Master of Science thesis

Short-term and subchronic phytotoxicity of low-weight carboxylic acids and their mixtures

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ABSTRACT

Presence of low-weight carboxylic acids (LWCA) in immature composts and anaerobically digested wastewater sludges is well established. When concentrations are high, phytotoxicity of the materials is exhibited, which hinders application of composts in agriculture or horticulture. Although there have been research concerning toxicity of individual LWCA, the mixture aspect has been poorly studied.

The research was aimed to study the toxicity of three LWCA, formic, acetic and propionic acids, to garden cress (*Lepidium sativum*) and Italian ryegrass (*Lolium multiflorium*). Test procedure consisted of short-term germination assays (48 h for cress and 72 h for ryegrass) and subchronic growth assays (21 d). The responses recorded in the assays were germination and shoot length in short-term assay and germination (7d and 21d) and biomass of the plants. The acids were tested individually as well as binary and trinary mixtures.

Dose-response relationships and EC values were modeled using three-parametric log-logistic model. Toxicity of the mixtures was evaluated using additive index method utilizing test concentrations in toxic units. Index vales were used to judge whether the toxicity of studied LWCA is simply additive, less than additive or greater than additive.

In the study $EC_{10,}$ EC_{50}, and EC_{90} values were generated for cress and ryegrass that can be used for assessing toxicity of composts or digestates. According to the additive index values the mixture toxicity of the LWCA was either additive or less than additive.

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TIIVISTELMÄ

Kompostia käytetään maaparannusaineena tai kasvualustan osana. Hajoava kompostiaines ja mädätysjäännös sisältävät kuitenkin lyhytketjuisia karboksyylihappoja, jotka ovat kasveille haitallisia. Karboksyylihapot kuitenkin hajoavat kompostimassasta sen stabiloituessa, joten niiden pitoisuudet ovat kompostin kypsymisen indikaattoreita.

Tutkimuksen tarkoituksena oli tutkia muurahais-, etikka- ja propionihapon fytotoksisuutta yksistään ja kahden tai kolmen hapon seoksissa. Testilajit olivat krassi (*Lepidium sativum*) ja raiheinä (*Lolium multiflorium*) ja vasteena itävyys, itujen pituus lyhytkestoisessa itävyyskokeessa (krassi 48 h ja raiheinä 72 h) sekä kasvien itävyys (7 vrk ja 21 vrk) ja painoon pitempiaikaisessa kasvatuskokeessa (21 vrk).

Annos-vaste suhteet ja EC50 arvot mallinnettiin kolmiparametrisen log-logistisen mallia kayttäen. Happojen seosvaikutukset arvioitiin additive index-menetelmän avulla.

Muurahais-, etikka- ja propionihapolle määriteltiin EC_{50}-, EC_{10}- ja EC_{90}-arvot krassille ja raiheinälle useille vasteille. Additive index arvojen perusteella voidaan päätellä, että näiden happojen kasvitoksisuus on summautuvaa tai antagonistista. Saatuja EC-arvoja voidaan soveltaa hyötykäytettävien massojen kasvitoksisuuden arviointiin.

Contents

1. INTRODUCTION

1.1 Nomenclature, structure and properties of low weight carboxylic acids

Low weight carboxylic acids (LCWA) are a common name for a number of short chained aliphatic carboxylic acids with maximum of six carbons (Cherrington et al., 1991). They are also generally known as volatile fatty acids, name that originates from the fact that fat molecules are esters of glycerol alcohol and three carboxylic acids. The general structure of carboxylic acid is R-COOH, where R can be single hydrogen or a carbon chain (Smith, 2006). The study was focused on the aliphatic carboxylic acids containing 1 to 3 carbons (Table 1).

Table 1. Structures of selected LWCA (Smith, 2006).

IUPAC name	Structure	Trivial name
Methanoic acid	H - COOH	Formic acid
Ethanoic acid	CH_3 - COOH	Acetic acid
Propanoic acid	CH_3 - CH_3 - COOH	Propionic acid

LWCA act as weak Brønsted acids that dissociate partly in water solutions into carboxyl anion and hydrogen cation according to reaction equation 1.

$$R - COOH + H_2O \rightarrow R - COO^- (aq) + H^+ (aq) \quad (1)$$

As a result, water solution is mildly acidic with pH varying according to dissociation constant of the acid (pK_a), which defines a point where fifty percent of the acid is dissociated. Dissociation constant is acid specific and is defined by its molecular properties, e.g. bond strength. Degree of the dissociation can be calculated using the Henderson-Hasselbach equation (equation 2.) if the pK_a- value of the acid and pH of the solution are known. However, the equation is accurate only when the solution is consisting of 1 acid and is relatively dilute (Harris 2003).

$$pH = pK_a + log_{10} \frac{[A^-]}{[HA]} \quad (2),$$

where $[A^-]$ = concentration of anionic form of acid and [HA] = concentration of undissociated form of acid

1.2 Occurrence and importance of low weight carboxylic acids in the environment

In the environment, LWCA are formed when organic matter is fermented or decomposed by microbes. They are usually formed in hypoxic and anaerobic processes, such as municipal sewage treatment, but they can be produced also in aerobic processes, such as composting and can be found in soil (Zucconi et al 1981, Hope 1986).

