over time (data not shown).

3.2 Effects on Reproductive Performance

In the first exposure study, there was an effect of treatment on the survival of male fish exposed to the highest concentration of the mixture (100% mixture dilution); all four males died within 48 hours of exposure. One further mortality occurred in males in the mixture treatment during

the second low dose study. In contrast, there was no effect on the survivorship of females, although two became egg bound (i.e. bloated and unable to release their eggs): one during each phase of the study. Data from these individuals were excluded (*12*).

3.2.1 Phase 1: Concentration-response Analysis

Figure 1 shows treatment-related effects on fecundity in each group during the first experiment. The analysis of this data (presented in Table 1) revealed that each pair spawned, on average, 4-5 times prior to exposure. After dosing began, however, the number of spawning pairs fell in a concentration-dependent manner and the mean number of spawning events that occurred pre- and post-exposure was significantly reduced in the 20 and 40% mixture treatments. In the 40% treatment, the mean number of spawning events was also significantly lower than in the solvent control. There was no clear effect of treatment on the number of eggs produced during each spawning event. However, the ratio between the total number of eggs produced pre- and post-exposure was reduced in the 10 and 20% mixture treatments, in relation to the solvent control. The effects on fecundity partly reflect the lower numbers of spawning pairs in the 20 and 40% mixture treatments, although the reproductive performance of the fish that continued to spawn throughout exposure was also impaired.

The analysis of male secondary sexual characteristics also revealed treatment-related effects. The number and prominence of the nuptial tubercles was reduced in the mixture treatments relative to the solvent control. Fatpad weight was also reduced in the 20 and 40% mixture treatments, although this effect was not statistically significant. Similarly, there was no significant effect of treatment on GSI or HSI. VTG was not measured in this experiment, as the induction was assumed to be maximal (or near maximal) in all treatment groups (*10*).

3.2.2 Phase 2: Mixture Effects at Low-Effect Concentrations

The results obtained from the first phase of the study revealed that there were significant effects on the majority of reproductive endpoints in fish exposed to the 20% mixture dilution, which contained each component at its EC50 for VTG induction. Hence, these concentrations were tested, both individually and in combination, in the second phase, to investigate whether the response to the mixture exceeded the response to each chemical individually.

Low Dose Experiment 1

In the first low dose study, two out of the six pairs in the mixture treatment failed to reproduce post-exposure and there was a significant reduction in the mean number of spawning events performed during the pre- and post-exposure periods (Figure 2A). These effects were in agreement with the results of the concentration-response analysis, but contrary to expectation, the proportion of spawning pairs was reduced to a greater extent by exposure to OP on its own, which led to a reduction in the mean number of spawnings. The spawning activity of the pairs that continued to spawn throughout the exposure to OP was also reduced. As a result, although there was no significant effect of OP treatment on the number of spawnings performed pre- and post-exposure, there was a significant difference between the number of spawnings performed in the OP treatment group relative to the solvent controls. There was evidence of a combined effect on the mean number of eggs produced per spawning (Figure 2B). The ratio between the total number of eggs produced pre- and post-exposure was significantly reduced by the mixture, and also by OP on its own (Table 1).

As anticipated on the basis of the concentration-response analysis (Phase 1), the expression of tubercles was inhibited in response to the mixture. This effect was statistically significant for the number of tubercles, but there was no evidence of mixture effects on fatpad weight, or on the somatic indices. The VTG concentrations in response to the individual mixture components

were indicative of an intermediate response, whilst the response to the mixture was maximal (data not shown).

The first study of mixture effects at low doses provided evidence of mixture effects on a range of reproductive endpoints. However, egg production, which is arguably the most relevant measure of fecundity, appeared to be reduced in response to OP on its own, as well to the mixture. The response to OP was unexpected, even accounting for the fact that the measured concentrations of this chemical were approximately 20% higher than nominal values. It was therefore concluded that OP had contributed disproportionately to the overall effect of the mixture and, hence, the concentration-dependent effects attributed to the mixture in the first phase of the study may have arisen solely in response to OP. As a result, it was decided to repeat the low dose study using modified test concentrations. The concentrations of the alklyphenols (NP and OP) were lowered to reduce the likelihood that they would contribute disproportionately to the overall effect of the mixture. In contrast, the concentrations of EE2, E2 and BPA were raised to maintain its overall estrogenic activity (See Table 1 for details).

Low Dose Experiment 2

In the second low dose experiment, one pair in each of the BPA and mixture treatments and two pairs in the OP treatment failed to spawn post-exposure. The mean number of spawnings during the post-exposure period was lowest in the mixture treatment group and the pairs that continued to spawn throughout the exposure reduced their rate of spawning by a half. However, there was no significant difference between the mean number of spawnings performed by each group. The mean number of eggs per spawning differed pre- and post-exposure to the mixture, as well as in response to BPA and to solvent alone. There was extensive variability in the total number of eggs produced by each group during each exposure period (Figure 3A). Analysis of the ratio of the total number of eggs produced pre- and post-exposure revealed a significant effect of the

mixture in relation to the solvent control. An effect was also observed in response to BPA. However, the magnitude of the difference in the ratios indicates that the combined effects of the mixture were more pronounced than the response to BPA. This suggests that the components of the mixture have acted in combination to reduce fecundity.

