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Review

Interactions between effects of environmental chemicals and natural stressors: A review

Martin Holmstrup^{a,*}, Anne-Mette Bindsbøl^a, Gertie Janneke Oostingh^b, Albert Duschl^b, Volker Scheil^c, Heinz-R. Köhler^c, Susana Loureiro^d, Amadeu M.V.M. Soares^d, Abel L.G. Ferreira^d, Cornelia Kienle^{c,e}, Almut Gerhardt^e, Ryszard Laskowski^f, Paulina E. Kramarz^f, Mark Bayley^g, Claus Svendsen^h, David J. Spurgeon^h

^a National Environmental Research Institute, Aarhus University, Department of Terrestrial Ecology, Vejløvej 25, DK-8600 Silkeborg, Denmark

^b Department of Molecular Biology, University of Salzburg, Hellbrunner Strasse 34, A-5020 Salzburg, Austria

^c Animal Physiological Ecology, Institute of Evolution and Ecology, University of Tübingen, Konrad-Adenauer-Str. 20, D-72072 Tübingen, Germany

^d Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

^e LimCo International, Oststrasse 24, 49477 Ibbenbüren, Germany

^f Institute of Environmental Sciences, Jagiellonian University, Gronostajowa 7, 30-387 Kraków, Poland

^g Zoophysiology, Department of Biological Sciences, Aarhus University, Building 131, DK-8000 Aarhus C, Denmark

^h Centre for Ecology and Hydrology, Maclean Building, Benson Lane, Crowmarsh Gifford, Wallingford, Oxon, OX10 8BB, United Kingdom

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ABSTRACT

Ecotoxicological effect studies often expose test organisms under optimal environmental conditions. However, organisms in their natural settings rarely experience optimal conditions. On the contrary, during most of their lifetime they are forced to cope with sub-optimal conditions and occasionally with severe environmental stress. Interactions between the effects of a natural stressor and a toxicant can sometimes result in greater effects than expected from either of the stress types alone. The aim of the present review is to provide a synthesis of existing knowledge on the interactions between effects of “natural” and chemical (anthropogenic) stressors. More than 150 studies were evaluated covering stressors including heat, cold, desiccation, oxygen depletion, pathogens and immunomodulatory factors combined with a variety of environmental pollutants. This evaluation revealed that synergistic interactions between the effects of various natural stressors and toxicants are not uncommon phenomena. Thus, synergistic interactions were reported in more than 50% of the available studies on these interactions. Antagonistic interactions were also detected, but in fewer cases. Interestingly, about 70% of the tested chemicals were found to compromise the immune system of humans as judged from studies on human cell lines. The challenge for future studies will therefore be to include aspects of combined stressors in effect and risk assessment of chemicals in the environment.

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* Corresponding author. Tel.: +45 8920 1573; fax: +45 8920 1414.

E-mail address: martin.holmstrup@dmu.dk (M. Holmstrup).

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1. Introduction

Ecotoxicological risk assessment of environmental chemicals is predominantly based on the results of laboratory studies where test organisms are exposed to a range of concentrations of single compounds. This approach is useful for the generation of dose–response relationships and the derivation of toxicity data such as the concentration causing 50% impairment of a life history trait (e.g. LC₅₀ or EC₅₀). In such laboratory experiments the test organisms are almost always kept in optimal conditions (temperature, moisture, food etc.) to optimize performance in the control treatment and isolate the effects of the chemical in question. This is the advantage of traditional laboratory testing, and methods have been greatly improved and standardised during the last decades (see e.g. Walker et al., 2001). However, in their natural settings organisms rarely experience optimal conditions. On the contrary for most of their lifespan, organisms are forced to cope with sub-optimal conditions with frequent exposure to severe environmental stress. These added environmental stressors may or may not alter the effects of chemical contaminants in comparison to the optimal laboratory test performed in well-controlled conditions. Risk assessment procedures therefore typically include safety factors (or uncertainty factors) in order to ensure conservative estimates of environmental concentrations that minimise the risk posed by the chemical to organisms in the field (Chapman et al., 1998). Underestimation of risk is of course problematic, but overestimation of risk may have considerable economic consequences that are also undesirable. In order to provide a scientific basis for improving the setting of safety factors, it therefore seems appropriate to supplement traditional ecotoxicological and toxicological testing with investigations on how natural stressors interact with chemical stressors. However, even though “multiple stress” (or “cumulative stressor”) approaches have received increasing interest in the last decades such studies are still relatively scarce, and up to now, there exist no comprehensive overview of the literature addressing this problem. To this end, a comparative analysis is lacking to unravel; (i) the frequency with which interactions between “natural” and chemical (anthropogenic) stressors occur, (ii) the predominance of synergistic (combined effects greater than expected) and antagonistic (combined effects smaller than expected) interactions, and finally (iii) the identification of chemicals and natural stressors with high (and low) likelihoods of interactions.

It is important to realise that interactions between natural stressors and toxic chemicals (toxicants) can occur at different levels. For example, physico-chemical properties of soil, water and air have influence on the bioavailability of toxicants, meaning that the same total environmental concentration of a toxicant causes different effects in different exposure scenarios (e.g. Newman and Unger, 2003). Likewise, physical conditions (e.g. temperature) or nutritional status (influencing feeding activity) may influence toxicokinetics. Lastly, toxicants may interfere with the physiology of an organism,

rendering this organism less tolerant to environmental stress caused by extreme levels of natural stressors (Højer et al., 2001; Noyes et al., 2009). The present review focuses on this last issue, namely physiology-based interactions between natural stressors and toxicants, and the mechanism behind such interactions. This also implies that we focus on organisms exposed to natural stressor extremes rather than general relationships between such parameters within their normal physiological range and toxicant effects. We have restricted the review to include only natural stressors that have been included in the NoMiracle project (see preface of this special issue). These include allergens and pathogens (in humans and animals), temperature extremes, drought (for terrestrial animals), oxygen depletion (aquatic species) and starvation. Interactions between chemicals and other natural stressors of ecological importance such as interspecific competition and predation are not included here, but have recently been reviewed by other authors (Relyea and Hoverman, 2006).

Studies of the interactions between the effects of natural stressors and chemicals have used a variety of statistical methods to demonstrate significant interactions. Some studies have used the classic ANOVA concept to test for statistically significant interactions between different stressors (e.g. Coors et al., 2008). Recently, the independent action (IA) model (Greco et al., 1995), developed to detect effect interactions between more than one chemical exposure has been developed to study interactions between natural and chemical stressors. Here, a mathematical model is fitted to dose–response data generating a response surface (Højer et al., 2001; Long et al., 2009). Although such data only have a phenomenological similarity to mixture toxicity studies, deviations from the model may be interpreted as synergistic or antagonistic interactions and many authors commonly use these terms for describing effects larger or smaller than expected from the effects of the single stressors. In this review we use the terms “synergism” and “antagonism” as a convenient way to indicate combinations of a set of stressors that result in greater or lesser effects than expected from the single exposures.

We have structured this review by first defining and discussing how each particular natural stressor influences animals in general. For each natural stressor we summarise and discuss the relevant published studies and, where enough knowledge is available, identify mechanisms explaining the occurrence of interactions. Finally, for each natural stressor we summarise the existing studies on interactions with toxicants and perform an overall judgement of the likelihood for interaction effects.

2. Interactions between effects of heat stress and chemicals

‘Heat’ in general is a relative term, since moderate temperatures might be relatively ‘cold’ for high temperature-adapted organisms but relatively ‘hot’ for cold-adapted organisms. We therefore define heat

as 'temperature above the normal operational temperature' of the respective organism. Of course, at the upper thermal tolerance limit, temperature itself is lethal for an organism, and so the studies cited in this paragraph deal with sublethal but stressful temperatures in combination with chemical stress.

Heat stress effects on organisms have been investigated for more than 50 years now, but studies on interactions of toxic compounds and heat stress are relatively new. At least three major reviews on the interactions between temperature and chemicals exist (Cairns et al., 1975; Mayer and Ellersieck, 1988; Heugens et al., 2001) with main focus on aquatic organisms. Readers are referred to these reviews to access older literature. In general, most of these studies revealed significant interactions of heat and chemical stress, but of these few studies characterised the interaction as being synergistic, antagonistic, or additive. Therefore, this aspect is emphasized as follows and summarised in Table 1.

2.1. Interactions with metals

The vast majority of studies on interactions of heat stress and chemicals have been conducted with heavy metals. However, results are not consistent for all members of this heterogeneous group of chemicals.

In four studies, explicit synergistic effects of metal and heat stress were found. Lannig et al. (2006) investigated the temperature-dependent stress response in oysters (*Crassostrea virginica*). The oysters were acclimated at 20, 24 and 28 °C and exposed to 50 µg Cd/L. Both increased temperature and cadmium, alone and in combination with one another, affected several physiological parameters and also the survival of the animals. Increased temperature amplified the effect of cadmium in a synergistic way. The authors stated that Cd exposure leads to elevated energy costs as well as impaired aerobic energy production due to a progressive mismatch between oxygen demand

Table 1
Overview of studies concerning interactions between heat stress and chemicals.

