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Highlighted Article

Is mixture toxicity measured on a biomarker indicative of what happens on a population level? A study with *Lemna minor*

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Abstract

For plants, pigment content has shown to be a remarkably consistent biomarker across chemicals with different modes of action. In this study, we evaluated the use of pigment content as endpoint in binary mixture toxicity studies compared to three growth endpoints on the floating plant *Lemna minor*. Six binary combinations of six herbicides with different mode of action were used. Data were tested against both the concentration addition (CA) and independent action (IA) reference models. For CA, two statistical approaches were used. The study showed that for some herbicide combinations the mixture toxicity measured on pigment content did not reflect the results measured on plant population growth, emphasizing the importance of measuring growth in parallel with biomarkers. CA explained the data just as well as IA, and the two different statistical models used to test the data in relation to CA showed very similar results.

Keywords: Mixture toxicity; Endpoints; Concentration addition; Independent action; Statistical models; Herbicides; *Lemna minor*

1. Introduction

Studies of the biological effect of mixtures of chemicals are an area of interest of companies and end users who aim at increasing the biological efficiency of chemicals, as well as of risk assessors trying to quantify the effect of chemical mixtures on non-target organisms. Previous studies have shown that the biological effect of a mixture can depend on which trait is measured, even when only growth measures are used (Cedergreen and Streibig, 2005). From a visual assessment of affected plants, it is clear that plant pigments change in different ways depending on the mode of action of the toxicant. Biomarkers such as pigment content, primary chlorophyll and carotenoids, are commonly used as endpoints for toxicity tests. Chlorophyll content is one of the recommended endpoints in the standard OECD-guideline for *Lemna*-tests (Sims et al., 1999) and is frequently used as an endpoint for other macrophyte species (Brain et al., 2004; Turgut and Fomin, 2002; Viet and Moser, 2003). In a study with the macrophyte *Myriophyllum aquaticum*, including 17 pesticides representing 12 different modes of action, eight endpoints were measured. The three pigment endpoints: chlorophyll *a*, *b* and carotenoids, were the only endpoints sensitive enough to determine an EC_{50} for all pesticides within the 2-week duration of the experiments (Turgut and Fomin, 2002). Hence, changes in pigment concentration seem to occur consistently across chemicals with different modes of action. Therefore, pigment changes could be an excellent endpoint when evaluating the joint toxicity of mixtures of chemicals with different modes of action. In addition, the use of chlorophyll fluorescence as a toxicity endpoint both for aquatic as for terrestrial plants is starting to emerge, and shows promising results in terms of correlations with biomass growth both for individual compounds and for chemical mixtures (Christensen et al., 2003; Juneau et al., 2003; Marwood et al., 2001).

Although many of these biomarkers are directly related to the health of the organism in relation to collection of energy or protection of the cell, the question still remains as to whether the joint effect of chemicals of different mode of

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action, measured using biomarkers such as pigment contents, corresponds to the joint effect of chemicals when measured on individual plants or plant population growth rates.

To evaluate the joint effects of chemicals, comparison of observed results must be compared to a reference model. Two fundamental concepts are generally evaluated and each be summarized as a model (Greco et al., 1995). The first model, Concentration Addition (CA), assumes that the chemicals have the same molecular site of action and therefore can be regarded as dilutions of one another. The joint effect of two chemicals is therefore expected to be equal to the effect of the sum of the chemicals, when the potency of the chemicals (the degree of "dilution") has been accounted for (Greco et al., 1995). If the effect of a chemical mixture can be described with CA the chemicals are said to be additive. Though the theory assumes similar molecular target sites, it has been suggested that CA could also apply to mixtures of chemicals that are able to cause a common toxicological response (Berenbaum, 1989). When it comes to integral endpoints such as death or growth, this may apply to almost all chemicals (Faust et al., 2003). The model of independent action (IA) is based on test-systems with binominal endpoints such as dead/alive and assumes that the chemicals act independently. Under these assumptions the fractional effects of individual chemicals (e.g. 50% response) are expected to be independent of each other in a probabilistic sense. This means that if two chemicals are added at a concentration that can kill 50% of the test-organisms when applied individually, chemical A will kill 50% of the organisms and chemical B will kill 50% of what is left, leaving 25% of the test organisms alive (Greco et al., 1995). While IA is considered of theoretical importance, there is debate concerning its practical relevance when it comes to assessing joint effects on continuous endpoints measured on whole organisms (Berenbaum, 1989; Greco et al., 1995). Although both models have proven to be valid in predicting joint effects of chemicals on algae and bacteria (Altenburger et al., 2000; Backhaus et al., 2000; Faust et al., 2001, 2003), CA is often the preferred model for risk assessment purposes. If a chemical mixture gives a higher effect than predicted by one of the two models they are said to be synergistic in relation to either CA or IA (or both). If the joint effect of two chemicals is lower than predicted, it is called antagonism. Evaluating different endpoints, one could expect that biomarkers that are directly affected by a chemical, such as chlorophyll often is by herbicides affecting photosynthesis, would follow the model of IA. Integrated endpoints, such as growth, could be more likely to follow the model of CA.