The analysis of male secondary sexual characteristics also provided evidence of mixture effects; both the number and prominence of the tubercles were significantly reduced in fish exposed to the mixture relative to the solvent control (Figure 3B and C). There was also a significant effect of the mixture on the HSI of male fish (Figure 3D). Analysis of the mean VTG levels in each treatment group (Figures 3E and F) revealed that there was no evidence of VTG induction in males exposed to NP and BPA, whereas there was a significant response to the mixture, as well as BPA, EE2 and E2 alone. In contrast, only females exposed to the mixture exhibited elevated VTG levels, clearly illustrating the capacity for estrogenic chemicals to act together to exert significant effects at low and individually ineffective concentrations.

4. DISCUSSION

The first experiment revealed that the mixture was acutely toxic to males at the highest concentration tested (Phase 1). It is not known why males are more sensitive to the effects of these chemicals than females, although this phenomenon has previously been reported in the literature (*16, 18*). Low mortality was observer in the subsequent studies, which permitted the analysis of chronic reproductive effects.

4.1 Phase 1: Concentration-response Analysis

The first phase revealed that the number of spawning pairs, spawning frequency and the rate of egg production responded to the mixture in a clear monotonic concentration-dependent manner. This was consistent with published data describing the effects of single chemicals, such as NP

(*12*), methoxychlor (*13*) and oestrone (*17*). Effects on tubercle expression were also observed. The pattern was consistent with that reported in response to NP (*12*), E2 (*19*) and EE2 (*20*). There were no significant effects on the weight of the fatpad or the somatic indices in this study, although the patterns observed indicated that differences between treatment groups may have become apparent at higher concentrations, after extended exposure and/or with greater sample sizes. Hence, the apparent differences in the sensitivity of these endpoints may be related, in part, to statistical power considerations. Nevertheless, when concentration-dependent effects on the reproductive endpoints were related to the VTG concentration-response characterised by Brian et al. (*10*) there was, in general, a 5-10 fold difference in sensitivity.

The difference in the relative sensitivities of these endpoints was also consistent with some of the literature describing the effects of single chemicals. For example, Harries et al. (*12*) reported a low dose for fecundity was 10-fold higher than that for VTG induction following exposure to NP and found that total egg production and the expression of tubercles exhibited similar sensitivities. In contrast, Harries et al. (*12*) reported that the lowest observable effects concentration (LOEC) for fatpad size was the same as that for VTG induction, indicating that this endpoint is highly sensitive to estrogenic effects. This is not consistent with the data presented here. There are further inconsistencies in the literature. For example, using a similar, but modified pair breeding assay based on gonadal recrudescence, effects on egg production were detected at concentrations of EE2 that were below the threshold of detectable effects for the induction of VTG (*20*). It is not known whether these contrasting data reflect the more integrative and complex nature of the reproductive endpoints in relation to VTG or whether they simply highlight the need for standardised test protocols to facilitate comparisons between studies.

4.2 Phase 2: Low Dose Experiment 1

In the first low dose study, it was anticipated that the concentration of each chemical in the 20% mixture dilution would fulfil the experimental criterion by exerting an effect when present in combination, but not when present individually. There was some risk that the concentration of the mixture may not have been sufficient as to induce detectable effects on all of the endpoints. However, it was considered unwise to increase the total mixture concentration, as the likelihood of an individual chemical causing a significant effect would also have increased: a significant response to one or more mixture components would make it impossible to determine whether individually undetectable responses have the capacity to act together to produce significant mixture effects.

The results of this low dose study were in line with expectations based on the concentration-response analysis (Phase 1) in that there was a significant reduction in the number of spawnings and the number of eggs produced by fish in the mixture treatment. However, there was also a reduction in the fecundity of fish exposed to OP alone. This indicates that OP is more potent in terms of fecundity than expected on the basis of its capacity to induce VTG. Hence, the relative sensitivities of these endpoints differs for OP in relation to the other components of the mixture, which did not affect fecundity, despite the fact that they were tested at concentrations that were equipotent for the induction if VTG. It is interesting to note that OP treatment did not affect the expression of male secondary sexual characteristics. Tubercle number was, however, significantly reduced in response to the mixture, which is consistent with the theory that estrogenic chemicals can act together at low-effect concentrations to elicit significant combined effects. The disparity between the effects of OP on fecundity and tubercle expression serves to highlight the fact that each chemical may influence each reproductive endpoint quite differently.

Treatment-related effects on fecundity occur as an integrated response to alterations in the behaviour and/or the physiology of males and females. These alterations may be mediated by

the estrogen receptor, but can also occur via alternative hormonal pathways, as well as by the disruption of metabolic, neurological and sensory processes. Differences in the relative sensitivity of the reproductive endpoints to OP compared with the other chemicals indicate that this chemical can exerts its effects on reproduction via more than one mechanism. The response pattern could be explained by general toxic response, which may have reduced spawning and egg production via the disruption of behavioural and/or physiological processes that are independent of the estrogen receptor, whilst endpoints that specifically depend on sex hormone function, such as the expression of secondary sexual characters, were not affected. Molecular evidence demonstrates that the alkylphenols influence a greater suite of genes than the pure estrogens (*21*). Alternative mechanisms for reproductive impairment by OP are discussed in Rasmussen et al. (*22*). In light of this, it is questionable as to whether the combined effects of chemical mixtures on reproductive performance can be described by CA with the same accuracy as demonstrated for VTG (*10*): from a strictly mechanistic perspective, the assumption that effects are mediated via a common mode of action is violated at higher levels of biological complexity.