Composts are widely used in modern agriculture and horticulture to maintain organic content and nutrient balance in the soil. The problem of compost material use is maturity of the compost, as LWCA are produced in the early stages of composting and degraded in later stages. Careless application of raw compost material can lead to crop failure (Zucconi et al 1981, Hope 1986).

LWCA are formed when municipal sewage sludge high in organic matter is digested anaerobically and, thus, presence of the acids can hinder the utilization of the digestate as soil improver. In opposition to compost material, utilization of sewage sludge is more difficult due to its contamination, especially by heavy metals, so utilization in landscaping and as a horticultural fertilizer is very tempting (Hope 1986).

LWCA occur also naturally in soils but rarely in concentrations high enough to cause phytotoxicity. Recent studies suggest that some LWCA may be beneficial in soils as they increase nutrients availability to plants and buffer toxic action of some heavy metals, such as Al and Zn. They also form a major part of the labile organic carbon source for the soil microbes (Jones et al 2003). It has also been observed that LWCA have a positive role in germination process of seeds and that the toxicity of LWCA varies between different types of soils (Chandrasekaran & Yoshida 1972, Cohn. et al 1987).

1.3 Phytotoxicity of low weight carboxylic acids and significance of pH

Despite being natural metabolic products with somewhat beneficial effects, high concentrations of LWCA can inhibit plant growth. This phytotoxicity of LWCA is a complex phenomenon. Acting as weak acids, LWCA dissolve partly in solutions releasing protons (H^+) and lowering the pH of the solution. Opposite to positive aspects expressed in the chapter before, this acidity itself may cause harmful effects in plants by lowering the availability of soil nutrients, mobilizing toxic metals and inhibiting protein function. High proton concentration can cause adverse effects also by changing the chemical potential of

the water which is important in early stages of germination, the imbibition (Bewley & Black 1994). There is evidence that proton concentrations above 1 mmol/l (pH below 3) are directly toxic to majority of plants (Taiz & Zeiger 1998, Fitter & Hay 1987). Harmful effects can also rise from the changes of ionic composition of the solution as high ionic concentrations can cause toxic effects and lead changes in the soil structure and chemistry (Taiz & Zeiger 1998).

However, it has been shown in earlier studies that increased acidity is not necessarily harmful to plants since some plants have been observed to exude organic acids to soil and increase the acidity near rhizospere. Rhizosphere dwelling microbes can also produce organic acids and tolerate them (Stevenson 1967, International Rice Institute 1970). Previous studies suggest that increased acidity is not the main cause of LWCA toxicity. The lipophilic carbon chain itself is perceived to cause most toxic effects and it has been observed that the toxicity of the LWCA increases as the carbon chain lengthens (Lee 1997, Himanen et al 2012).

1.4 Mixture effect and its evaluation

It is generally recognized that in terms of toxicity, the sums of toxic components do not always add up. The basic concept of mixture toxicity, the joint action principle, was first introduced by Bliss in 1939. The principle was later defined further by Finney who formulated a method for testing toxicity of chemical mixtures using harmonized means of the EC_{50} values for individual components of the mixture. The method was considered very detailed and too complicated, but it created the basis for Marking and Dawson to derive a quantitative index for the toxicity of mixtures of chemicals later on. They suggested additive, greater than additive and smaller than additive effects and the index was expressed as zero, positive or negative value, respectively. This was achieved by assigning zero as the simple additive toxicity point and deriving linearity for greater and less than additive toxicity. This method relies on using toxic units (TU) formed from actual concentrations. By definition, toxic unit is the sum of the toxic strength of individual compounds of the mixture (Marking 1985).

Calculation of toxic units is represented in equation 3 (Marking 1985).

$$TU = \frac{1}{n} \times EC_{50\,A} + \frac{1}{n} \times EC_{50\,B} \ldots + \frac{1}{n} \times EC_{50\,n} \quad (3)$$

where TU is the toxic unit, n is the number of components in the mixture and EC_{50} is the EC_{50} value of the individual component.

The toxicity testing is performed using toxic units and the results are converted back to concentrations by simple multiplication to obtain $EC_{50,mixture}$ values in addition to the $EC_{50,individual}$ values. From these values we can calculate the sums of toxic action with equation 4 (Rand & Petrocelli 1984).

$$S = \frac{A_{mixture} + B_{mixture}}{A_{Individual} + B_{Individual}} \ldots + \frac{M_{mixture}}{M_{Individual}} \quad (4)$$

where S is the sum of toxic action, $M_{mixture}$ is the $EC_{50,mixture}$ value of component M and $M_{individual}$ is the $EC_{50,individual}$ value of component M.