Toxicant	Test organism	Species	Life stage	Interaction	Reference
<i>Heavy metals</i>					
Cadmium	Mussel	<i>Crassostrea virginica</i>	Adult	Synergistic	Lannig et al. (2006)
Cadmium	Mussel	<i>Crassostrea virginica</i>	Adult	Synergistic	Cherkasov et al. (2006)
Cadmium	Water flea	<i>Daphnia magna</i>	Adult	Synergistic	Heugens et al. (2003)
Cadmium	Crayfish	<i>Orconectes immunis</i>	Adult	Synergistic	Khan et al. (2006)
Cadmium	Clam	<i>Crassostrea virginica</i>	Adult	Synergistic	Cherkasov et al. (2007)
Cadmium	Woodlouse	<i>Porcellio scaber</i>	Adult	Antagonistic	Abdel-Lateif et al. (1998)
Cadmium	Fish	<i>Danio rerio</i>	Embryo	Synergistic	Hallare et al. (2005)
Nickel	Fish	<i>Danio rerio</i>	Embryo	Synergistic	Scheil and Köhler (2009)
Mercury	Springtail	<i>Folsomia candida</i>	Adult	Synergistic	Slotsbo et al. (2009)
Copper	Snail	<i>Viviparus bengalensis</i>	Adult	Synergistic	Gupta and Khangarot (1981)
Copper	Amphipod	<i>Gammarus pulex</i>	Adult	Synergistic	Bat et al. (2000)
Copper	Mussel	<i>Dreissena polymorpha</i>	Adult	Synergistic	Rao and Khan (2000)
Copper	Copepod	<i>Diaptomus clavipes</i>	Adult	Synergistic	Boeckman and Bidwell (2006)
Copper	Water flea	<i>Daphnia pulex</i>	Adult	None	Boeckman and Bidwell (2006)
Copper	Fish	<i>Ictalurus punctatus</i>	Adult	Antagonistic	Perschbacher (2005)
Copper	Crayfish	<i>Orconectes immunis</i>	Adult	Synergistic	Khan et al. (2006)
Copper	Fish	<i>Pagrus major</i>	Adult	None	Furuta et al. (2008)
Copper	Fish	<i>Paralichthys olivaceus</i>	Adult	None	Furuta et al. (2008)
Zinc	Amphipod	<i>Gammarus pulex</i>	Adult	Synergistic	Bat et al. (2000)
Zinc	Crayfish	<i>Orconectes immunis</i>	Adult	Synergistic	Khan et al. (2006)
Zinc	Earthworm	<i>Lumbricus terrestris</i>	Adult	Synergistic	Khan et al. (2007)
Lead	Amphipod	<i>Gammarus pulex</i>	Adult	Synergistic	Bat et al. (2000)
Lead	Crayfish	<i>Orconectes immunis</i>	Adult	Synergistic	Khan et al. (2006)
Lead	Earthworm	<i>Lumbricus terrestris</i>	Adult	Synergistic	Khan et al. (2007)
<i>Polycyclic aromatic hydrocarbons (PAH)</i>					
Anthracene	Fish	<i>Lepomis macrochirus</i>	Adult	None	McCloskey and Oris (1991)
<i>Surfactants</i>					
4-nonylphenol	Earthworm	<i>Dendrobaena octaedra</i>	Adult	Synergistic	Jensen et al. (2009)
<i>Pesticides</i>					
Fenarimol	Amphipod	<i>Monoporeia affinis</i>	Adult	Synergistic	Jacobson et al. (2008)
Diazinon	Fish	<i>Danio rerio</i>	Embryo	Synergistic	Osterauer and Köhler (2008)
Chlorpyrifos	Fish	<i>Danio rerio</i>	Embryo	None	Scheil and Köhler (2009)
Chlorpyrifos	Insect	<i>Chironomus tentans</i>	Adult	Synergistic	Lydy et al. (1999)
Imidachloprid	Fish	<i>Danio rerio</i>	Embryo	None	Scheil and Köhler (2009)
Terbufos	Shrimp	<i>Palaemonetes</i> sp.	Adult	Synergistic	Brecken-Folse et al. (1994)
Terbufos	Fish	<i>Cyprinodon variegatus</i>	Adult	Synergistic	Brecken-Folse et al. (1994)
Terbufos	Fish	<i>Oncorhynchus mykiss</i>	Adult	Synergistic	Howe et al. (1994)
Terbufos	Amphipod	<i>Gammarus pseudolimnaeus</i>	Adult	Synergistic	Howe et al. (1994)
Trichlorfon	Shrimp	<i>Palaemonetes</i> sp.	Adult	Synergistic	Brecken-Folse et al. (1994)
Trichlorfon	Fish	<i>Cyprinodon variegatus</i>	Adult	Synergistic	Brecken-Folse et al. (1994)
Trichlorfon	Fish	<i>Oncorhynchus mykiss</i>	Adult	Synergistic	Howe et al. (1994)
Trichlorfon	Amphipod	<i>Gammarus pseudolimnaeus</i>	Adult	Synergistic	Howe et al. (1994)
M-parathion	Insect	<i>Chironomus tentans</i>	Adult	Synergistic	Lydy et al. (1999)
Pentachlorobenzene	Insect	<i>Chironomus tentans</i>	Adult	Synergistic	Lydy et al. (1999)
Profenofos	Fish	<i>Pimephales promelas</i>	Adult	Synergistic	Baer et al. (2002)
Pyrethrine	Lizard	<i>Anolis carolinensis</i>	Adult	Antagonistic	Talent (2005)
GCSC-BtA	Insects	Various species	Adult	Synergistic	Zhu et al. (2006)

and oxygen supply and increasing mitochondrial dysfunction with rising temperature. Another study (Cherkasov et al., 2006) also conducted with *C. virginica* revealed similar results. Scheil and Köhler (2009) tested the effect of nickel (as nickel chloride) on the embryogenesis of *Danio rerio*. Increasing temperatures led to a drastic reduction in hatching of embryos if applied together with high Ni concentrations. The effect was related to a postulated increase of Ni uptake at higher temperature in combination with a detrimental effect of Ni on the activity of the proteases which are needed for hatching. Slotsbo et al. (2009) conducted experiments on heat stress in combination with mercury exposure in the collembolan *Folsomia candida* and found a highly significant synergistic interaction between the effects of mercury and heat stress. They attributed these effects to the fact that Hg may deplete cellular anti-oxidation systems resulting in the production of reactive oxygen species and peroxidation of membrane lipids. This may have led to a destabilization of membrane function leading to destabilising high membrane fluidity at high temperatures.

All other studies remain rather descriptive concerning observed interactions (Table 1), or just report on interactions but do not attribute them to be synergistic, antagonistic, or additive. Most authors related an observed increase of metal toxicity with increasing temperature to the increased uptake and accumulation of the metal by the organism.

For cadmium, some studies showed increasing toxicity at increasing temperatures. In a study from Heugens et al. (2003), *Daphnia magna* was exposed to Cd at standard and higher temperatures, leading to an increase of the Cd toxicity at increasing temperatures. The authors were able to demonstrate a correlation between increased Cd accumulation in the daphnids and increased Cd toxicity at higher temperatures. Hallare et al. (2005) investigated Cd toxicity in *D. rerio* embryos and larvae and found increasing effects of Cd at increasing temperatures. Khan et al. (2006) reported on increased oxygen consumption due to Cd exposure in the crayfish *Orconectes immunis* if kept at a standard temperature, and an amplified effect at higher temperatures. However, the authors did not investigate the underlying mechanisms of this effect. Cherkasov et al. (2007) showed that Cd accumulated faster in gills and hepatopancreas of the oyster *C. virginica*, if exposure took place at higher temperatures. Khan et al. (2007) found an increased oxygen consumption in the earthworm *Lumbricus terrestris* at an elevated temperature compared to a standard temperature if the earthworms were exposed to Cd.

In contrast to the findings mentioned above, other studies revealed a lower effect of Cd at higher temperatures than at control temperature, or a temperature-independent effect. Abdel-Lateif et al. (1998) noticed a decrease of Cd toxicity (measured as reduction of growth rate) at higher temperatures in a study with *Porcellio scaber*. The reduction in growth rates as a result of Cd exposure was masked by an increase of the growth rates at higher temperatures. The authors speculated that, on one hand, elevated temperatures are known to increase the rate of all physiological processes in the isopods, whereas cadmium, on the other hand, tends to disturb metabolism, which results in the opposite effect. In other studies with the oyster *C. virginica* and other bivalves, temperature-independent accumulation patterns of Cd were found (Frayse et al., 2000; Cherkasov et al., 2006; Lannig et al., 2006). This discrepancy may be related to the differences in exposure regimes in different experiments. At shorter exposure times, Cd accumulation rates in oysters were shown to be slow and largely temperature-independent whereas, at longer exposure times, a strong temperature-dependent increase in accumulated Cd levels was observed (Cherkasov et al., 2006; Lannig et al., 2006; Cherkasov et al., 2007).

Studies on copper toxicity and interactions with temperature have shown increasing temperatures to increase the toxicity of Cu. Gupta and Khangarot (1981) investigated the freshwater pond snail, *Viviparus bengalensis* and found a positive correlation between temperature and Cu toxicity. This was also demonstrated by Bat et al. (2000) for *Gammarus pulex* with a three-fold decrease of the 96 h-LC₅₀

from 0.08 mg/L at 15 °C to 0.028 mg/L at 25 °C. Rao and Khan (2000) studied Cu toxicity in the zebra mussel (*Dreissena polymorpha*). The authors found that increasing temperatures increased the toxicity of Cu to the mussels, and they attributed this effect to higher metabolic rates at higher temperatures leading to a higher exposure to the metal. A study of Boeckman and Bidwell (2006) revealed increasing Cu toxicity in *Diaptomus clavipes* (but not in *Daphnia pulex*) to depend on exposure temperature. Perschbacher (2005), however, found an inverse relationship between Cu toxicity and temperature. In his experiments, Cu was less toxic to catfish (*Ictalurus punctatus*) if applied at increasing temperatures. To explain this rather uncommon inverse relationship, the author suggested hampered functioning of copper tolerance mechanisms at lower temperatures (Perschbacher, 2005). Khan et al. (2006) also investigated Cu toxicity to the crayfish *O. immunis*, showing increasing toxicity of Cu at increasing temperatures. In contrast to this, Furuta et al. (2008) did not find significant effects of water temperature on the Cu toxicity to the Japanese flounder, *Paralichthys olivaceus*, and red sea bream, *Pagrus major*. Other metals such as zinc and lead have been studied, both with increasing effect at increasing temperatures in *G. pulex* (Bat et al., 2000), *O. immunis* (Khan et al., 2006) and *L. terrestris* (Khan et al., 2007).

Thus, metal toxicity generally increases with rising temperature (Table 1). This notion is largely in accordance with the earlier analysis of Heugens et al. (2001). As discussed by some of the above-mentioned authors, other factors, like animal age, animal size, or their developmental stage might influence the sensitivity of organisms to metals, which may complicate the comparability of different studies.

2.2. Interaction with polycyclic aromatic hydrocarbons

Only a single study with a PAH, namely anthracene, and increased temperature seems to exist. In this study, McCloskey and Oris (1991) investigated the effect of water temperature on the photo-induced toxicity of anthracene to bluegill sunfish (*Lepomis macrochirus*). The authors did not find a significant effect of temperature (20 °C versus 30 °C) but claimed to see a trend towards lower toxicity at higher temperatures, possibly due to increasing rates of PAH desorption from biological membranes at higher temperatures.

2.3. Interactions with surfactants

Surfactants are an important and widely used group of chemicals. Nevertheless, only a single study on the interactions of a surfactant with increasing temperature seems to exist. Jensen et al. (2009) found synergistic interactions between 4-nonylphenol (4-NP) and high temperatures in the earthworm *Dendrobaena octaedra*. In the experiments, the LC₅₀ decreased from 308 mg 4-NP/kg dry soil at 25 °C to 40 mg 4-NP/kg dry soil when the animals were exposed at 33 °C. The authors suggested that this effect was a result of protein denaturation caused by high temperatures as well as by 4-NP, which, in combination, may overwhelm cellular repair mechanisms.

2.4. Interactions with pesticides

Pesticide toxicity at above-optimum temperatures has been investigated in a number of studies. This is important because the seasonal pattern of pesticide application during the summer months makes exposure under heat stress a realistic scenario.

Brecken-Folse et al. (1994) studied the toxicity (measured as LC₅₀) of the two organophosphate insecticides terbufos and trichlorfon to grass shrimps (*Palaemonetes* sp.) and sheepshead minnows (*Cyprinodon variegatus*). The toxicity of both insecticides increased with increasing temperatures. The same result was found by Howe et al. (1994) for rainbow trout (*Oncorhynchus mykiss*) and the amphipod *G. pseudolimnaeus*. Lydy et al. (1999) investigated the effects of temperature on the toxicity of m-parathion, chlorpyrifos, and pentachlorobenzene to

Chironomus tentans. For all the three pesticides, a positive correlation between toxicity and increasing temperature was found which was accompanied by increased uptake of the chemicals at higher temperatures. In a study of Baer et al. (2002), profenofos was found to be most toxic (measured as LC₅₀ and acetylcholinesterase activity inhibition) to fathead minnows (*Pimephales promelas*) at high temperature. This effect was more drastic if the oxygen concentrations in water were reduced. In contrast, Talent (2005) reported pyrethrin in combination with high temperature (35 °C) to be less toxic to lizards (*Anolis carolinensis*) than at lower temperatures (15 °C). To explain his findings, Talent (2005) discussed that the primary reason for the variation of the toxicity of pyrethrins and pyrethroids to poikilothermic animals is the higher susceptibility of neurons to excitation at lower temperatures as a result of increased sodium transport through sodium channels. A study of Zhu et al. (2006) dealt with the toxicity of Germany–China Scientific Cooperation–*Bacillus thuringiensis*–Abamectin (GCSC–BtA, a new type of biocide developed by conjugating a toxin from *B. thuringiensis* with abamectin from *Streptomyces avermitilis*) on arthropod pests under different temperature conditions. Several insect and mite species were exposed to the biocide at temperatures between 15 °C and 30 °C. With the exception of a single species, in which no effect of temperature was found, all other tests showed increasing toxicity of the biocide, if it was applied at high temperatures.