4.3 Phase 2: Low Dose Experiment 2

Although the first low dose study provided evidence of combined estrogenic effects on tubercle number and the mean number of eggs per spawning, a reduction in fecundity was observed in response to OP alone, as well as the mixture. This meant that it was not possible to draw any conclusions regarding the potential for combined effects on fecundity. Moreover, the patterns observed revealed that the relative sensitivity of the reproductive endpoints varied, depending on the toxicological properties of the chemical in question. This meant that the risk of reproductive effects could not be predicted accurately using the VTG response data, as originally assumed in the design of the low dose study, by the implementation of the 5-fold rule. However, as only one concentration of each chemical was tested, the extent to which the relative sensitivities shift for

each compound was unclear. This meant that it was necessary to adopt a "best guess" approach to predict individually ineffective test concentrations that would be capable of exerting effects when present in combination for the design of the second lo dose study.

Exposure to the mixture at the revised concentrations was associated with reduced fecundity, in terms of the ratio between the total number of eggs produced pre- and post-exposure, but again, a significant effect was observed in response to one of the components on its own. Nevertheless, the effects observed in response to the mixture were more pronounced, indicating a combined response. Mixture effects were also evident from the analysis of tubercle expression and somatic indices. Collectively, these data provide strong evidence in support of the theory that chemicals can act together at low and apparently ineffective concentrations to affect reproduction in fish. Mixture effects on VTG induction were also apparent. Males exposed to the mixture exhibited significantly higher VTG levels than any of the other treatment-groups. The female VTG data were discordant with the effects on males because there was no evidence of a response to any of the individual mixture components. However, there was a 10-fold increase in the VTG levels of females exposed to the mixture. This response pattern provides a perfect illustration of the "something from 'nothing'" phenomenon.

The VTG responses of male and female fish are of further interest because they demonstrate the potential for sex differences in chemical potency. For example, E2, EE2 and BPA were highly estrogenic in terms of VTG induction in male fish, but were ineffective in females. There was also evidence of discordance between treatment-related effects on VTG and reproduction. For example, Figure 3E shows that the concentrations of E2 and EE2 that were tested in the second low dose study were extremely potent in terms of VTG induction, but were ineffective in terms of reproduction. In contrast, the first low dose study revealed that OP is capable of reproductive impairment at concentrations that are close to the threshold of detectable effects for VTG

induction. Despite these inconsistencies, there was no evidence that the analysis of VTG underestimates the risk of reproductive effects, which justifies its use as a biomarker for predicting potentially adverse effects at higher levels of biological complexity.

The evidence presented here has important ecological implications because it demonstrates that mixtures of chemicals can pose a significant threat to population-level processes, even when the components are present at low and individually ineffective concentrations. Accurate assessment of this risk will require a greater understanding of the way in which these chemicals interact: whilst there is convincing evidence that estrogenic chemicals behave according to the principles of CA to induce specific endpoints, such as VTG (*10, 23, 24*), it remains unclear whether this holds true for more holistic parameters. For example, reproductive performance is dependent upon the appropriate coordination of the entire hypothalamo-pituitary-gonadal (HPG) axis and, hence, chemicals acting at any level of this axis, via any mechanism, could have adverse effects on reproduction. Thus, it is possible that some chemicals may be estrogenic to the liver, leading to VTG induction, but act via different mechanisms to exert effects at other sites (receptors or enzyme systems). As a result, different types of estrogenic chemicals are likely to exert their effects on reproductive performance quite differently.

In view of the complex and integrative nature of the reproductive responses, it is likely that the principles of CA only apply to mixtures of "true" estrogens, such as E2 and EE2, which exert their effects via similar mechanisms of action (*21*). In contrast, chemicals such as OP and BPA, which appear to exert their effects via more than one pathway, may act together according to the "general solution" scenario proposed by Berenbaum (*25*). Further investigation is required to assess the combined effects of chemicals that act on reproduction via different mechanisms. This may be extended to include the interactive effects of chemicals with different properties, such as anti-estrogens and androgens, as well as those that exert their effects independently of

the endocrine system. The potentially confounding effects of environmental variables, such as temperature and exposure duration, should also be assessed to determine the hazard posed by mixtures in real exposure situations. Further exploration of these issues will improve our understanding of the hazard posed by mixtures and thereby contribute to the development of more effective methods for assessing environmental risk.