After the sums of toxic action are derived, the actual additive index values can be calculated according to equations 5 and 6 (Rand & Petrocelli 1984).

$$S \leq 1, AI = \frac{1}{S} - 1 \quad (5)$$

$$S \geq 1, AI = S(-1) + 1 \quad (6)$$

where S is the sum of toxic action and AI is the additive index.

The additive toxicity of components in a mixture can be assessed using theindex values or can be further evaluated whether they truly differ from zero or not using Marking and Dawson's method for evaluating the significance of index values. In the method the deviation from zero is determined by substituting the EC_{50} values for 95% confidence intervals to equation 4 to obtain a range for the additive indices. The range is calculated according to equations 7 and 8 (Rand & Petrocelli 1984).

$$Upper\ range = \frac{M_{\text{mixture,2,5\% conf.int}}}{M_{\text{individual,97,5\% conf.int}}} \quad (7)$$

$$Lower\ range = \frac{M_{\text{mixture,97,5\% conf.int}}}{M_{\text{individual,2,5\% conf.int}}} \quad (8)$$

where $M_{mixture,\ 2,5\%\ conf.int}$ and $M_{mixture,\ 97,5\%\ conf.int}$ are the lower and upper limits of the confidence interval for EC_{50} mixture value of the component M, $M_{individual,\ 2,5\%\ conf.int}$ and $M_{individual,\ 97,5\%\ conf.int}$ are the lower and upper limits of the confidence interval for EC_{50} individual value of component M.

Mixtures with the additive index range overlapping zero are judged to express additive toxicity and mixtures with ranges that do not overlap zero are judged to have greater or less than additive toxicity (Rand & Petrocelli 1984).

1.5 Objectives of the research

The phytotoxicity of formic, acetic and propionic acids is well reported separately but mixture toxicity and possible additive toxicity effect has remained undetermined. Previous study has not supplied useful EC values usable in practical applications.

The main objectives of this study were:

1. To obtain EC_{50} values for individual formic, acetic and propionic acids
2. To evaluate the additive toxicity of three LWCA in binary and trinary mixtures using additive index method.
3. Take a closer look at the mode of action of LWCA to plants and evaluate the significance of pH in phytotoxicity.
4. To calculate EC values for the acids mixtures that can be applied in evaluation of the substrate phytotoxicity

2. MATERIALS AND METHODS

2.1 Preliminary experiments and buffers

Due to the proton donating qualities of the LWCA, the acidity of the test solutions increase with increase in concentrations of the acid. To rule out the impact of acidity on germination, an effort was made to find a suitable buffer solution that could be used in the acid solutions to prevent additional inhibition of germination and growth.

Buffers within the suitable pH range were citric acid, potassium hydrogen phthalate, and Clark & Lubs buffer solution which contains potassium phosphate as acting reagent. The prospective buffers were tested in germination bioassay in several pH-values: citric acid buffer was tested at pH-values 4, 5 and 6, potassium hydrogen phthalate buffer at pH 4, 5 and 6, and Clark & Lubs buffer solution at pH 5 and 6.

The bioassays were conducted in similar way as the short-term toxicity assay described in 2.3. Unfortunately all the buffer solutions proved to inhibit germination and shorten the shoot lengths considerably, therefore, none of them was qualified for the assay procedure.

2.2 Testing strategy – from single to complex

The basis of the additive index method lies in the toxic unit value (TU) which is the equivalent to the EC_{50} value of the compound. TU value determinates the setting for the next phase of testing. In this study the existence of preliminary data for the phytotoxicity of formic, acetic and propionic acids by Himanen et al. (2012) gave a good head start by providing knowledge of the range in which the acids operated. The chosen concentrations are listed in table 2.

EC_{50} values obtained in the study were used as basis for preparation of the binary mixtures. The EC_{50} values were converted to toxic units and solutions for short-term and subchronic assays were prepared in accordance with concentration series of 1/10 TU, 1/4 TU, 1/2 TU, 1 TU, 2 TU and 4TU.

The final stage of the study was assaying the mixture of all three acids. For that, the concentration series was modified to accommodate all three components. The resulting

Table 2. Concentrations of formic, acetic and propionic acids used in short-term and subchronic assays.