Jacobson et al. (2008) investigated and found combined effects of temperature and the fungicide fenarimol on the baltic amphipod *Monoporeia affinis*. Elevated temperature and fenarimol interacted synergistically and increased the number of females with dead eggs, impaired sexual maturation in males and females, lowered the number of fertilised females, reduced the fecundity, and altered embryogenesis. Osterauer and Köhler (2008) investigating hatching rate of zebrafish (*D. rerio*) embryos found the combination of high temperature stress and diazinon to interact synergistically. On the other hand, Scheil and Köhler (2009) did not find increased toxicity of chlorpyrifos and imidacloprid when investigating embryogenesis of *D. rerio* at high temperature.

3. Interactions between effects of freezing temperatures and chemicals

Cold-hardy organisms are traditionally divided into freeze avoiding or freeze tolerant species (Zachariassen, 1985; Ramløv, 2000).

Freeze avoiding species die if their body fluids freeze. They survive sub-zero temperatures by supercooling their body fluids or dehydrating until the melting point of their body fluids is lower than the ambient temperature. Freeze tolerant species, on the other hand, can survive freezing of their extracellular body fluids. Since both freeze tolerance and freeze avoidance often depend on the synthesis and accumulation of cryoprotective molecules (various carbohydrates and antifreeze proteins) and also on cell membrane adjustments, it is expected that toxicants interfering with these processes will significantly reduce survival at low temperatures.

3.1. Interactions with heavy metals

With the exception of lead, a variety of heavy metals have been shown to interact synergistically with frost survival (Table 2). Bindesbøl et al. (2005, 2009a) showed that a synergistic interaction occurred between freezing temperatures and environmentally realistic copper concentrations in the earthworm *D. octaedra*, and proposed that changes in membrane phospholipid fatty acids (PLFAs) might provide a mechanistic explanation for the decreased freeze tolerance. These changes in membrane phospholipids can probably be explained by copper's ability to induce lipid peroxidation, where especially phospholipids containing two or more double bonds are particularly susceptible to oxidation by free radicals and other highly reactive species (Valko et al., 2005). This synergistic interaction between copper and freezing temperatures has also been observed in the earthworm *Aporrectodea caliginosa* (Holmstrup et al., 1998) and the springtail *Protaphorura armata* (Bossen, 2001).

Synergistic interactions between mercury and freezing temperatures have been observed with significantly reduced ability of *D. octaedra* to survive at –6 °C (Bindesbøl et al., 2009b). The LC₅₀ decreased from approximately 40 mg/kg dry tissue at the control temperature of 2 °C to less than 10 mg/kg at –6 °C. Likewise, mercury reduced the cold shock tolerance of the springtail *F. candida* as well as reducing the beneficial effect of rapid cold hardening in this species (Holmstrup et al., 2008). Reduced freeze tolerance was observed when different tissues of the larvae *Eurosta solidaginis* and *Chilo suppressalis* (insects) were exposed to mercury (Philip et al., 2008; Izumi et al., 2006). These authors explained the reduced freeze tolerance by the ability of mercury to block aquaporins, which are integral proteins channelling trans-membranous

Table 2
Overview of studies concerning interactions between cold stress and chemicals.

Toxicant	Test organism	Species	Life stage	Interaction	Reference
<i>Heavy metals</i>					
Copper	Earthworm	<i>Dendrobaena octaedra</i>	Adult	Synergistic	Bindesbøl et al. (2005, 2009a)
Copper	Earthworm	<i>Dendrobaena octaedra</i>	Cocoon	Synergistic	Holmstrup et al. (1998)
Copper	Earthworm	<i>Aporrectodea caliginosa</i>	Cocoon	Synergistic	Holmstrup et al. (1998)
Copper	Springtail	<i>Protaphorura armata</i>	Adults	Synergistic	Bossen (2001)
Nickel	Earthworm	<i>Dendrobaena octaedra</i>	Adult	Synergistic	Bindesbøl et al. (2009b)
Mercury	Earthworm	<i>Dendrobaena octaedra</i>	Adult	Synergistic	Bindesbøl et al. (2009b)
Mercury	Springtail	<i>Folsomia candida</i>	Adult	Synergistic	Holmstrup et al. (2008)
Mercury	Insect	<i>Chilo suppressalis</i>	Larvae tissue	Synergistic	Izumi et al. (2006)
Mercury	Insect	<i>Eurosta solidaginis</i>	Larvae tissue	Synergistic	Philip et al. (2008)
Lead	Earthworm	<i>Dendrobaena octaedra</i>	Adult	None	Bindesbøl et al. (2009b)
<i>Polycyclic aromatic hydrocarbons (PAH)</i>					
Pyrene	Springtail	<i>Protaphorura armata</i>	Adult	Antagonistic	Sjursen and Holmstrup (2004)
Pyrene	Earthworm	<i>Dendrobaena octaedra</i>	Adult	None	Bindesbøl et al. (2009b)
Phenanthrene	Earthworm	<i>Dendrobaena octaedra</i>	Adult	Antagonistic	Bindesbøl et al. (2009b)
<i>Pesticides</i>					
Abamectin	Earthworm	<i>Dendrobaena octaedra</i>	Adult	None	Bindesbøl et al. (2009b)
Carbendazim	Earthworm	<i>Dendrobaena octaedra</i>	Adult	None	Bindesbøl et al. (2009b)
<i>Other toxicants</i>					
Oil dispersant	Mussel	<i>Mytilus edulis</i>	Adult	Synergistic	Aarset and Zachariassen (1983)
Nonylphenol	Earthworm	<i>Dendrobaena octaedra</i>	Adult	None	Jensen et al. (2009)
Surfactants	Insect	<i>Cacopsylla pyricola</i>	Adult	Synergistic	Horton et al. (1996)

transport of water and cryoprotective osmolytes such as glycerol (Borgnia et al., 1999). Blocking of aquaporins may increase the risk of intracellular freezing in freeze tolerant organisms. Using radiotracer techniques, Izumi et al. (2006) showed that the transport of both water and glycerol was almost eliminated by mercury, suggesting an explanation for the reduced freeze tolerance. Copper ions have been shown to inhibit the water and glycerol permeability of aquaporins in human erythrocytes (Zelenia et al., 2004) suggesting that also this metal's ability to block aquaporins might contribute to the synergistic interaction between this chemical and frost survival.

Zachariassen et al. (2004) proposed that metals could affect the cryoprotective mechanism of antifreeze proteins (AFP) which improve the supercooling capacity by a phenomenon known as thermal hysteresis (Zachariassen, 1985). Both AFPs and metallothioneins contain high amounts of cysteine, which means that exposure to metals may deplete the cysteine pool and thereby reduce the exposed organism's ability to produce AFPs. Pedersen et al. (2006) explored this idea and found that AFP production was reduced in the freeze avoiding meal worm (*Tenebrio molitor*) when exposed to metals (copper, zinc and cadmium) at summer temperatures (20 °C), but not at winter temperatures (4 °C).

It seems reasonable to suggest that the mechanistic explanation behind the observed synergistic interactions of heavy metals and freezing temperatures (across species) is at least partly due to membrane damage. This was supported by Taulavuori et al. (2005), who hypothesised that heavy metals increase the risk of frost injury due to membrane alterations in plants at northern high latitudes. Further, Pukacki (2004) found a reduction in the PLFAs 18:2 and 18:3 of cell membranes in needles of Scots pine (*Pinus sylvestris*) near a copper smelter in Glogow, Poland, which likely could lead to a reduced freeze tolerance in those tissues. In addition, such reductions in freeze tolerance were recorded in a study by Sutinen et al. (1996), who observed that needles of *P. sylvestris* were more susceptible to frost near a copper-nickel smelter in Russia than those further away from the smelter.

3.2. Interactions with polycyclic aromatic hydrocarbons

Only two studies concerning the interactions between freezing temperatures and PAHs exist (Bindesbøl et al., 2009b; Sjørnsen and Holmstrup, 2004). In the study by Bindesbøl et al. (2009b), the earthworm *D. octaedra* was exposed to increasing concentrations of pyrene and phenanthrene, respectively, and cold acclimated for 6 weeks before the worms were exposed to freezing temperatures (–6 °C). The worms were significantly more susceptible to phenanthrene at the control temperature than at the freezing temperature. This tendency was also observed with pyrene although in this case, results were not statistically significant. This could indicate antagonistic interactions between the effects of PAHs and low temperature. The authors suggested that PAHs might increase the fluidity of cellular membranes which may be an advantage during freezing. Sjørnsen and Holmstrup (2004) also reported a higher survival in pyrene exposed *P. armata* at –3 °C compared to springtails exposed to pyrene at 5 and 20 °C. This might support the above suggested mechanistic explanation, that a PAH-induced increased fluidity of cell membranes counteracts the expected mortality observed at non freezing temperatures.

3.3. Interactions with surfactants

Horton et al. (1996) found that spraying pear psylla (*Cacopsylla pyricola*; Hemiptera) with four different surfactants individually, caused a dramatic decrease in the survival of frost. Spraying with water also decreased freeze survival, but to a lesser extent than with surfactants. Temperatures causing 50% mortality increased from below –18 °C in control and water sprayed animals to between

–2.6 and –12.7 °C in surfactant treated animals. *C. pyricola* is a cold-hardy, freeze avoiding species, able to supercool to temperatures well below –20 °C during winter, and freezing of their body fluids is lethal. The increased mortality in water and surfactant exposed animals is probably a result of inoculative freezing as described by Salt (1963). Spraying with surfactant might also increase the water permeability of the cuticle of *C. pyricola* increasing the risk of inoculative freezing.

Aarset and Zachariassen (1983) observed that an oil dispersant reduced freeze tolerance of the blue mussel, *Mytilus edulis*. Jensen et al. (2009) found that the freeze tolerance of *D. octaedra* was not reduced after exposure to the surfactant nonylphenol. Whereas the negative impact of surfactants on survival through increased freeze inoculation is evident in freeze avoiding species, this phenomenon does not reduce frost survival in a freeze tolerant species such as *D. octaedra*.

3.4. Interactions with pesticides

Only two pesticides, abamectin and carbendazim, have been tested with respect to effects on freeze tolerance and neither of these influenced frost survival in *D. octaedra* at –6 °C (Bindesbøl et al., 2009b). This is probably due to their specific mode of action, which is not linked to the freezing response. Abamectin inhibits the gamma-aminobutyric acid induced neurotransmission and causes paralysis in parasites (Campbell et al., 1983) and carbendazim works by inhibiting the development of fungi, probably by interfering with spindle formation at mitosis. These modes of action are presumably the same in *D. octaedra*, and both chemicals were inherently toxic, but with no effect on freeze tolerance.