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Table 1

	Spawning pairs		Mean no. spawnings		Mean no. eggs per spawning		Ratio of total egg no.
Treatment	Post:pre exposure	Ratio	Pre-exposure	Post-exposure	Pre-exposure	Post-exposure	Post:pre exposure
Phase 1							
Water control	4:4	1.0	4.5 (2.8-7.1)	3.3 (1.9-5.6)	374 (312-437)	461 (357-567)	0.88 (0.64-1.15)
Solvent control	4:4	1.0	4.5 (2.8-7.1)	3.3 (1.9-5.6)	349 (316-383)	425 (359-491)	0.87 (0.71-1.02)
10% Mixture	3:3	1.0	5.0 (3.0-8.3)	2.0 (0.9-4.5)	421 (359-483)	387 (218-560)	0.37 (0.19-0.52)
20% Mixture	2:4	0.5	4.8 (3.0-7.5)	**1.3 (0.5-3.0)**	382 (333-432)	349 (200-502)	0.24 (0.13-0.33)
40% Mixture	1:4	0.3	4.8 (3.0-7.5)	**0.3 (0.0-1.8)**	485 (436-534)	661 (n.d)	0.07 (n.d.)
100% Mixture	0:4	0.0	4.5 (2.8-7.1)	-	319 (276-364)	-	-
Phase 2 (i)							
Water control	4:4	1.0	3.8 (2.6-6.2)	2.3 (1.2-4.3)	414 (330-499)	516 (383-652)	0.74 (0.52-0.97)
Solvent control	6:6	1.0	3.5 (2.3-5.4)	2.8 (1.8-4.6)	580 (503-658)	585 (480-692)	0.82 (0.65-0.99)
E2 (25ng/l)	3:3	1.0	3.0 (1.6-5.8)	3.0 (1.6-5.8)	538 (318-764)	329 (96-573)	0.63 (0.21-1.26)
EE2 (0.6ng/l)	3:4	0.8	3.3 (1.9-5.6)	2.3 (1.2-4.3)	672 (572-772)	608 (451-767)	0.63 (0.45-0.81)
BPA (150μg/l)	4:4	1.0	3.5 (2.1-5.9)	3.3 (1.9-5.6)	499 (382-617)	418 (299-539)	0.79 (0.53-1.12)
NP (7μg/l)	3:4	0.8	3.3 (1.9-5.6)	2.3 (1.2-4.3)	535 (470-602)	567 (502-633)	0.73 (0.61-0.83)
OP (45μg/l)	2:4	0.5	2.8 (1.5-5.0)	0.5 (0.1-2.0)	603 (535-672)	628 (209-1066)	0.19 (0.06-0.29)
Mix. (202μg/l)	4:6	0.7	4.0 (2.7-6.0)	**1.3 (0.7-2.7)**	562 (496-628)	**303 (150-461)**	0.19 (0.09-0.26)
Phase 2 (ii)							
Water control	4:4	1.0	3.8 (2.3-6.2)	3.3 (1.9-5.6)	351 (317-385)	375 (333-416)	0.92 (0.8-1.05)
Solvent control	4:4	1.0	4.0 (2.5-6.5)	5.0 (3.2-7.8)	385 (360-410)	**278 (225-332)**	0.92 (0.74-1.1)
E2 (70ng/l)	4:4	1.0	4.0 (2.5-6.5)	4.8 (3.0-7.5)	322 (250-395)	358 (297-419)	1.31 (1.02-1.74)
EE2 (1.5ng/l)	4:4	1.0	4.0 (2.5-6.5)	3.5 (2.1-5.9)	332 (290-374)	312 (233-393)	0.83 (0.6-1.04)
BPA (500μg/l)	3:4	0.8	4.0 (2.5-6.5)	3.0 (1.7-5.8)	298 (238-359)	**177 (123-231)**	0.46 (0.29-0.63)
NP (5μg/l)	4:4	1.0	4.0 (2.5-6.5)	4.5 (2.8-7.1)	299 (263-334)	300 (253-347)	1.13 (0.93-1.37)
OP (5μg/l)	2:4	0.5	4.0 (2.5-6.5)	2.3 (1.2-4.3)	289 (217-362)	312 (241-384)	0.6 (0.41-0.84)
Mix. (511μg/l)	3:4	0.8	4.0 (2.5-6.5)	1.5 (0.7-3.3)	338 (270-407)	**205 (143-267)**	0.23 (0.13-0.31)

Table 1 (continued)

	Secondary sexual characteristics				Somatic indices	
Treatment	Fatpad Index		Tubercle no.	Tubercle prom.	HSI	GSI
Phase 1						
Water control	6.3	(4.8-8.2)	17.5 (13.8-22.1)	3.5 (2.46-3.87)	2.22 (1.69-2.74)	1.22 (0.77-1.67)
Solvent control	5.3	(3.0-9.2)	24.0 (19.6-29.3)	3.5 (2.46-3.87)	2.47 (1.91-3.02)	1.21 (0.81-1.61)
10% Mixture	5.8	(3.9-8.6)	12.8 (9.7-16.8)	2.0 (1.09-2.91)	2.02 (1.77-2.27)	1.29 (0.90-1.68)
20% Mixture	4.5	(3.1-6.5)	8.3 (5.9-11.6)	1.3 (0.55-2.27)	2.36 (1.36-3.36)	1.45 (1.11-1.80)
40% Mixture	4.4	(2.5-7.5)	8.8 (6.3-11.2)	1.5 (0.72-2.49)	1.83 (1.42-2.23)	0.88 (0.36-1.40)
100% Mixture	-		-	-	-	-
Phase 2 (i)						
Water control	4.4	(1.6-12.0)	23.8 (19.4-29.0)	3.3 (2.2-3.8)	1.78 (0.57-2.99)	1.38 (1.17-1.59)
Solvent control	3.5	(1.7-7.5)	19.8 (16.6-23.7)	3.2 (2.3-3.6)	1.93 (1.71-2.16)	1.17 (0.98-1.36)
E2 (25ng/l)	3.2	(1.3-7.8)	18.8 (15.0-23.5)	3.5 (2.5-3.9)	1.52 (1.34-1.70)	1.12 (0.77-1.46)
EE2 (0.6ng/l)	5.7	(4.6-7.1)	19.0 (15.2-23.8)	3.0 (2.0-3.6)	1.90 (1.58-2.23)	1.29 (1.19-1.38)
BPA (150μg/l)	8.0	(6.5-10.0)	18.8 (15.0-23.5)	3.0 (2.0-3.6)	2.11 (1.76-2.47)	1.30 (1.00-1.61)
NP (7μg/l)	4.1	(1.9-9.1)	19.0 (15.2-23.8)	3.0 (2.0-3.6)	1.38 (0.96-1.81)	1.65 (1.31-1.99)
OP (45μg/l)	7.5	(3.7-15.1)	18.3 (14.5-23.0)	2.8 (1.7-3.5)	2.54 (1.44-3.64)	1.02 (0.56-1.49)
Mix. (202μg/l)	3.9	(2.0-7.9)	11.8 (9.4-14.9)	1.7 (1.0-2.5)	1.77 (1.50-2.05)	1.10 (0.86-1.34)
Phase 2 (ii)						
Water control	5.2	(3.5-7.5)	21.0 (17-26)	3.8 (2.7-4)	1.70 (1.46-1.94)	1.36 (1.25-1.47)
Solvent control	6.8	(5.2-9.0)	20.5 (16.5-25.5)	3.5 (2.5-3.9)	2.01 (1.52-2.49)	1.58 (1.06-2.10)
E2 (70ng/l)	7.1	(6.6-7.7)	21.3 (17.2-26.3)	3.5 (2.5-3.9)	2.88 (2.31-3.46)	1.67 (1.37-1.97)
EE2 (1.5ng/l)	5.5	(3.2-9.4)	19.0 (15.2-23.8)	3.3 (2.2-3.8)	2.30 (1.28-3.31)	1.70 (1.49-1.92)
BPA (500μg/l)	4.3	(2.1-8.9)	14.8 (11.4-19)	2.8 (1.7-2.5)	2.79 (2.28-3.30)	1.62 (1.57-1.67)
NP (5μg/l)	4.9	(3.2-7.4)	21.8 (17.6-26.8)	3.8 (2.7-4)	2.63 (1.92-3.34)	1.95 (1.70-2.20)
OP (5μg/l)	3.5	(2.3-5.3)	23.0 (18.7-26.2)	3.8 (2.7-4)	2.10 (1.61-2.60)	1.85 (1.31-2.39)
Mix. (511μg/l)	2.3	(1.6-3.2)	7.0 (4.6-10.7)	1.0 (0.3-2.2)	3.60 (2.66-4.54)	0.83 (0.18-1.47)