Acid	Cress	Ryegrass
	Short-term assays (mmol/l)	
Formic	0, 0.4, 0.8, 1.6, 3.2, 9.6 0, 0.5, 1, 2, 4, 12	0, 0.4, 0.8, 1.6, 3.2, 9.6 0, 0.5, 1, 2, 4, 12
Acetic	0, 0.3, 0.6, 1.2, 2.4, 9.6 0, 0,5 1, 1.5, 3, 12	0, 0.4, 0.8, 1.6, 3.2, 9.6 0, 0.5, 1, 2, 4, 12
Propionic	0, 0.3, 0.6, 1.2, 2.4, 9.6	0, 0.3, 0.6, 1.2, 2.4, 9.6
	Subchronic assays (mmol/kg, dw)	
Formic	0, 5, 10, 20, 40, 80	0, 10, 30, 60, 120, 150
Acetic	0, 5, 10, 20, 40, 80	0, 5, 25, 50, 75, 100
Propionic	0, 2, 5, 10, 30, 60	0, 5, 10, 15, 30, 60

concentration series for short-term and subchronic assays was 1/12 TU, 1/6 TU, 1/3 TU, 1 TU, 1 1/2 TU and 3 TU.

2.3 Short-term toxicity assays

The short-term toxicity experiments were conducted with two species, monocotyledon Italian ryegrass (*Lolium multiflorum*) and dicotyledon garden cress (*Lepidium sativum*).

A series of 5 or 6 dilutions were made according to Table 2, deionized water was used as control. All the solutions were prepared from pro analysi (98 - 99,5 m%) quality stock solutions and diluted with deionized water. The solutions were prepared before the first replicate and stored for a maximum of two months in darkness at room temperature. Every replica contained three parallels of each concentration and six positive controls. Each assay was replicated three separate times.

Ten milliliters of the pure acid solution, acid mixture or control were added on plastic (PS, styrofoam) Petri dish (Ø = 9 cm) lined with filter paper (Whatman No. 1, Ø = 7 cm) and 20 seeds of the test species were spread over the paper. The closed dishes were incubated in the darkness at 25 ± 1°C for 48 hours for cress and 120 hours for ryegrass. After the incubation time the number of germinated seeds was counted and the length of the seedlings was measured to the nearest millimeter.

2.4 Subchronic assays

The subchronic growth experiments were conducted with Italian ryegrass (*L. multiflorum*) and garden cress (*L.sativum*) according to the method described in Himanen et al. (2008). One hundred and fifty millilitres of the pure acid solution, acid mixture or control (deionized water) were added to 1.6 l of the inorganic growth substrate that was a mixture (6+1, w/w) of coarse-grained sand (Ø = 0.5 - 1.2 mm, Maxit puhallushiekka, Maxit Oy Ab, Helsinki, Finland) and the quartz sand (Ø = 0.05 - 0.2 mm) (NFQ Nilsiän kvartsi, SP Minerals Oy Ab, Halluna, Finland).

The plastic pots (Ø = 9.5 cm, V = 0.39 l) were filled in layers, with peat at the bottom and top and sand in between. Approximately one cm layer of moistened *Sphagnum* peat (Kekkilä Kasvuturve B2, Kekkilä Oyj, Finland) was added to the bottom of the pot and compressed lightly. Onto that, 300 g sand mixture was weighed with analytical balance (d = 0.1 g) to the closest 1 g and 25 seeds were sown on top of the sand mixture and covered with another one cm layer of peat. Sewn pots were watered with approximately fifty millilitres of tap water so that a few droplets appeared on the saucer and placed in to the greenhouse. The first few days the pots remained covered with clear plastic (PE-LD, low density polyethene) sheet to prevent excess evaporation. The covers were removed when shoots began to emerge and pots were watered for the first time after a couple days since the removal of the covers.

The pots were incubated in experiment greenhouse in total 21 days at target temperature of 25 °C (16-40 °C), light regime of 16/8 h light/dark at 7000 – 9000 lux. Temperature was monitored throughout the experiments using digital thermometer which recorded the minimum and maximum temperatures. The temperatures were recorded on each irrigation occasion during the assays and are listed in appendix 2. Light intensity was measured approximately thirty cm above the table at several points around the table with light meter (LI-250A Light Meter, Li-Cor, USA) facing up towards the lamps.

The temperatures varied much in accordance to the amounts of sunlight, light from the lamps, and the outdoor temperatures. Also elongation of daytime towards the midsummer, coinciding with presence of large uncovered windows, had some effect on the assays. However, this effect was present in all of the assays with one exception. Due to scheduling

issues, the last of the subchronic assays (the third replica of mixture assays) was conducted in a temperature controlled toxicity testing room where the only source of light was the greenhouse lamps (approximately 6000 lux) set to same light-dark regime as in the experiment greenhouse. As the room was temperature controlled, the temperature in the assay in question remained lower and closer to desired 25 °C than at the experiment greenhouse.

Pots were watered with fertilizer solution (Kekkilä Kukkaravinne NPK (12-6-9), Kekkilä Oyj, Eurajoki, Finland). In the beginning of the experiment (for the first 12-14 days depending on the irrigation cycle), fertilizer concentration of the irrigation water was 1 g/l and in the end it was increased to 2 g/l. Irrigation was performed according to demand approximately in every second days.