4. Interaction between effects of desiccation and chemicals

As with cold-hardy organisms, drought tolerance often depends on physiological adaptations such as the accumulation of sugars and polyols and adjustments of membrane composition (Bayley and Holmstrup, 1999; Holmstrup et al., 2002). It is therefore expected that toxicants interfering with these processes will significantly reduce survival of drought. Further, dehydration will reduce the volume of liquid water in the organism thereby increasing the concentration of chemicals and the risk of toxic damage.

4.1. Interaction with heavy metals

Combinations between drought and metals have shown both synergistic interactions and no interaction (Table 3). Most studies so far have investigated the effect of copper on drought tolerance but no clear trend has been found. Drought tolerance was reduced by copper in the earthworm *A. caliginosa* (Friis et al., 2004) as well as in cocoons of both *D. octaedra* and *A. caliginosa* (Holmstrup et al., 1998). Furthermore, copper significantly reduced the drought tolerance of the collembolan *F. candida* in a study by Holmstrup (1997), whereas no interaction was observed between copper and drought in the same species in a study by Sørensen and Holmstrup (2005). This difference in interactions in *F. candida* may be explained by the drought exposure levels used. Holmstrup (1997) exposed *F. candida* to 300 mg Cu/kg dw for one week at 20 °C, followed by exposure to different drought stresses ranging from 99.6% relative humidity (RH) to 96.8% RH for seven days. The synergistic interaction became apparent at drought levels lower than 97.8% RH, whereas no interaction with copper occurred at higher drought exposures. In the study by Sørensen and Holmstrup (2005) *F. candida* were exposed to increasing concentrations of copper and cadmium, followed by drought stress at 97.8% RH for seven days. This drought level is less severe than those showing synergistic interactions with copper in the study by Holmstrup (1997), and might explain the lack of synergistic interaction in this later study.

Table 3
Overview of studies concerning interactions between desiccation and chemicals.

Toxicant	Test organism	Species	Life stage	Interaction	Reference
<i>Heavy metals</i>					
Copper	Earthworm	<i>Dendrobaena octaedra</i>	Cocoons	Synergistic	Holmstrup et al. (1998)
Copper	Earthworm	<i>Aporrectodea caliginosa</i>	Cocoons	Synergistic	Holmstrup et al. (1998)
Copper	Earthworm	<i>Aporrectodea caliginosa</i>	Adults	Synergistic	Friis et al. (2004)
Copper	Springtail	<i>Folsomia candida</i>	Adults	Synergistic	Holmstrup (1997)
Copper	Springtail	<i>Folsomia candida</i>	Adults	None	Sørensen and Holmstrup (2005)
Cadmium	Springtail	<i>Folsomia candida</i>	Adults	None	Sørensen and Holmstrup (2005)
<i>Polycyclic aromatic hydrocarbons (PAH)</i>					
Pyrene	Springtail	<i>Protaphorura armata</i>	Adult	Synergistic	Sjursen and Holmstrup (2004)
Pyrene	Springtail	<i>Folsomia candida</i>	Adult	Synergistic	Skovlund et al. (2006)
Pyrene	Springtail	<i>Folsomia candida</i>	Adult	Synergistic	Sørensen and Holmstrup (2005)
Pyrene	Springtail	<i>Folsomia fimetaria</i>	Adult	Synergistic	Sjursen et al. (2001)
Flourene	Springtail	<i>Folsomia fimetaria</i>	Adult	Synergistic	Sjursen et al. (2001)
Flourene	Springtail	<i>Folsomia candida</i>	Adult	Synergistic	Sørensen and Holmstrup (2005)
Flouranthene	Springtail	<i>Folsomia fimetaria</i>	Adult	Synergistic	Sjursen et al. (2001)
Flouranthene	Earthworm	<i>Lumbricus rubellus</i>	Adult	None	Long et al. (2009)
Dibenzothiophene	Springtail	<i>Folsomia fimetaria</i>	Adult	Synergistic	Sjursen et al. (2001)
Acridine	Springtail	<i>Folsomia fimetaria</i>	Adult	None	Sjursen et al. (2001)
Dibenzofuran	Springtail	<i>Folsomia fimetaria</i>	Adult	None	Sjursen et al. (2001)
Carbazole	Springtail	<i>Folsomia fimetaria</i>	Adult	None	Sjursen et al. (2001)
<i>Surfactants</i>					
Nonylphenol	Springtail	<i>Folsomia candida</i>	Adult	Synergistic	Skovlund et al. (2006)
Nonylphenol	Springtail	<i>Folsomia candida</i>	Adult	Synergistic	Sørensen and Holmstrup (2005)
Nonylphenol	Springtail	<i>Folsomia candida</i>	Adult	Synergistic	Holmstrup (1997)
Nonylphenol	Springtail	<i>Folsomia candida</i>	Adult	Synergistic	Højer et al. (2001)
LAS	Springtail	<i>Folsomia candida</i>	Adult	Synergistic	Sørensen and Holmstrup (2005)
LAS	Springtail	<i>Folsomia candida</i>	Adult	Synergistic	Holmstrup (1997)
<i>Pesticides</i>					
DDT	Springtail	<i>Folsomia candida</i>	Adult	None	Skovlund et al. (2006)
Cypermethrin	Springtail	<i>Folsomia candida</i>	Adult	None	Sørensen and Holmstrup (2005)
Dimethoate	Springtail	<i>Folsomia candida</i>	Adult	None	Sørensen and Holmstrup (2005)
Lindane	Springtail	<i>Onychiurus quadricellatus</i>	Adult	Synergistic	Demon and Eijssackers (1985)

Friis et al. (2004) exposed the earthworm *A. caliginosa* to a sublethal copper concentration (150 mg/kg) at different drought levels in soil, obtaining soil water potentials from pF 1.5 (wet) to pF 5 (very dry). They found that drought tolerance decreased in copper exposed worms. At drought levels where mortality occurred in controls (pF 4.0–4.5), copper increased the mortality rate 2 to 3-fold. *A. caliginosa* enters diapause during summer when the water potential is sufficiently reduced (Gerard, 1967), during which process the worm encloses itself into an estivation cell, which minimises water loss during drought. Friis et al. (2004) found that the development of estivation cells was significantly impaired in copper exposed worms, and that worms without estivation cells were more prone to drought-induced mortality than worms with intact estivation cells. However, the lack of estivation cells was not the only explanation for the observed synergistic interaction. An additional explanation was that the internal copper concentration was also higher with increased drought stress, possibly causing more severe acute effects (Friis et al., 2004). The authors suggested that the observed increase in copper burden at high drought stress could be a result of the loss of copper regulation during severe dehydration and that the mechanistic explanation of the observed synergy was probably a combination of both behavioural and physiological responses.

4.2. Interaction with polycyclic aromatic hydrocarbons

Sjursen et al. (2001) tested the effects of seven PAHs on the drought tolerance of the springtail *F. fimetaria*. The springtails were exposed to a drought level of 98.2% RH or 100% RH (control) after exposure to increasing concentrations of the different PAHs. Synergistic effects between the PAH and drought could be seen for fluorene, pyrene, fluoranthene, dibenzothiophene and carbazole, whereas dibenzofuran and acridine did not reduce the drought tolerance. A

synergistic interaction between pyrene and drought was also observed in two other studies with springtails (Sørensen and Holmstrup, 2005; Skovlund et al., 2006). Skovlund et al. (2006) tested the effect of drought and pyrene using a full factorial design with six sub-lethal pyrene concentrations between 0 and 150 mg/kg dry soil and six drought levels from 100% RH to 97% RH. The synergistic interaction became apparent at drought levels lower than 98.2% RH and at the highest tested pyrene concentration of 150 mg/kg. At a drought level of 97.8% RH, used in the study by Sørensen and Holmstrup (2005), the synergistic interaction with pyrene also became clear at a concentration of 150 mg/kg dw or more. They suggested that the reduced drought tolerance was due to disrupted membrane functionality, because PAHs are known to interact with membranes. Functional cell membranes are crucial for the osmoregulatory changes associated with dehydration (Bayley and Holmstrup, 1999), and membrane disturbance might detrimentally influence these processes, which in turn would increase mortality.

Long et al. (2009) tested the effects of fluoranthene on survival and reproduction during drought exposure in the earthworm *L. rubellus*. This was tested using a full factorial design with five fluoranthene concentrations and four drought treatments, including controls. Survival was not significantly affected by any of the exposure treatments. Cocoon production, however, was significantly reduced by both drought and fluoranthene, but no synergistic interactions were observed between the two stressors that seemed to work in concert by simple additive effects. These results are in contradiction to the results with springtails, where synergistic interactions were observed for almost all tested PAHs and drought. This may be explained by the rather low degree of drought stress used in the study by Long et al. (2009), which in itself will probably not cause the worms to dehydrate, as was the case with the collembolans discussed above.

4.3. Interactions with surfactants

Højer et al. (2001) showed that exposure to nonylphenol caused a reduction in the drought tolerance of *F. candida*. The intensity of the two stressors was varied in a full factorial design with six nonylphenol concentrations (0–62.5 mg/kg dw) and seven drought levels (99.7–97.0% RH). The synergistic interaction became more pronounced at a drought level of 97.9% RH and below. The relative humidity causing 50% mortality increased from approximately 98% RH with no nonylphenol exposure to 98.6% RH in animals exposed to a nonylphenol concentration of 60 mg/kg dw. Furthermore, it was shown that nonylphenol caused a small but significant increase in water permeability across the integument, as well as inhibiting the synthesis of drought protective sugars. Three other studies (Sørensen and Holmstrup, 2005; Holmstrup, 1997; Skovlund et al., 2006) have confirmed the highly synergistic interaction between nonylphenol and drought in *F. candida*. A synergistic interaction was also recorded between drought and the surfactant linear alkylbenzene sulphonate (LAS) in *F. candida* (Holmstrup, 1997; Sørensen and Holmstrup, 2005), though not to the same extent as observed with nonylphenol. This difference between the abilities of nonylphenol and LAS to create synergistic interactions with drought within the same species may be explained by a difference in lipophilicity, with nonylphenol being more lipophilic than LAS. Furthermore, the molecular structure of nonylphenol is similar to membrane phospholipids in the sense that both have a non-polar carbon chain and a polar head-region which supposedly could become embedded in and likely interfere with cellular membranes.

4.4. Interactions with pesticides

Skovlund et al. (2006) tested the effect of the pesticide residue, DDE, on the drought tolerance of *F. candida* applying a full factorial design. No interaction between the two stressors was observed, even at the highest drought level exposure of 97% RH. This was also evident in a study with the same species by Sørensen and Holmstrup (2005), where no interaction was observed between two insecticides (dimethoate and cypermethrin) and drought, even though a severe effect on reproduction was observed. On the other hand, Demon and Eijsackers (1985) found that springtails (*Onychiurus quadricellatus*) that had been subjected to previous drought incidents were much more sensitive to the insecticide lindane than control animals.