Legends

Table 1. Treatment-related effects on fecundity and the expression of secondary sexual characteristics. The number of spawning events and the number of eggs per spawning are presented as means. Total egg production is presented in terms of the ratio between the mean number of eggs produced pre- and post-exposure. The values given in brackets are 95% confidence intervals. Where possible, comparisons were made between reproductive performance pre- and post-exposure: significant effects are highlighted in bold. Comparisons were also made across each treatment group: underlined values represent a significant effect of treatment relative to the solvent controls.

Figure 1. Treatment-related effect on cumulative total egg production during the concentration-response analysis (Phase 1). During the pre-exposure period, the rate of egg production was similar across each treatment group. Concentration-dependent effects on egg production are apparent within a few days of exposure. The statistical analyses of these data are presented in Table 1.

Figure 2. Treatment-related effects on the mean number of spawnings (A) and the mean number of eggs produced per spawning (B) during the first low dose study (Phase 2). Error bars represent 95% confidence intervals. The open and shaded bars represent the pre- and post-exposure data. The concentrations tested were 25ng/l E2, 0.6ng/l EE2, 150μg/l BPA, 7μg/l NP and 45μg/l OP. The comparison of spawning and egg production pre- and post-exposure revealed a significant effect of mixture treatment. However, there was also a significant effect of OP treatment on spawning activity in relation to the solvent controls.

Figure 3. Evidence of combined effects in the second low dose study, when the test concentrations were revised to 70ng/l E2, 1.5ng/l EE2, 500μg/l BPA, 5μg/l NP and 5μg/l OP. The total numbers of eggs produced by each group during the pre- and post-exposure periods (A) are represented by the open and shaded bars, respectively. Analysis of the ratio of egg production revealed a significant effect of exposure to the mixture and, to a lesser extent, BPA. There was also evidence of mixture effects on the expression of tubercles (B and C) and HSI (D). Analysis of the 95% confidence limits revealed that, in each case, there was no effect of individual chemical treatment, whereas there was a significant response to the mixture relative to the solvent control. The analysis of VTG in males (Figure 3E) revealed a significant effect of E2, EE2, BPA and the mixture treatment relative to the solvent controls. The effect of the mixture was significantly higher than that of the single chemicals. There was no evidence of VTG induction in females (Figure 3F) exposed to the single chemicals, whereas there was a marked mixture effect.