The numbers of germinated seeds were counted at the 7th day and again at the end of assay. The experiments were ended after 21 days of exposure by cutting the shoots on ground level. The shoots were counted and weighed on analytical balance (d = 0.0001 g) to the closest 0.1mg to obtain wet weight of the biomass, and dried in 70 °C overnight and weighed again to the closest 0.1mg to obtain dry weight of the biomass.

For the pH measurements, samples from the sand mixtures were taken before and after the growth experiments. The samples at the end were compilation samples obtained by mixing sand mixture scraped from the three replicate pots after cutting off the shoots. The samples were collected to a zipper bag and stored in a freezer at -18 °C.

2.5 Measurement of pH

The pH-value of the growth substrates before and after the experiments were measured directly using apH meter (pHenomenal pH1000L, VWR International Oy, Germany, accuracy ±0.005 ±1 digit, calibrated daily) with the attached electrode designed for measuring pH straight from the solid media (VWR general electrode, Art.No. 662-1805). According to the standardized method (ISO 2005), pH in growth substrate is measured as a pH-value in substrate-water extract (5:1) deionized, therefore, the correlation of the methods was tested on several samples in the preliminary experiments.

The results can be compared by calculating back the proton concentration from the pH-values according to equation 9 and dividing them by 5 to simulate the effect of the extraction procedure and dilution. For calculation simplicity the pH-value of the deionized water was presumed to be 7. The values were then calculated back to pH-values and compared against each other.

$$pH = \log [H^+] \rightarrow [H^+] = 10^{-pH} \quad (9)$$

where $[H^+]$ is the proton concentration and pH is the measured pH-value of the sample.

The comparison was made for 10 different samples from various stages of the assays and compared by SPSS using pairwise comparison. The result showed high correlation between the results with a p value of 0.982, so the straight measurement method was deemed fully comparable with the extraction method.

For comparison with pH data using the water extraction method, a simple correction factor was devised by taking the mean from the difference between the corresponding pH-values measured in different methods.

2.6 Modeling of the toxicological values

Dose response modeling and computing of the EC_{50} -values was performed using R program (version 2.53) drc package. All results were modeled using three parametric log-logistic function model (LL.3) defined by equation 10. This model was selected since it presumes that the distribution of the data is symmetric across the LC50 value on both tails and eventually reaches zero (Ritz & Streibig 2005). Germination modeling was performed with binomial LL.3.

$$f(x) = c + \frac{1-c}{1+\exp(b(\log(x)-e))} \quad (10)$$

SPSS program (version 22) was used in pH-value comparisons (pairwise comparison tool) and Microsoft® Excel 2010 was used in processing the data from the assays to appropriate format and to calculate mixture EC_{50} values.

3. RESULTS

3.1 Short-term bioassays

Data collected in the bioassays was processed with R to model dose-response relationships and to obtain EC_{10}, EC_{50} and EC_{90} values. Also corresponding standard errors for EC_{10}, EC_{50} and EC_{90} values and 95% confidence intervals for EC_{50} were recorded and the whole data table can be found in Appendix 1.

3.1.1 Phytotoxicity of individual acids

EC_{50} values in short-term assays for shoot length of cress were 1.8, 1.5 and 1.0 mmol/l in formic, acetic and propionic acid, respectively (table 3). These results are in conforming to previous observations (Himanen et al. 2012) on growing toxicity as the LWCA chain lengthens. However the EC_{50} values for cress germination and ryegrass shoot length and germination vary from this perception. EC_{50} values obtained for shoot length of ryegrass were 1.7, 3.3 and 1.3 mmol/l for formic, acetic and propionic acid, respectively.

EC_{50} values for germination for cress were 2.9, 3.2 and 3.3 mmol/l and for ryegrass 5.16, 7.12 and 2.92 mmol/l for formic, acetic and propionic acid, respectively (table 3).

Table 3. EC_{50} values obtained in short-term assays for cress and ryegrass in individual formic, acetic and propionic acids. The endpoints were seed germination and shoot length. EC_{10} and EC_{90} are in parenthesis. EC values are expressed in mmol/l.

Acid	**EC_{50} (mmol/l)**			
	Cress		**Ryegrass**	
	Germination	**Shoot length (mm)**	**Germination**	**Shoot length (mm)**
Formic acid	2.9 (2.2 – 4.0)	1.8 (1.0 - 3.3)	5.2 (3.1 - 8.5)	1.7 (0.6 - 5.2)
Acetic acid	3.2 (2.3 – 4.7)	1.5 (0.8 - 3.2)	7.1 (4.9 - 10.4)	3.3 (1.8 - 6.2)
Propionic acid	3.3 (1.8 – 5.5)	1.0 (0.4 - 2.8)	2.9 (2.2 - 3.9)	1.3 (0.7 - 2.5)