5. Interactions between effects of oxygen depletion and chemicals

Oxygen in water can vary temporally and spatially and these changes are related to organisms' activities (e.g. respiration, photosynthesis), atmospheric gas exchanges, temperature, or related to eutrophication phenomena (Dodds, 2002). Oxygen depletion can occur during summer in the hypolimnion of eutrophic lakes and in streams dominated by organic matter degradation (Schwoerbel, 1992). Even oxygen levels of 40 to 50% of saturation affect the aquatic biocoenosis considerably, e.g. by reduction in biodiversity.

Few studies are published on the combined effects of chemical stressors and oxygen depletion in aquatic ecosystems. However, as indicated by van der Geest et al. (2002), periods of the lowest oxygen concentrations often coincide with the presence of the highest contaminant concentrations. For example, in the Meuse River toxicant concentrations in 1996 were relatively high in periods of low dissolved oxygen (DO) (van der Geest et al., 2002). The authors postulated that, besides the direct adverse effects of toxicants (Becker, 1987; Nebeker et al., 1996), low oxygen concentrations influence the response of aquatic insects to environmental contaminants since a variety of physiological processes in the organism may be affected by oxygen deficiency (Penttinen and Holopainen, 1995). Impairments of physiological processes have been observed for fish (Behrens and

Steffensen, 2007) and amphibians (James, 2003), which also encounter periods of oxygen depletion e.g. during hibernation (Boutilier et al., 1997). Thus, combined effects of oxygen depletion and pollutants are a likely scenario.

5.1. Interaction with heavy metals

Ferreira et al. (2008) evaluated the combined effects of cadmium chloride and low levels of DO on *D. magna*. Two different response patterns were observed, depending on the endpoint. For survival, a synergistic pattern was found, and for feeding activity, an antagonistic response was observed. The synergistic pattern found could be explained by the formation of reactive oxygen species (ROS) in exposures to low oxygen levels and high levels of cadmium. Hypoxia in organisms can lead to a gradual reduction of electrons in the mitochondrial chain, leading to an unbalanced formation of ROS (Chandel and Schumacker, 2000). Metal accumulation can also induce oxidative stress by increasing the formation of H₂O₂, O₂ and OH⁻ (Dautrempuits et al., 2002; Livingstone, 2001) and thus further add to this effect.

Kienle et al. (2008) studied the effects of combined exposure to nickel chloride and oxygen depletion on behaviour and vitality patterns of zebrafish (*D. rerio*) larvae. Locomotor activity decreased with increasing nickel concentrations and high oxygen saturation levels, but at lower oxygen concentrations (<4.19 mg O₂/L), nickel induced a stimulating effect on locomotor activity. As an overall result, a significant antagonistic pattern was observed when nickel was combined with oxygen depletion.

Sampaio et al. (2008) studied the exposure of the neotropical fish *Piaractus mesopotamicus* to combined hypoxia and copper, and observed a more significant disturbance in the antioxidant defences than at exposures to these factors applied alone. Combined exposures enhanced ROS formation and decreased the activity of several enzymes involved in oxidative stress processes, including superoxide dismutase, catalase and glutathione peroxidase. Further, under combined stressor exposure, the number of red blood cells increased. These results were explained by the stimulation of erythropoiesis by metals and the compensation when at hypoxic conditions by increasing the number of red blood cells, as a mechanism to meet with the increased oxygen demands.

Depledge (1987) studied the combined effects of hypoxia and copper on survival in the shore crab *Carcinus maenas* and observed that natural stressors synergistically enhanced copper toxicity. A reduction of life span was also observed when these factors were combined. Similar results were reported by Weber et al. (1992) who studied the effects of sublethal (20 ppb) or lethal (200 ppb) copper concentrations combined with hypoxic conditions in *M. edulis*. Compared to control situations where 100% survival was observed (no copper and normoxia), both 200 ppb Cu and anoxia (singly) drastically decreased survival, which further deteriorated when the animals were subjected to both stresses combined. The cellular effects of combined anoxia and 200 ppb Cu increased and generally correlated with opposite changes in hemolymph ion levels, indicating disruption of transmembrane concentration gradients.

Acute exposure of mayfly larvae (*Ephoron virgo*) to copper at reduced oxygen concentrations (50% oxygen saturation at 20 °C) resulted in increased mortality compared to the normoxic treatment (van der Geest et al., 2002). The authors explained this fact by a possible increase in gill movements at lower oxygen concentrations, as observed by Philipson and Moorhouse (1974) for aquatic insects. Also, copper may directly lead to an increase in ventilation rate (e.g. Gerhardt and Palmer, 1998). Both mechanisms would result in a higher volume of toxicant-containing water passing across the absorbent membranes of the gill surface with subsequent adverse effects. Toxicants could also cause damages to the gill structure and thereby influence the respiratory efficiency, which has been described for copper in several organisms, such as mussels (Viarengo et al., 1993), fish (Heath, 1991; Pilgaard et al., 1994) and crustaceans (Hebel

et al., 1999; Nonnotte et al., 1993). Similar patterns were observed by Eriksson and Weeks (1994) who exposed adult *Corophium volutator* to combinations of copper and reduced oxygen concentrations resulting in higher mortality at <30% oxygen saturation and copper, as well as a decreased egg production.

The combined effects of cadmium and low DO on the survival, hematology, erythrocytic volume regulation and haemoglobin-oxygen affinity were studied in the marine bivalve *Scapharca inaequalvis* (Weber and Lykke-Madsen, 1990). Upon an exposure to 50 ppb Cd and low DO levels for six weeks a reduction of the survival rates of *S. inaequalvis* was observed.

5.2. Interaction with pesticides

Ferreira et al. (2008) evaluated the acute (survival) and chronic (feeding) effects from the combination of carbendazim and low levels of DO using the cladoceran *D. magna*. For survival a synergistic interaction pattern was observed, as toxicity was higher than expected, but for feeding rate an antagonistic interaction was observed (Table 4).

Synergistic interactions were observed when the cladoceran *D. pulex* was exposed to carbaryl and low oxygen levels (Hanazato and Dodson, 1995). The combined toxicity was enhanced in endpoints related to growth, reproduction and juvenile development. Moreover, when *Chaiborus* kairomones was introduced in addition to the combination carbaryl and oxygen depletion, further synergistic effects were observed.

Oxygen depletion (50% oxygen saturation at 20 °C) did not cause increased toxicity of diazinon to larvae of the mayfly *E. virgo* compared to treatments with 100% oxygen saturation in acute exposures (van der Geest et al., 2002). The authors suggested that low oxygen reduced the formation of the oxygenated metabolite, diazoxon, which is the main responsible for the toxic effect.

Very few vertebrate studies on combined effects of pesticides and oxygen depletion exist. In a modelling study Sekine et al. (1996) estimated the combined effects of oxygen depletion, decreased salt concentration and pesticide exposure on mullet, based on acute experimental data. These authors predicted that a decrease in salt concentration (from sea water to river water) would result in a seven-fold higher mortality compared to a decrease in oxygen concentration (from oxygen saturated water to water with 1–3 mg oxygen/L). Combinations of decreased salt concentrations and oxygen depletion were predicted to cause three-fold higher mortality than a decrease in salt concentration only. Also, the threshold body level for effects of the pesticide pyridaphenthion on fish mortality would be reduced by decreasing the salt concentration and depleting oxygen.

5.3. Interaction with other chemical compounds

Gupta et al. (1983) exposed adult ghost knife fish (*Notopterus notopterus*) to several phenolic compounds (phenol (P), 2,4-dinitrophenol (DNP), pentachlorophenol (PCP)) singly and in various mixtures of the compounds. The susceptibility of *N. notopterus* to the

Table 4
Overview of studies concerning interactions between oxygen depletion and chemicals.

Toxicant	Test organism	Species	Life stage	Endpoint	Interaction	Reference
<i>Pesticides</i>						
Carbendazim	Daphnid	<i>Daphnia magna</i>	Neonate	Mortality	Synergistic	Ferreira et al. (2008)
Carbendazim	Daphnid	<i>Daphnia magna</i>	Fourth instar	Feeding rate	Antagonistic	Ferreira et al. (2008)
Carbaryl	Daphnid	<i>Daphnia pulex</i>	All stages	Growth, reproduction, development	Synergistic	Hanazato and Dodson (1995)
Diazinon	Insect	<i>Ephoron virgo</i>	Larvae	Mortality	None	van der Geest et al. (2002)
<i>Heavy metals</i>						
Cadmium	Daphnid	<i>Daphnia magna</i>	Neonate	Mortality	Synergistic	Ferreira et al. (2008)
Cadmium	Daphnid	<i>Daphnia magna</i>	Fourth instar	Feeding rate	Antagonistic	Ferreira et al. (2008)
Copper	Insect	<i>Ephoron virgo</i>	Larvae	Mortality	Synergistic	van der Geest et al. (2002)
Copper	Crab	<i>Carcinus maenas</i>	Adult	Mortality	Synergistic	Depledge (1987)
Copper	Shrimp	<i>Corophium volutator</i>	Adult	Egg production, survival	Synergistic	Eriksson and Weeks (1994)
Copper	Fish	<i>Lepomis macrochirus</i>	Adult	Physiological measures	None	Heath (1991)
Copper	Fish	<i>Piaractus mesopotamicus</i>	Juvenile	Antioxidant defences	Synergistic	Sampaio et al. (2008)
Nickel	Fish	<i>Danio rerio</i>	Larvae	Locomotor activity	Antagonistic interaction	Kienle et al. (2008)
Cadmium	Mussel	<i>Scapharca inaequalvis</i>	Adult	Mortality and respiratory measures	Antagonistic interaction	Weber and Lykke-Madsen (1990)
Copper	Mussel	<i>Mytilus edulis</i>	Adult	Mortality	Synergistic	Weber et al. (1992)
Copper	Mussel	<i>Mytilus edulis</i>	Adult	Hemolymph ion levels	Synergistic	Weber et al. (1992)
Copper	Mussel	<i>Mytilus edulis</i>	Adult	Hemolymph osmolytes	None	Weber et al. (1992)
<i>Other chemical compounds</i>						
Ammonia	Prawn	<i>Penaeus monodo</i>	Juvenile	Mortality	Not stated	Allan et al. (1990)
Ammonia	Fish	<i>Oncorhynchus mykiss</i>	Juvenile	Mortality	Synergistic	Magaud et al. (1997)
Ammonia	Fish	<i>Sparus auratus</i>	Juvenile	Growth and survival	Not stated	Wajsbrot et al. (1991)
Anthracene	Fish	<i>Lepomis macrochirus</i>	Juvenile	Mortality	Not stated	McCloskey and Oris (1991)
Phenol	Fish	<i>Campostoma anomalum</i>	Adult	Loss of equilibrium	Synergistic	Hlohowskyj and Chagnon (1991)
Phenol (P)	Fish	<i>Notopterus notopterus</i>	Adult	Mortality	Synergistic	Gupta et al. (1983)
2,4-dinitrophenol (DNP)	Fish	<i>Notopterus notopterus</i>	Adult	Mortality	Synergistic	Gupta et al. (1983)
Pentachlorophenol (PCP)	Fish	<i>Notopterus notopterus</i>	Adult	Mortality	Synergistic	Gupta et al. (1983)
(PCP + DNP)/P (antagonistic)	Fish	<i>Notopterus notopterus</i>	Adult	Mortality	Synergistic	Gupta et al. (1983)
(DNP + P)/PCP (additive)	Fish	<i>Notopterus notopterus</i>	Adult	Mortality	Synergistic	Gupta et al. (1983)
(P + DNP)/PCP (synergistic)	Fish	<i>Notopterus notopterus</i>	Adult	Mortality	Synergistic	Gupta et al. (1983)
Pulp and paper effluent	Fish	<i>Oncorhynchus mykiss</i>	Juvenile	Swimming performance	None	Landman et al. (2006)
Pulp and paper effluent	Fish	<i>Oncorhynchus mykiss</i>	Juvenile	Metabolism	None	Landman et al. (2006)
Pulp and paper effluent	Fish	<i>Oncorhynchus mykiss</i>	Juvenile	Hematology	Synergistic	Landman et al. (2006)
Pulp mill and sewage effluent	Insect	<i>Baetis tricaudatus</i>	Larvae	Behaviour	None	Lowell and Culp (1999)
Pulp mill and sewage effluent	Insect	<i>Baetis tricaudatus</i>	Larvae	Mayfly grazing intensity	None	Lowell and Culp (1999)
Pulp mill and sewage effluent	Insect	<i>Baetis tricaudatus</i>	Larvae	Mortality	None	Lowell and Culp (1999)

toxicants increased up to 6-fold with decreasing oxygen concentrations (from 7.8–5.0 mg O₂/L) although low oxygen conditions were not detrimental *per se* suggesting potentiation of toxicity.