Figure 1

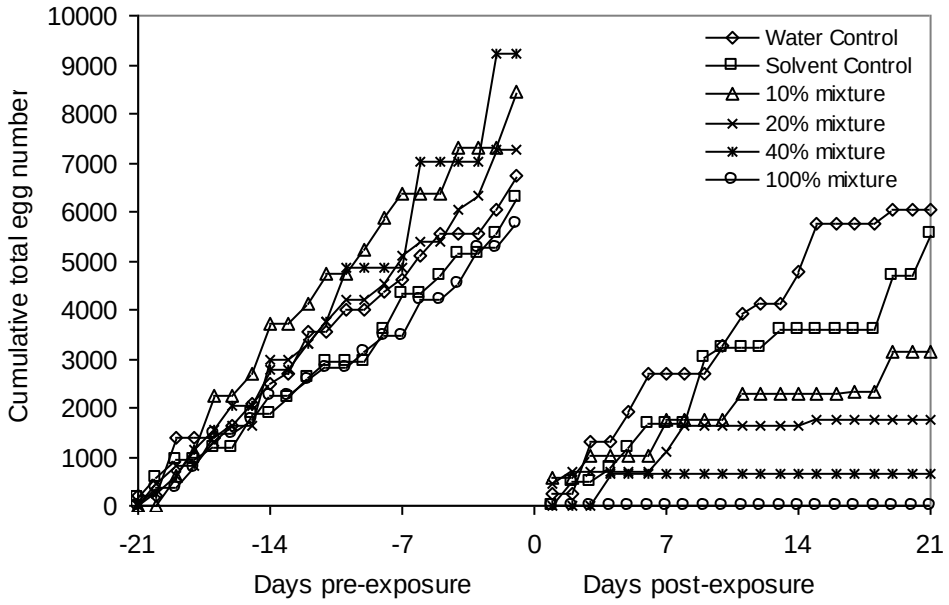
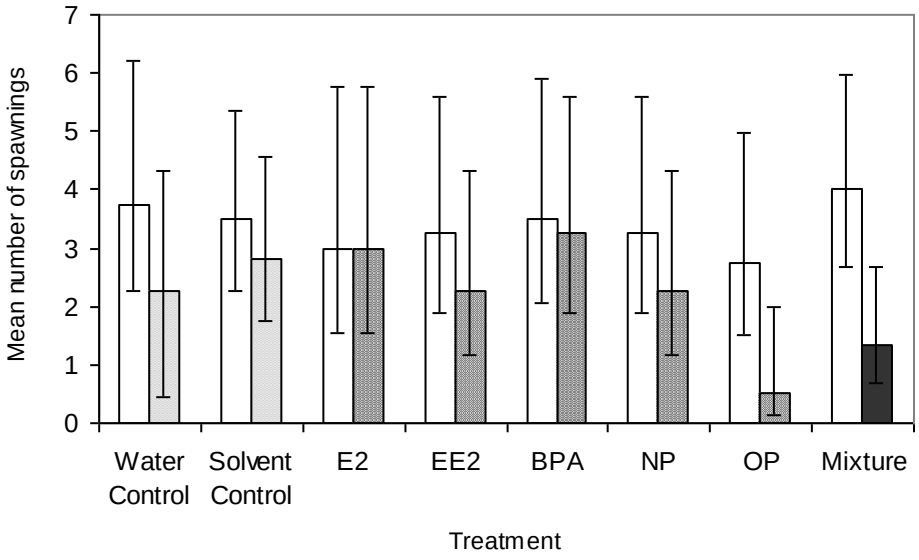
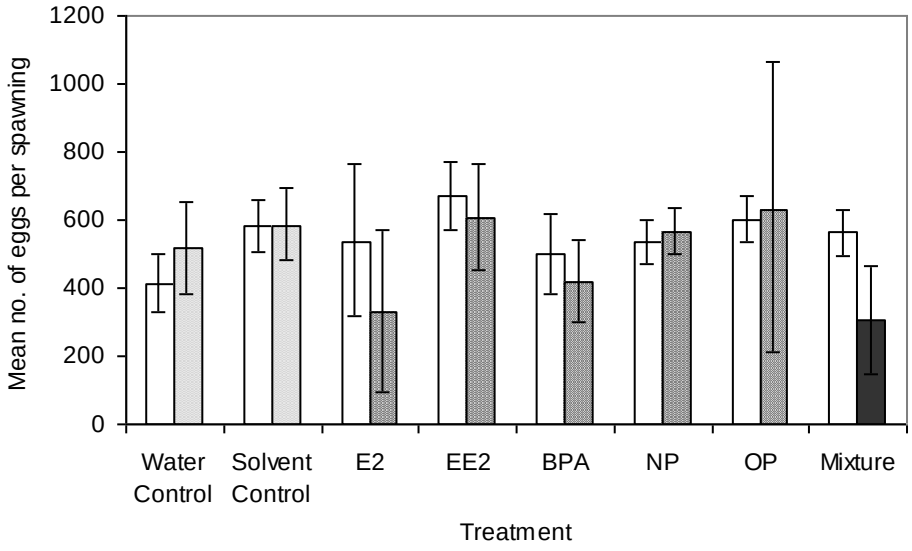

Cumulative total egg number
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-21
-14
-7
0
7
14
21
Days pre-exposure
Days post-exposure
Water Control
Solvent Control
10% mixture
20% mixture
40% mixture
100% mixture

Figure 2

A


Mean number of spawnings
0
1
2
3
4
5
6
7
Water Control
Solvent Control
E2
EE2
BPA
NP
OP
Mixture
Treatment