The aim of the present study was to evaluate the joint effect of nine binary mixtures of herbicides with different mode of action on three growth endpoints and two pigment endpoints. The dose–response surface data was statistically analyzed for synergy and antagonism both in relation to CA and IA, to evaluate which model best describes the data for the different endpoints. In addition, data was evaluated at an EC_{50} level in relation to CA using isobolograms to visually quantify the degree of a potential synergistic or antagonistic interaction. Thereby the congruency of two recently published statistical methods for evaluating binary dose–response surfaces was evaluated (Jonker et al., 2005; Sørensen et al., 2007).

2. Materials and methods

2.1. Lemna test

The *L. minor* test closely follows the guidelines given by the International Organization for Standardization (International Organization for standardization, 2004), with a few modifications. The *L. minor* plants were collected in Copenhagen in 1999, surface sterilized according to Landolt and Kandeler (1987) and the resulting sterile clone was kept in Ehrlenmeyer flasks containing "K"-medium (Maeng and Khudairi, 1973). Test conditions were held at pH 5, 24 °C and a continuous photon flux density of 85–120 $\mu mol\,m^{-2}\,s^{-1}$ (PAR). The flasks and medium were sealed with cotton wool and autoclaved before the weekly transfer of plants to new media. Regular tests on $K_2Cr_2O_4$, and on 3,5-dichlorophenol (CAS-Nr.: 591-35-5) as part of the ISO-ring test, have proven the Copenhagen *L. minor* clone equally sensitive to plants from standard clones. The K-medium was used, as it has proven to give the highest growth rates in the used experimental setup.

For the *Lemna* tests, three mixture ratios were used. Each concentration–response curve consisted of 6 dilutions, each in three replicates. There were 6–12 controls that were shared by all five concentration–response curves. Nine binary combinations of the following six herbicides were made: acifluorfen, diquat, glyphosate, mecoprop, mesotrione and terbuthylazine. Details on the herbicides are given in Table 1. The herbicide combinations were: acifluorfen/diquat, acifluorfen/mesotrion, diquat/mesotrione, glyphosate/mecoprop, glyphosate/mesotrione, glyphosate/terbuthylazine, mecoprop/mesotrion, mecoprop/terbuthylazine and mesotrion/terbuthylazien. Experiments using glyphosate/mecoprop, mecoprop/mesotrion and mecoprop/terbuthylazine combinations were repeated to explore the reproducibility of the results. The dose ranges of the herbicides were chosen based on previous experiments where area-specific growth rates were measured (Cedergreen and Streibig, 2005). The experiments were performed in 6-well tissue culture (TC) test plates (CM. Lab. Aps, Vordingborg, Denmark) and were initiated by transferring one *L. minor* frond to 10 ml K-medium (Maeng and Khudairi, 1973) containing a selected concentration of herbicide. The plants were photographed with a digital camera alongside a 1×1 cm white plastic square, and total frond surface area was determined by pixel counts using the computer program Photoshop 5.0 (Adobe). Flasks were then placed in a growth cabinet at 24 °C and a continuous photon flux density of 85–120 $\mu mol\,m^{-2}\,s^{-1}$ (PAR). Six samples of plants, similar to the incubation plants were photographed, dried at 80 °C for 24 h and weighed, to determine the start surface area/dry weight ratio. After 7 days, the *L. minor* plants were photographed and frond surface area and frond number were determined. The plants were then dried and weighed. Relative growth rates were calculated according to: $(\ln A_T - \ln A_0)/T$, where A_T is the surface area, frond number or dry weight at time T and A_0 is the surface area, frond number or dry weight at the start of the experiment. The three replicates per dose were pooled for dry weight determination in order to obtain sufficient plant material for determining a reliable dry weight on a five decimal balance (AX26 Coparator, Mettler Toledo, Glostrup, Denmark).

2.2. Pigment analysis

The analysis of chlorophyll *a* and *b* and carotenoids were done on ethanol extractions according to Lichtenthaler (1987). One to two