Hlohowskyj and Chagnon (1991) observed a reduction in tolerance to progressive hypoxia in the central stoneroller minnow (*Campostoma anomalum*) following sublethal exposure to phenol. This fact may have a major impact on the survival of this species in some streams, as many of the streams in which this fish species occurs often experience periods of low DO (<2.5 mg O₂/L) (Tramer, 1977).

When exposing juvenile rainbow trout (*O. mykiss*) to several concentrations of DO (1.7–2.9 mg/L) as well as several un-ionized ammonia concentrations (0–0.5 mg/L) synergistic effects of the stressors could be observed (Magaud et al., 1997). As a possible reason for the high toxicity of the combined stressors, an inhibition of the oxygen-binding ability of haemoglobin caused by ammonia was postulated (Sousa and Meade, 1977). Reduced dissolved-oxygen levels significantly increased the acute toxicity of ammonia to individuals of the juvenile school prawn *Penaeus monodon* (Allan et al., 1990) as well as to juvenile gilthead seabream (*Sparus aurata*) (Wajsbrodt et al., 1991).

Exposure of bluegill sunfish (*L. macrochirus*) to the PAH anthracene at concentrations from <0.01 to 20.88 µg/L and different oxygen concentrations (5.0, 6.9 and 8.1 mg O₂/L) resulted in a nonlinear relationship between oxygen concentration and LC₅₀ values, with a significantly increased toxicity of anthracene at 6.9 mg O₂/L (McCloskey and Oris, 1991). Opercular ventilation rates were inversely related to oxygen concentration, suggesting that as dissolved-oxygen concentration decreases, respiratory stress increases. Additionally, they were directly related to the anthracene concentration, due to the reason that the photo-oxidation of anthracene into toxic photoproducts increases with increasing oxygen concentration. In this situation gill membrane damage is expected to increase. Consequently if those responses are combined, the highest toxicity is expected at intermediate oxygen concentrations where the relative contributions of respiratory stress and oxygen-dependent photo-induced toxicity of anthracene to animal mortality are highest (McCloskey and Oris, 1991).

Landman et al. (2006) studied the combined effects of pulp and paper effluent and low DO on the swimming performance, metabolism, and hematology of *O. mykiss*. Effects were evaluated when exposed to thermomechanically bleached kraft pulp and paper mill effluent (TMP/BKME) for 4 weeks and their critical swimming speed, oxygen consumption, and hematology were evaluated. Additionally, the authors examined the effects of 15% v/v TMP/BKME exposure at 2.5 and 5.0 mg O₂/L for 4 weeks. There was an interaction between DO and effluent exposure, but only when considering the hematocrit level, where effluent caused a significant increase at levels of 5 mg/L and decrease at 2.5 mg/L DO. However, exposure to combinations of low DO levels and effluent indicated that the presence of this particular effluent has minimal potential to modify the responses of fish to hypoxia.

Lowell and Culp (1999) investigated the cumulative effects of multiple effluent and low dissolved-oxygen stressors (5 mg O₂/L) on the mayfly species *Baetis tricaudatus* at low temperatures (4.5 °C). The low DO level used in this experiment led to altered microhabitat positioning behaviour (with an increased movement of the mayfly larvae into regions of higher current velocity), reduced grazing intensity, and decreased survival. This is particularly important as oxygen concentrations of 5 mg/L are comparable with those that have been measured in cold water (≤5 °C) downstream of pulp mill and municipal effluent outfalls in similar habitats as used by *B. tricaudatus* in northern rivers.

6. Influence of starvation on effects of chemicals

In comparison to other natural stressors, effects of nutritional conditions on toxicity of chemicals are relatively poorly studied.

Interactions between toxicants and starvation may be potentially complex. On the one hand, there is evidence that exposed organisms may be less resistant to starvation than unexposed; while on the other hand organisms exposed to contaminated food frequently decrease their consumption rate, most probably as a direct effect of “unpalatability” of such food. The latter mechanism allows organisms to avoid excessive consumption of toxicants (Depta et al., 1999) but if there is no alternative food source, starvation may result (Laskowski and Hopkin, 1996). Consequently, increased mortality in animals exposed to contaminated food may occur not only due to intoxication but also starvation, as shown by Kostić et al. (2008) for the effect of plant-derived compounds on the larvae of the moth *Lymantria dispar*.

Another aspect of the effect of food limitation on toxicity is that nutritional conditions during an organism's development may have profound influence on overall life history characteristics (Metcalfe and Monaghan, 2001). Even if an organism recovers from the transient food deprivation in early life stages, this may still have permanent effect on the adult individual, and even on offspring raising the possibility that epigenetic mechanisms may play a role in determining effects. An interesting example of the interaction between a short period of fasting and pesticide toxicity was given by Campero et al. (2008) who exposed damselfly (*Coenagrion puella*) larvae to endosulfan after a short-term starvation. Although the larval post-starvation development period increased, the full mass compensation was reached only in pesticide-treated females. However, the pesticide significantly reduced phenoloxidase activity and haemocyte numbers (Campero et al., 2008).

A negative interaction between food deprivation and exposure to organic compounds (PCB) was observed in Arctic charr (*Salvelinus alpinus*) (Jørgensen et al., 1999). After oral dosage of 1 mg Aroclor 1260/g body weight the animals were kept under different food regimes or starved. In the starved population the PCB concentration was highest, lipid content in the liver and muscle was reduced, the highest fin erosion was found, and activity of biochemical biomarkers (P450 (CYP1A) and EROD) was induced. Similar effects of starvation on mobilization of PCBs into liver have been found in studies conducted with birds of prey (Wienburg and Shore, 2004).

Even if significant effects of food limitation on toxicity of chemicals have been documented, they are by no means unambiguous. Starving animals can be either more sensitive to toxicants, as in dogwhelks (*Nucella lapillus*) exposed to cadmium-contaminated water (Leung and Furness, 2001) or just the opposite, as shown for carps (*Cyprinus carpio*) which were less resistant to copper at high food availability than when the food was supplied at a more limited rate (Hashemi et al., 2008). Increased sensitivity to toxicants of starving animals was shown in lake chubsuckers (*Erimyzon sucetta*) exposed to ash-contaminated sediment (Hopkins et al., 2002). The opposite result was found in a study by Spadaro et al. (2008) who did not reveal any difference in copper LC₅₀ between starving and fed amphipods (*Melita plumulosa*).

Starvation can also interact with toxic chemicals in another way; intoxicated organisms have been shown to be more sensitive to the lack of food. Thus, Stone et al. (2001) showed that starving ground beetles (*Pterostichus oblongopunctatus*), originating from an area heavily polluted with zinc, cadmium and lead, survived significantly shorter than those living in uncontaminated regions. Such an outcome might be expected if additional energy is used by intoxicated organisms for detoxification processes, as predicted by Sibly and Calow (1989). For lipophilic compounds, such as a number of pesticides, it has been shown that post-exposure starvation results in mobilization of toxicants, which may lead to postponed intoxication (Thompson et al., 1977).

Despite rather limited literature on interactions between nutritional status of organisms and toxicity of chemicals, it can be concluded that in most cases significant interactions were found (Table 5). The effect of starvation is, however, hard to predict as it can

Table 5
Overview of studies concerning interactions between starvation and chemicals.

Toxicant	Test organism	Species	Life stage	Interaction	Reference
Cadmium	Mollusc	<i>Nucella lapillus</i>	Adult	Synergistic	Leung and Furness (2001)
Copper	Fish	<i>Cyprinus carpio</i>	Adult	None	Hashemi et al. (2008)
Contaminated sediment (Fe, Mn, Cu, Pb, Zn and PAHs)	Amphipod	<i>Melita plumulosa</i>	Juvenile	Synergistic	Spadaro et al. (2008)
Coal combustion wastes (As, Cd, Cr, Cu, Se, Sr, V)	Fish	<i>Erimyzon sucetta</i>	Adult	Synergistic	Hopkins et al. (2002)
Chlorinated hydrocarbons (pesticides)	Bird	<i>Pelecanus occidentalis</i>	Juvenile	Not stated	Thompson et al. (1977)
PCB	Fish	<i>Salvelinus alpinus</i>	Adult	Synergistic	Jørgensen et al. (1999)

provoke both an increase and decrease in sensitivity to pollutants. This, together with the scarcity of hard data, calls for further studies on this issue, which represents a highly relevant ecological scenario.

7. Influence of pathogens on effects of chemicals

The influence of pathogens, in a broad sense, on effects of chemicals is an area of research where human toxicology and environmental toxicology have many common characteristics. In fact, some aspects of the immune system are highly conserved among animal species from invertebrates to humans (Alper et al., 2008; Stuart and Ezekowitz, 2008). The understanding of the human immune system is probably more advanced than that of animal species and therefore more amenable to experimentation because its responses to pathogens (and the nature of the pathogens themselves) are so well known. This opens up for the use of molecular techniques in the study of human cell lines whereas studies on animal models are often of a more phenomenological nature. In order to give the best possible overview of how pathogens and environmental toxicants may interact we have included a section describing this problem from a human health perspective. Both areas of research, ecotoxicology and human toxicology, can be improved by integration and utilization of experience from these respective fields.