B


Mean no. of eggs per spawning
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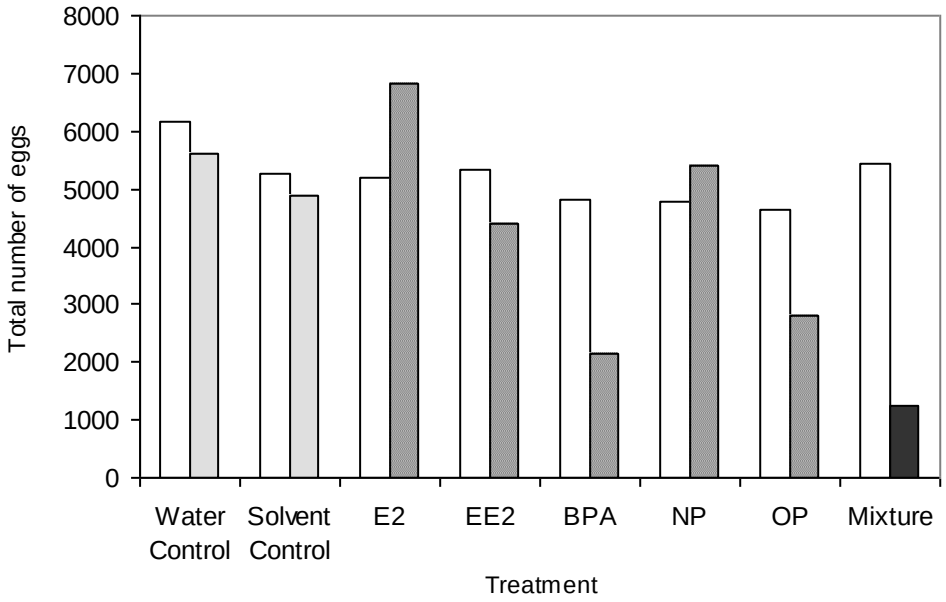
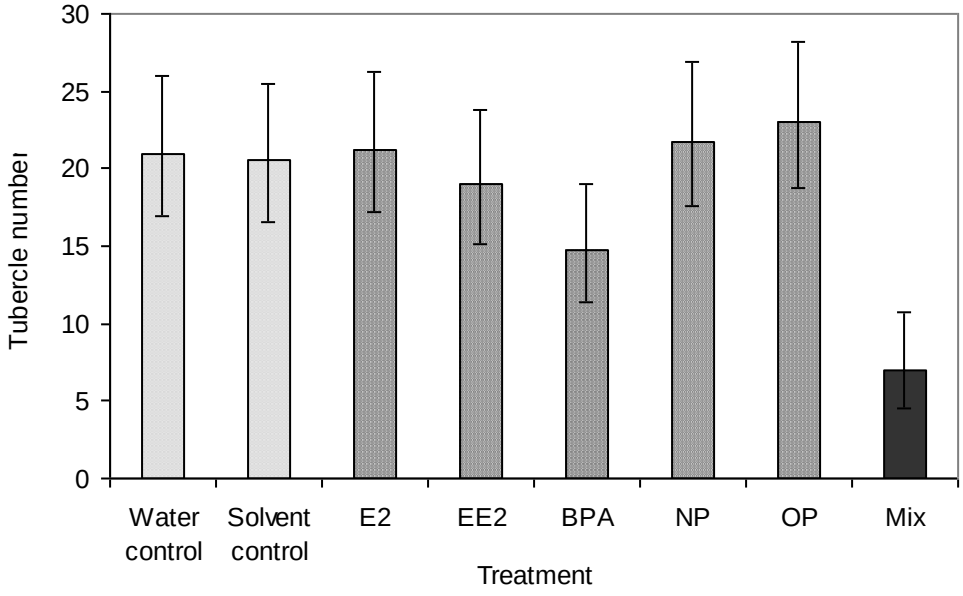
200
400
600
800
1000
1200
Water Control
Solvent Control
E2
EE2
BPA
NP
OP
Mixture
Treatment

Figure 3

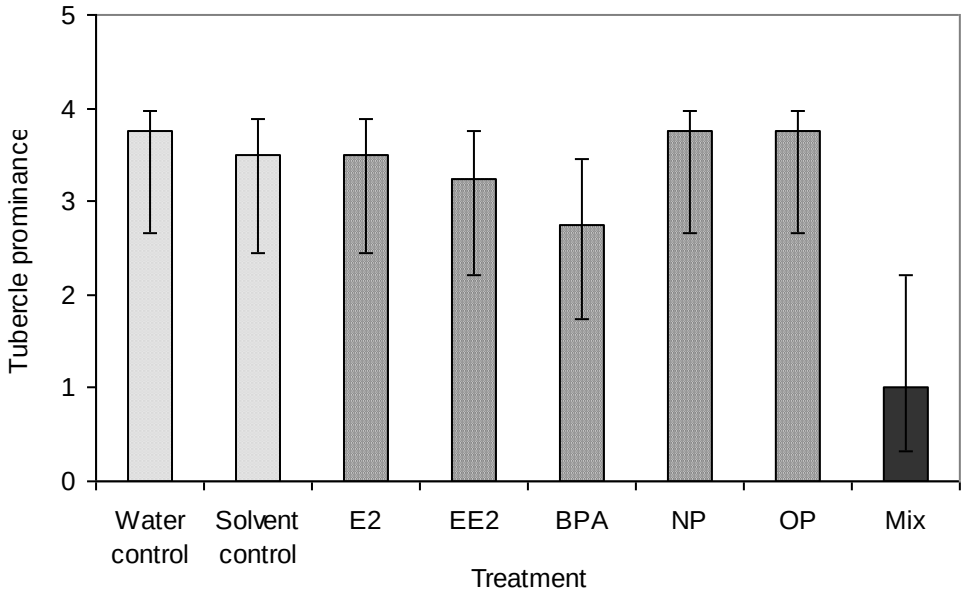
A


8000
7000
6000
5000
4000
3000
2000
1000
0
Total number of eggs
Water Control
Solvent Control
E2
EE2
BPA
NP
OP
Mixture
Treatment

B


30
25
20
15
10
5
0
Tubercle number
Water control
Solvent control
E2
EE2
BPA
NP
OP
Mix
Treatment

C


Tubercle prominance
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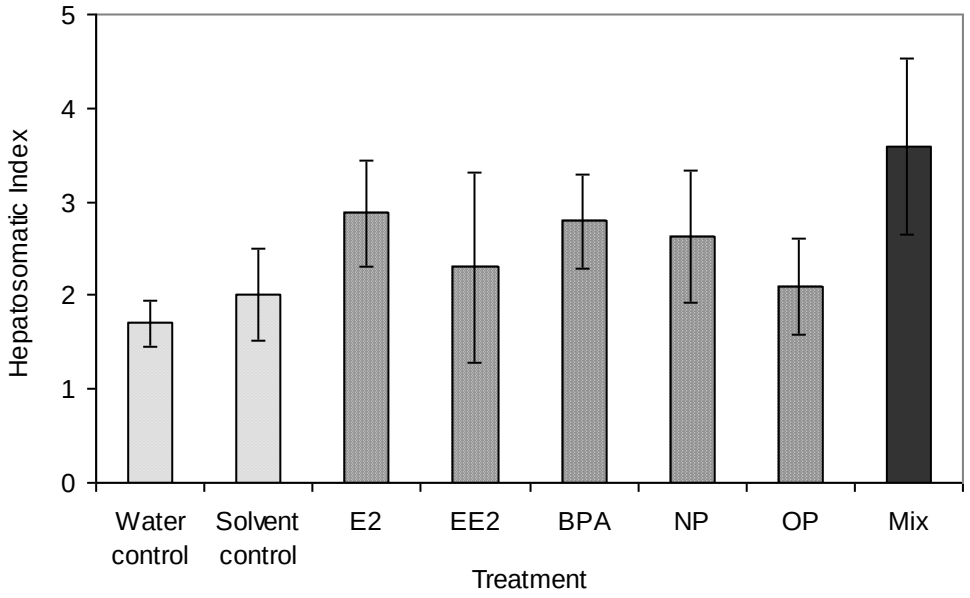
4
3
2
1
0
Water control
Solvent control
E2
EE2
BPA
NP
OP
Mix
Treatment