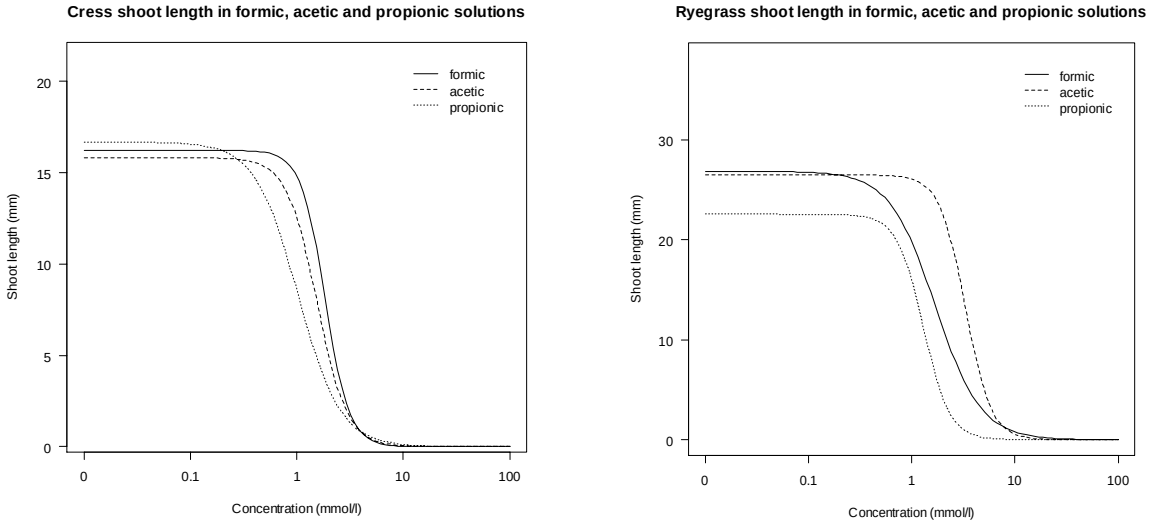

Figure 1. Dose-response curves for shoot length of cress and ryegrass in formic, acetic and propionic acid. Log logistic model (LL.3).

As expected, cress follows the general observation that toxicity of the acid increases as the carbon chain of the acid lengthens. However, ryegrass differs from this observation and suggests that formic acid is more toxic than acetic acid. This phenomenon is unusual and did not occur in other assay sets, so there's a possibility this result is biased.

3.1.2 Phytotoxicity of binary mixtures of LWCA

Evaluation of the acid mixture effect begun with assaying three binary mixtures: formic and acetic acid (F + A), formic and propionic acid (F + P) and acetic and propionic acid (A + P) mixed in proportions of toxic units rather than concentrations. As the assay yielded results in toxic units, modeling was also done in toxic units, which were then converted back to corresponding concentrations (mmol/l). Also EC_{10} and EC_{90} values and 95% confidence intervals were extracted from the model (appendix 1).

EC_{50} values with EC_{10} - EC_{90} intervals are summarized in table 4 for cress and ryegrass. From the data it can be seen that toxicity of formic and acetic acids increases when the acids were tested in mixture with propionic acid. The trend is observable for both species and end points.

Comparison of EC values for two end points suggest that germination is less sensitive parameter to acids than shoot length. All in all, the data shows no great variation between

the two assayed species in sensitivity, though there might be some indication that cress is more sensitive in terms of shoot length while ryegrass is more sensitive in terms of germination.

The difference between the dose-response curves of the three sets of binary mixtures for cress and ryegrass is presented in figure 2. It is evident in both species that mixtures containing propionic acid are more toxic than mixtures of formic and acetic acids.

To evaluate the presumed additive toxic action of the acids in binary mixtures, the additive indices (AI) together with their ranges were calculated and are summarized in table 5. As described earlier, the mixture effect is determined rather by the range (95 % confidence interval) than the AI value itself. If this range overlaps zero, effect is judged to be additive. Ranges in negative values point to less than additive effect while range in positive values to greater than additive effect. In this case, indices suggest that toxicity of LWCA in binary acid mixtures is mainly additive, although some cases show greater or lesser than additive effect.

Table 4. Short-term EC_{50} values for cress and ryegrass shoot length and germination in binary mixtures. EC_{10} and EC_{90} in parenthesis. F = formic acid, A = acetic acid, P = propionic acid.