7.1. The influence of inflammatory stress factors on the immunomodulatory effects of chemicals in human cell based *in vitro* systems

Humans are exposed to a multitude of environmental pollutants, of which several were found to play a role in the development of diseases, including cancer, chronic obstructive pulmonary disease (COPD) and allergic asthma (Cazzola et al., 2007; D'Amato et al., 2005; Kriek et al., 1998; Kyrtopoulos, 2006; Liu et al., 2009; Viegi et al., 2006). While overall toxicity and carcinogenicity are detectable by well-established standard assays (Das et al., 2007; Holden and Schreiner, 1983; Leist et al., 2008; Martin and Clynes, 1993; Tiffany-Castiglioni et al., 2006), effects on immunity can both be subtle and highly variable, which makes it difficult to address this issue (Krzystyniak et al., 1995). Several studies have shown that for some chemicals, significant immune effects occur at chemical doses well below those where acute cellular toxicity is observed (Oostingh et al., 2008, 2009; Röder-Stolinski et al., 2008a,b). These data suggest that the results of assays detecting cell viability might lead to an underestimation of chemical effects on human health. Many pollutant related diseases are linked to immune alterations and several proposals have been made for testing the immunotoxic potential of environmental pollutants in both animal (Luster et al., 1988; Van Loveren et al., 1988) and human systems (Luster et al., 1988; Tryphonas, 2001; Van Loveren et al., 1988). However, the selection of relevant endpoints remains a key problem when monitoring immune effects of pollutants. The immune system can be activated or suppressed, and these effects can be general or limited to specific branches of the immune system. In addition, immune responses may be diverted from one type to another (e.g. from inflammation to allergic responses) and effects may depend on the health status of the individual. It is easy to

imagine that ill people could be more susceptible to and experience detrimental health effects due to exposure to pollutants, but it may also be that cells that are pre-activated due to an ongoing immune defence, can deal more efficiently with chemicals.

The latter factor, i.e. the difference between chemical induced immunomodulation in a naïve or activated immune system, has not been studied in detail in the past. Most *in vivo* immunological experiments are performed on healthy, adult animals, or are aimed at the analysis of a specific disease. However, immunotoxic effects may change when the immune system is compromised due to an ongoing disease or when young individuals, that have not fully developed their immune system, are tested (Descotes, 2006; Krzystyniak et al., 1995; Richter-Reichhelm et al., 2002). In this context, immunotoxicity can be defined as any modulation (activation, suppression or deviation) of immune responses by chemicals that cannot be related to the infection with a certain type of pathogen.

A limited number of studies exist in which cellular stress factors were added to different cell culture systems to compare the immunomodulatory effects of chemicals on activated and naïve cells (Oostingh et al., 2008, 2009). Different activators of the immune system were applied, depending on the cell types used. For the stimulation of a human bronchial epithelial cell line, A549, human tumour necrosis factor- α (TNF- α) was used. This cytokine is secreted by a multitude of cells at the early stages of inflammation. TNF- α can cause cell activation by binding to the TNF receptor, which is expressed by most cell types. This makes TNF- α a crucial "alarm" signal during infection. Primary human T cells or a cell line derived from a human T-cell lymphoma (Jurkat cells), were stimulated using phytohemagglutinin (PHA). This compound is a lectin derived from kidney beans, which is widely used as a polyclonal T-cell activator, and acts by binding to N-acetylgalactosamine, expressed on T cells. PHA is a tetramer that binds simultaneously to several T cells and this aggregation results in cell activation and proliferation. Finally, the bacterial endotoxin, lipopolysaccharide (LPS) was used to activate monocytes in peripheral blood mononuclear cells (PBMCs) derived from human blood. LPS is derived from the cell wall of gram-negative bacteria and binds to toll like receptor (TLR) 4, which belongs to the pattern recognition receptors through which the immune system is able to sense the presence of microorganisms. TLR4 is expressed on a multitude of cells, including monocytes and macrophages, which both play an important role in innate immunity.

These three stress factors affect different cells and via different pathways. The use of multiple stress factors on different cell types, in combination with exposure to chemicals, provides valuable information with respect to the specificity of the observed immunomodulatory effects.

One requirement that has to be fulfilled by all immunological responses is the communication between immune cells to coordinate the immune defence. Cytokines, small proteins secreted by many different cells, play a key role in intercellular communication. The pattern of secreted cytokines reflects ongoing immune activities and can therefore be used to determine the immunomodulatory effects caused by chemicals (House, 1999). Reporter gene studies were performed in order to test the effect of chemicals and cellular stress factors on the proximal promoter of different cytokine promoters that

were stably transfected in Jurkat cells or A549 cells (Oostingh et al., 2008, 2009; Röder-Stolinski et al., 2008a,b). In these reporter cell lines, the gene for luciferase is expressed under control of various cytokine promoters and other regulatory elements, which allows the screening of a large number of chemicals and mixtures using one single robust assay. In addition, cytokine secretion by these cells and by PBMCs or T cells was determined as a measure for immunomodulatory effects. Cell viability was tested as well to ensure that the concentrations of chemicals used were not cytotoxic.

In general, it was found that approximately 70% of all chemicals tested had a clear immunomodulatory effect at non cytotoxic concentrations (Table 6). Since the immune system is not designed to interact with chemicals that are not associated with pathogens, the high number of chemicals with effects on the immune system was surprising. In general, the presence of cellular stress factors (TNF- α , PHA, LPS) increased the immunomodulatory effects in naive cells. The data do imply that an activated immune system can be more prone to react to chemical pollutants and that the concentrations of pollutants that might have an impact on human health are lower when the person exposed is ill implying that people suffering from infections as well as immune-compromised people are more prone to negative effects of chemicals. Thus, TIER testing of chemical induced immunomodulatory effects should not be limited to animal studies only, also since new research suggests that *in vitro* tests allow for a higher throughput and thereby strongly enhance the amount of information obtained.

7.2. Interactions between pathogens/parasites and toxicants: animal studies

Parasites represent one of the most important natural, biotic environmental stress factors that influence populations in the field. They may alter the physiology and metabolism of animals and in consequence may influence important life-history characteristics such as survival, growth and fecundity. Parasitism can modify intra- and interspecies competition for resources ('indirect parasite-mediated competition'), by rendering infected individuals less competitive (Relyea and Hoverman, 2006). The infection with parasites may also influence the choice of infected prey by predators. Furthermore, parasite pathogenity can increase with host environmental stress (Gérard et al., 2008).

Other stress factors, such as chemicals in the form of heavy metals and pesticides, may potentially exacerbate the harmful effects of infection. On the other hand, a higher vulnerability of parasites to contaminants than their hosts may cause decreases in the infection rate; this effect is one explanation of hormesis, where low levels of chemicals may increase the performance of a species (Boonstra et al., 2005). There are reports showing that cestode infections of eel (*Anguilla anguilla*) may modify metal metabolism/storage pro-

cesses in the tissues of the host and reduce the chromium and nickel body burden of the host (Eira et al., 2009).

The main system protecting an animal against infections is the immune system; excellent reviews on direct effects of chemicals on immune function (immunotoxicity) in invertebrates are given by Galloway and Depledge (2001) and Galloway and Handy (2003). Here, two kinds of studies are considered: firstly, the impact of pathogens on animals inhabiting polluted areas (including laboratory experiments); secondly, interactions between pesticides and bio-control agents. An overview of relevant studies is shown in Table 7.

Environmental contaminants and infectious diseases are thought to be major factors contributing to global amphibian declines. Forson and Storfer (2006) exposed long-toed salamanders (*Ambystoma macrodactylum*) to *Ambystoma tigrinum* virus and sublethal concentrations of atrazine. They observed ameliorating effects of low atrazine concentration on infected individuals, whereas higher concentrations initiated metamorphosis at a smaller size, with potential negative consequences to fitness. Similar interaction were found by Kiesecker (2002), who found that stress in the form of pesticide exposure (atrazine and malathion) decreased wood frog (*Rana sylvatica*) tadpoles' ability to resist infection with a trematode, *Ribeiroia* sp., resulting in higher parasite loads and a higher risk of limb deformities. On the contrary, Puglis and Boone (2007) did not find interactive effects among the insecticide carbaryl and a widespread waterborne pathogen *Saprolegnia ferax* on the hatching and survival of bullfrog (*Rana catesbeiana*) tadpoles. In a fish species, the Chinook salmon *Oncorhynchus tshawytscha*, both the pyrethroid insecticide esfenvalerate and the organophosphate chlorpyrifos caused additive effects on the induction of the stress proteins hsp60, hsp70, and hsp90 when each compound was applied in combination with the infectious hematopoietic necrosis virus (IHNV) (Eder et al., 2007).

Evidence of anthropogenic stressors interacting with parasites in animals is mounting but most studies have been carried out with vertebrate species; data on simultaneous effects of pathogens and contaminants from invertebrate studies are still scarce. Heinonen et al. (2000) found only minor differences in toxicokinetics of 2,4,5-trichlorophenol and benzo(a)pyrene between infected and uninfected clams (*Pisidium amnicum*). At the same time, studies concerning energetic response of the same species of clam to different temperatures have shown that the general level of heat output was lower in infected clams (Heinonen et al., 2003).

Clear evidence of parasite-toxicant interactions was shown in a study by Coors et al. (2008). They tested the performance of the water flea, *D. magna*, exposed to carbaryl and two of its endoparasites, the bacterium *Pasteuria ramosa* and the microsporidium *Flabelliforma magnivora*, as invertebrate host-parasite models. The virulence of both parasites was enhanced by sub-lethal concentrations of the pesticide causing increased host mortality. Furthermore, host castration induced by *P. ramosa* was accelerated by carbaryl exposure. The authors explained the observed effects by decreased host resistance due to direct or indirect immunosuppressive activity of carbaryl. Another study on the cost of resistance to parasites using parasite susceptible and resistant strains of freshwater snails (*Biomphalaria glabrata*) investigated the response to cadmium intoxication (Salice and Roesijadi, 2002). Mortality due to cadmium of the parasite-resistant strain was higher in comparison with the parasite-susceptible strain. These findings were consistent with the hypothesis that the cost of being resistant to parasitic infection may be an increased sensitivity to cadmium.

Other examples of invertebrate host-pathogen interactions are studies on factors used as biological control of crop pests. Kramarz et al. (2007) showed that the snail *Canthareus aspersus* (former *Helix aspersa*) exposed to cadmium and treated with a high dose of parasitic nematodes (*Phasmarhabditis hermaphrodita*), commonly applied as a biological control agent, accumulated cadmium to higher concentrations than control snails. When a third stressor was applied, maize

Table 6

Overview of the chemicals that either had no immunomodulatory (IM) effect, a non-significant (NS) immunomodulatory effect or a significant effect as tested by the reporter gene assay using different stable transfected cell lines with promoter elements for a range of different cytokines or transcription factors.

No IM effect	NS IM effect	Significant IM effect
Imidacloprid	3,4-DCA	Carbendazim
Permethrin	Thiacloprid	Diazinon
Prochloraz		Ivermectin
		Fluoranthene
		Diclofenac
		Chlorpyrifos
		Piperonylbutoxid
		NiCl ₂
		Irgasan

Data from Oostingh et al. (2008, 2009), Röder-Stolinski et al. (2008a,b).

Table 7
Overview of studies concerning interactions between pathogens and chemicals in wildlife.