D


Hepatosomatic Index
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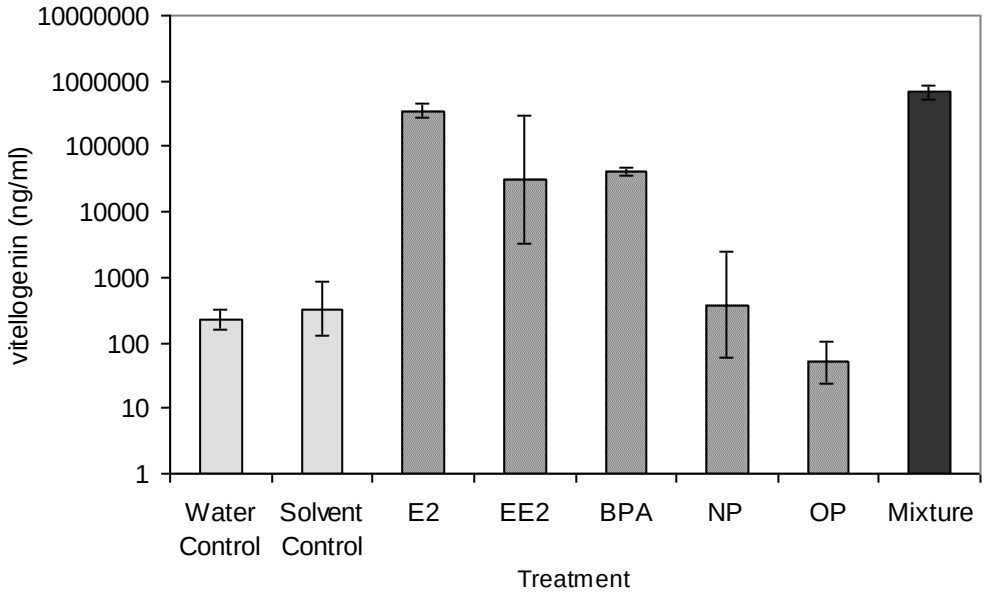
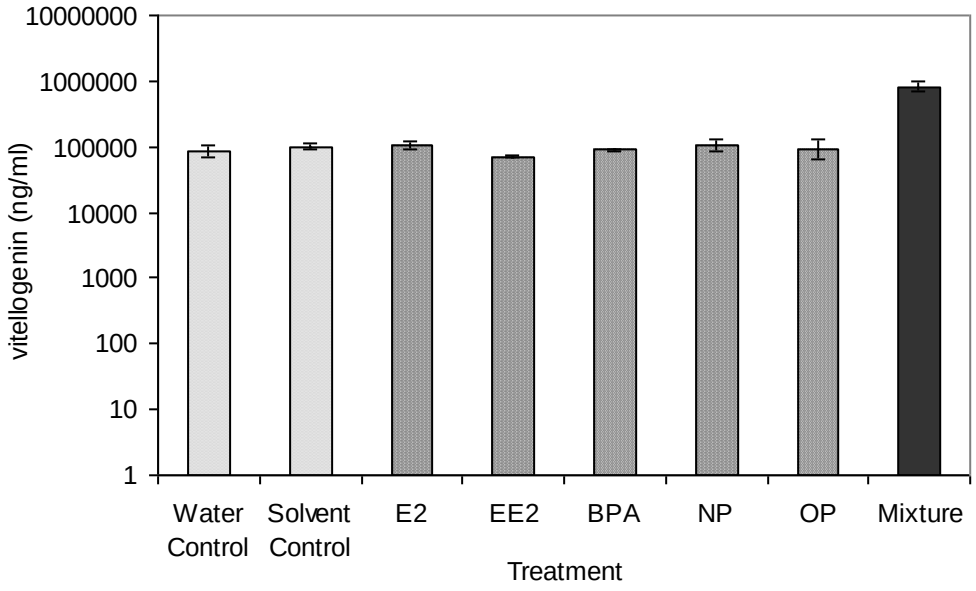
4
3
2
1
0
Water control
Solvent control
E2
EE2
BPA
NP
OP
Mix
Treatment

E


10000000
1000000
100000
10000
1000
100
10
1
vitellogenin (ng/ml)
Water Control
Solvent Control
E2
EE2
BPA
NP
OP
Mixture
Treatment

F


10000000
1000000
100000
10000
1000
100
10
1
vitellogenin (ng/ml)
Water Control
Solvent Control
E2
EE2
BPA
NP
OP
Mixture
Treatment

Brief: Mixtures of estrogenic chemicals act together to affect reproductive parameters, even when each component is present at a low and individually ineffective concentration.