Acid / Mixture	**Germination**			**Shoot length**		
	F + A	**F + P**	**A + P**	**F + A**	**F + P**	**A + P**
			Cress - EC_{50} (mmol/l)			
Formic	5.1 (2.2 -11.7)	3.6 (1.8 - 7.4)	- -	1.2 (0.5 - 2.8)	0.9 (1.2 - 5.1)	- -
Acetic	4.1 (1.8 - 9.5)	- -	3.3 (1.5 - 7.2)	1.0 (0.4 - 2.2)	- -	0.7 (0.4 - 1.5)
Propionic	- -	2.3 (1.1 -4.6)	2.5 (1.2 - 5.5)	- -	0.5 (0.8 - 3.2)	0.6 (0.3 - 1.1)
			Ryegrass - EC_{50} (mmol/l)			
Formic	3.0 (1.9 - 4.6)	2.0 (1.4 - 2.7)	- -	1.5 (0.8 - 3.0)	1.1 (0.6 - 1.9)	- -
Acetic	4.4 (2.8 - 6.7)	- -	3.2 (1.9 - 5.2)	2.2 (1.2 - 4.3)	- -	1.7 (1.0 - 2.7)
Propionic	- -	1.7 (1.2 - 2.3)	1.9 (1.1 - 3.1)	- -	0.9 (0.5 - 1.7)	1.0 (0.6 - 1.6)

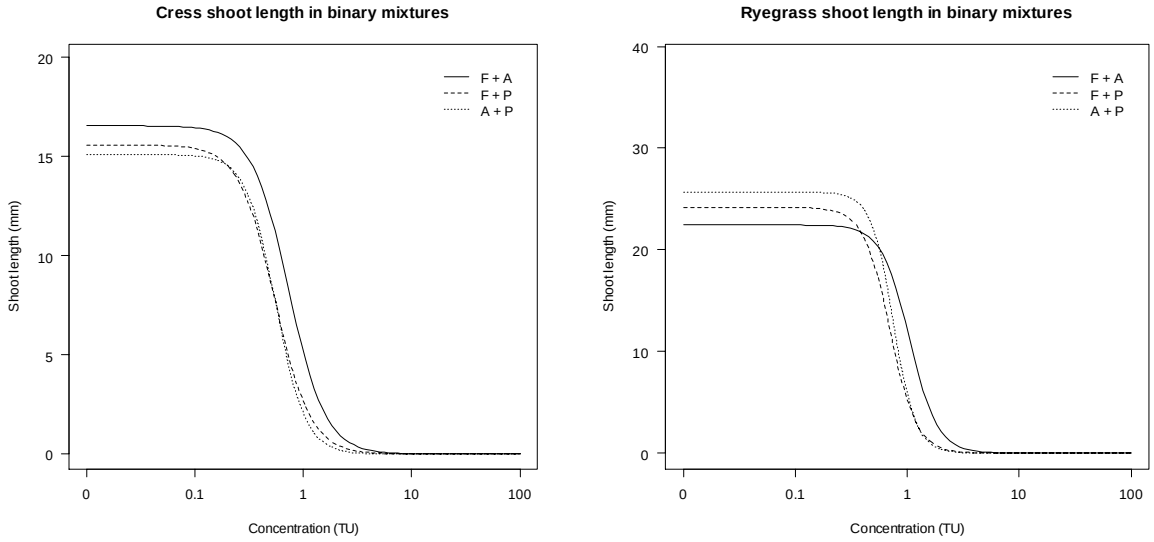

Figure 2. Dose response curves for cress and ryegrass shoot length in binary acid mixture. The data were obtained with log logistic model (LL.3). F = formic acid, A = acetic acid, P = propionic acid.

Table 5. Sums of toxic actions (*S*) and additive indices (*AI*) of binary mixtures for germination and shoot length of cress and ryegrass. Acids: F = formic acid, A = acetic acid, P = propionic acid. Type of toxic outcome: Ad = additive, GtAd = greater than additive, LsAd = less than additive.

Mixture	**Factor**	**Cress**		**Ryegrass**	
		Germination	**Shoot length**	**Germination**	**Shoot length**
F + A	**S**	1.27	1.25	1.19	1.58
	AI	-0.27	-0.25	-0.19	-0.58
	95% conf in. of AI	(-0.30 - -0.06)	(0.85 - -0.07)	(-0.61 - 0.09)	(-1.37 - -0.16)
	Toxicity	LsAd	Ad	Ad	LsAd
F + P	**S**	1.30	1.01	0.95	1.35
	AI	-0.03	-0.01	0.05	-0.35
	95% conf in. of AI	(-0,16 – 0,08)	(-2.35 - -1.43)	(-0.34 - 0.44)	(-0.86 - 0.03)
	Toxicity	Ad	LsAd	Ad	Ad
A + P	**S**	0.85	1.02	1.1	1.26
	AI	0.17	-0.02	-0.10	-0.26
	95% conf in. of AI	(0,01 – 0,55)	(-0.19 - 0.13)	(-0.85 - 0.26)	(-0.68 - 0.06)
	Toxicity	GtAd	Ad	Ad	LsAd

3.1.3 Phytotoxicity of trinary mixtures of LWCA

Mixture effect of three acids (F+A+P) was evaluated using solutions of formic, acetic and propionic acids mixed in proportions of TU values that were obtained in the individual acid assays. As in binary mixtures, the trinary mixture results were modeled using toxic units and transformed back to concentrations (mmol/l) afterwards. EC_{50} values of the mixture were modeled for each acid against the whole toxic action of the mixture. EC_{10} and EC_{90} values and 95% confidence intervals were calculated using the model (appendix 1).