Toxicant	Pathogen	Species	Test organism	Species	Life stage	Interaction	Reference
<i>Heavy metals</i>							
Cadmium	Nematode	<i>Phasmarhabditis hermaphrodita</i>	Snail	<i>Canthareus aspersus</i>	Adult	Synergistic	Kramarz et al. (2007)
Nickel	Bacterium	<i>Bacillus thuringiensis</i>	Insect	<i>Pterostichus oblongopunctatus</i>	Adult	Synergistic	Kramarz et al. (2008)
<i>Pesticides</i>							
Imidacloprid	Nematode	<i>Steinernema glaseri</i> (Steiner)	Insect	<i>Cyclocephala hirta</i> LeConte	Larva	Synergistic	Koppenhöffer et al. (2000)
Imidacloprid	Nematode	<i>Heterorhabditis bacteriophora</i>	Insect	<i>Cyclocephala hirta</i> LeConte	Larva	Synergistic	Koppenhöffer et al. (2000)
Imidacloprid	Nematode	<i>Steinernema kushidai</i> Mamiya	Insect	<i>Cyclocephala hirta</i> LeConte	Larva	None	Koppenhöffer et al. (2000)
Imidacloprid	Nematode	<i>Steinernema glaseri</i> (Steiner)	Insect	<i>Cyclocephala borealis</i> Arrow	Larva	Synergistic	Koppenhöffer et al. (2000)
Imidacloprid	Nematode	<i>Heterorhabditis bacteriophora</i>	Insect	<i>Cyclocephala borealis</i> Arrow	Larva	Synergistic	Koppenhöffer et al. (2000)
Imidacloprid	Nematode	<i>Steinernema kushidai</i> Mamiya	Insect	<i>Cyclocephala borealis</i> Arrow	Larva	None	Koppenhöffer et al. (2000)
Imidacloprid	Nematode	<i>Steinernema glaseri</i> (Steiner)	Insect	<i>Popillia japonica</i> Newman	Larva	Synergistic	Koppenhöffer et al. (2000)
Imidacloprid	Nematode	<i>Heterorhabditis bacteriophora</i>	Insect	<i>Popillia japonica</i> Newman	Larva	Synergistic	Koppenhöffer et al. (2000)
Imidacloprid	Nematode	<i>Steinernema kushidai</i> Mamiya	Insect	<i>Popillia japonica</i> Newman	Larva	None	Koppenhöffer et al. (2000)
Imidacloprid	Nematode	<i>Steinernema feltiae</i>	Insect	<i>Bemisia tabaci</i>	Larva	Synergistic	Cuthbertson et al. (2003)
Buprofezin	Nematode	<i>Steinernema feltiae</i>	Insect	<i>Bemisia tabaci</i>	Larva	None	Cuthbertson et al. (2003)
Teflubenzuron	Nematode	<i>Steinernema feltiae</i>	Insect	<i>Bemisia tabaci</i>	Larva	None	Cuthbertson et al. (2003)
Nicotine	Nematode	<i>Steinernema feltiae</i>	Insect	<i>Bemisia tabaci</i>	Larva	None	Cuthbertson et al. (2003)
Carbaryl	Bacterium	<i>Pasteuria ramosa</i>	Water flea	<i>Daphnia magna</i>	Adult	Synergistic	Coors et al. (2008)
Carbaryl	Microsporidium	<i>Flabelliforma magnivora</i>	Water flea	<i>Daphnia magna</i>	Adult	Synergistic	Coors et al. (2008)
Atrazine	Trematode	<i>Ribeiroia</i> sp.	Frog	<i>Rana silvatica</i>	Juvenile	Synergistic	Kiesecker (2002)
Malathion	Trematode	<i>Ribeiroia</i> sp.	Frog	<i>Rana silvatica</i>	Juvenile	Synergistic	Kiesecker (2002)
Atrazine	Virus	<i>Ambystoma tigrinum</i> virus	Salamander	<i>Ambystoma macrodactylum</i>	Juvenile	Synergistic	Forson and Storfer (2006)
Carbaryl	Fungus	<i>Saprolegnia ferax</i>	Frog	<i>Rana catesbeiana</i>	Juvenile	None	Puglis and Boone (2007)
Chlorpyrifos	Bacterium	<i>Bacillus thuringiensis</i>	Insect	<i>Pterostichus oblongopunctatus</i>	Adult	Synergistic	Kramarz et al. (2008)
Esfenvalerate	Virus	Infectious hematopoietic necrosis virus	Fish	<i>Oncorhynchus tshawytscha</i>	Juvenile	None	Eder et al. (2007)
Chlorpyrifos	Virus	Infectious hematopoietic necrosis virus	Fish	<i>Oncorhynchus tshawytscha</i>	Juvenile	None	Eder et al. (2007)
<i>Biopesticides</i>							
<i>Bacillus thuringiensis</i>	Nematode	<i>Phasmarhabditis hermaphrodita</i>	Snail	<i>Canthareus aspersus</i>	Adult	Synergistic	Kramarz et al. (2007)
<i>PAHs</i>							
2,4,5h-trichlorophenol	Trematode	<i>Bunodera luciopercae</i>	Mussel	<i>Pisidium amnicum</i>	Adult	None	Heinonen et al. (2000)
Benzo(a)pyrene	Trematode	<i>Bunodera luciopercae</i>	Mussel	<i>Pisidium amnicum</i>	Adult	None	Heinonen et al. (2000)

expressing Bt toxin (toxin produced by the bacterium *B. thuringiensis*), also regarded as a biological substitute of chemical crop protection, growth rate of infected snails was affected. The interaction between two kinds of biological control factors: biopesticide based on Bt toxin and entomopathogenic nematodes, *Heterorhabditis bacteriophora* or *Steinernema glaseri*, was investigated by Koppenhöffer and Kaya (1997). The scarab grubs *Cyclocephala hirta* and *C. pasadenae* exhibited additive mortalities or greater than additive mortalities when they were exposed to combinations of Bt toxin and nematodes, suggesting a synergistic interaction. Probably the grub's mid-gut was affected in a similar but milder way than in normal *B. thuringiensis* infections; as a result, the homeostasis of the grubs' internal environment was disturbed rendering it more susceptible to nematode attacks.

To protect crops against pests more efficiently, conventional pesticides may be used together with biological control agents. Koppenhöffer et al. (2000) revealed synergistic interactions of imidacloprid applied together with entomopathogenic nematodes (*S. glaseri* and *H. bacteriophora*) against white grubs (*C. hirta*, *C. borealis* and *Popillia japonica*). The major factor responsible for synergistic interactions between the pesticide and nematodes appears to be the general disruption of normal nerve function due to imidacloprid resulting in drastically reduced activity of the grubs.

A set of commercial pesticides (imidacloprid, buprofezin, teflubenzuron and nicotine) and their interaction with entomopathogenic nematode, *S. feltiae*, on the sweet potato whitefly, *Bemisia tabaci*, were subject of a study by Cuthbertson et al. (2003). Among all tested pesticides, only imidacloprid in combination with nematodes increased the mortality of *B. tabaci* larvae significantly.

Kramarz et al. (2008) gave an example of a rather complicated influence of mixtures of chemical contaminants (chlorpyrifos and

nickel) and biopesticide (*B. thuringiensis*) on a beetle, *P. oblongopunctatus*. The most interesting result of the study was a gender depending response in the case of changes in body mass. Hence, in terms of body mass, males seemed to be more vulnerable to mixtures of the toxicants used in this experiment, whilst body mass of females decreased only under single toxicant influence. Therefore, reproduction expressed as the number of larvae was affected by interactions of all examined factors.

8. Conclusions and future directions

It is clear from this review that synergistic interactions between the effects of various natural stressors and toxicants are not uncommon phenomena (Fig. 1). Although we did not perform any formal meta-analysis of the data compiled, and given that researchers tend to be biased towards publishing "positive" results, the body of evidence is overwhelming and indicates that the influence of natural stressors is indeed a problem that should be considered in risk assessment procedures (see also paper by Laskowski et al. in this issue for a formal meta-analysis). Thus, in more than 50% of the reviewed studies synergistic interactions were reported. Antagonistic interactions were also detected, but in fewer cases. From a risk assessment perspective, it could be argued that occurrence of synergistic interactions is the most significant and of concern.

It would have been valuable if very potent combinations of chemicals and natural stressors could be identified. Such information could improve current risk assessment considerably. Unfortunately, the available data is at present too diverse, both with respect to the adopted test species and in the variety of combinations of sets of stressors, to allow such an analysis. To date, no systematic experiment

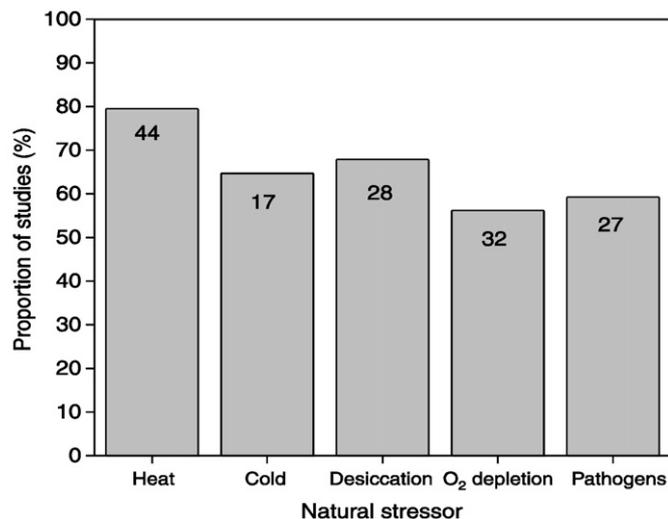


Fig. 1. Proportion of studies in which synergistic interactions were reported. The figures indicated in columns show the total number of studies included in the analysis.

with sufficient volume has been carried out in order to achieve this, partly because appropriate experimental protocols have only recently been developed.

Although the available data is limited and scattered, some more specific experience may be derived from the present synthesis. A major finding is that all of the natural stressors analysed may interact with chemicals. Heat stress seems to be particularly important but also cold, desiccation, oxygen depletion, starvation and pathogens are important natural stressors for animals exposed to chemicals in their environment. Interestingly, evidence from human cell lines suggests that many chemicals compromise the immune system of humans at sub-cytotoxic concentrations. This indicates that ill people are more sensitive to toxic chemicals in their environment than healthy individuals.

Another important point is that strong synergistic effects generally occur in situations where the particular natural stressor is intense enough to induce a strong physiological adaptive response. This suggests that risk assessment should consider extreme environmental events, despite their low frequency of occurrence making them seem irrelevant at a first glance. The ongoing climatic changes with the increasing frequency of extreme weather events such as heat waves and prolonged droughts highlights the relevance of this point. From an ecological viewpoint, the rationale is that catastrophic events can have long-lasting consequences from which populations may have difficulty in recovering.

It was not the aim of this article to specifically elaborate risk assessment procedures that include natural stressors, and much more research is probably needed before this is feasible. However, recent developments of protocols on natural stressors/chemicals testing and data interpretation (for example in the NoMiracle project) have made such techniques available which could stimulate improvements of current risk assessment procedures taking natural stressors into account. Further, the overview we have provided with this synthesis may be used to set out suggestions for future studies. These suggestions are summarised in three core problems that need further exploration:

- i) Which are the most potent combinations of natural and chemical stressors? Comparative studies surveying a prioritized list of relevant chemicals and natural stressors could identify which combinations of stressors would call for more restrictive (if synergistic interactions are likely), and which would warrant unchanged or less restrictive safety factors.
- ii) The importance of sequence in exposure to stressors has hardly received any attention in ecotoxicological research and should

be addressed in the future. This is surprising since at least three scenarios may be of relevance, and may have very different consequences. Thus, organisms may be exposed to the toxicant first, then natural stressor (e.g. after pesticide application), or the opposite, and different stressors may occur simultaneously.

- iii) Short-term laboratory tests may have little relevance for ecosystems or human health and need to be supplemented with field validation studies for example using mesocosm studies (Van den Brink et al., 2005). However, short-term laboratory tests can rapidly provide valuable data that can be used to develop testable hypotheses about how combinations of natural and chemical stressors work under more realistic conditions.

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