EC_{50} values of the mixture show that shoot length is more sensitive than germination. Additionally, ryegrass might be less sensitive to acid toxicity than cress (table 6). The EC_{50} values also indicate that propionic acid is more toxic than formic or acetic acid.

The dose response curve of the trinary acid mixture for cress and ryegrass is presented in figure 3. However, as cress and ryegrass produce different biomasses in control environment, relationships are not comparable.

Table 6. Short-term EC_{50} values from assay of trinary mixtures for cress and ryegrass germination and shoot length in acid mixture containing formic, acetic and propionic acid. EC_{10} and EC_{90} in parenthesis. F = formic acid, A = acetic acid, P = propionic acid.

Acid / Mixture	**EC_{50} (mmol/l)**			
	Cress		**Ryegrass**	
	Germination	**Shoot length**	**Germination**	**Shoot length**
	F + A + P	**F + A + P**	**F + A + P**	**F + A + P**
Formic	1.6 (1.22 – 2.0)	0.60 (0.3 - 1.4)	1.6 (1.2 - 2.0)	0.80 (0.5 - 1.3)
Acetic	1.27 (0.99 – 1.64)	0.50 (0.2 - 1.1)	2.3 (1.8 - 3.0)	1.1 (0.7 - 1.9)
Propionic	0.98 (0.76 – 1.26)	0.40 (0.2 - 0.9)	1.4 (1.0 - 1.8)	0.70 (0.4 - 1.1)

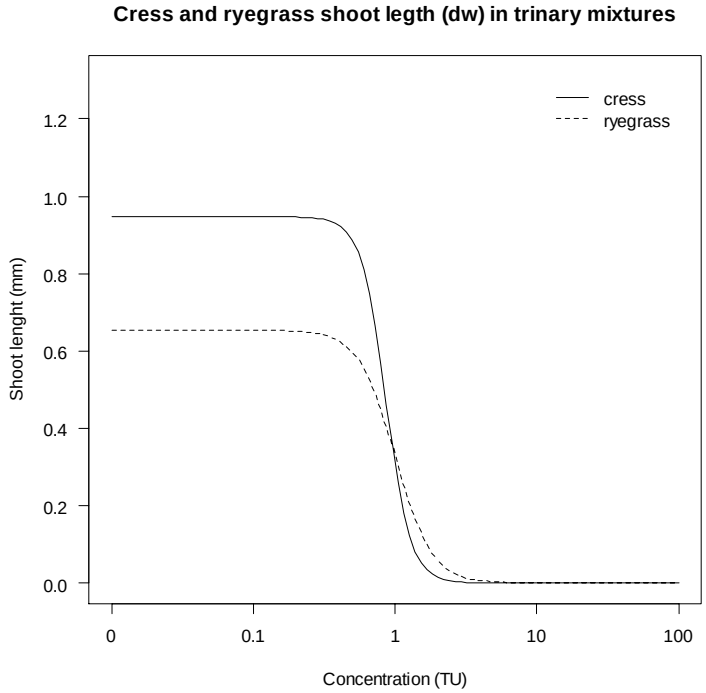

Figure 3. Dose- response curves for cress and ryegrass shoot length in trinary acid mixture. The data were obtained using log-logistic model (LL.3).

To evaluate the possible additive toxic action of the trinary mixtures, the additive indices *AI* together with their 95% confidential intervals were calculated and are presented in table 8. *S* and *AI* values listed suggest greater than or additive toxicity for germination and less than additive toxicity for shoot length.

Table 8. Sums of toxic action (S) and additive indices (AI) of trinary mixture for cress and ryegrass shoot length and germination. Range in parenthesis. Acids: F = formic acid, A = acetic acid, P = propionic acid. Type of toxic outcome: Ad = additive, GtAd = greater than additive, LsAd = less than additive.

Species	Response	S	AI	AI range	Toxicity
Cress	**Germination**	1.23	-0.23	(-0.30 - -0.17)	LsAd
	Shoot length	1.07	-0.07	(-0.06 - -0.07)	LsAd
Rye	**Germination**	1.11	-0.11	(-0.51 - 0.11)	Ad
	Shoot length	1.33	-0.33	(-0.03 - -0.52)	LsAd

3.2 Subchronic bioassays

3.2.1 Phytotoxicity of individual acids

Results of the subchronic assays showed similar increase in toxicity with increase in carbon chain length (table 9). Germination of cress and ryegrass seeds, after exposure time

of 7d and 21d generally followed this trend, with the sole exception of 7d germination of ryegrass where EC_{50} value for acetic acid was higher than for formic acid, 78.4 and 64.4 mmol/kg, respectively. For plant growth expressed as biomass, the EC_{50} values were 35.6, 24.2 and 11.4 mmol/kg for formic, acetic and propionic acid, respectively. Corresponding values for ryegrass biomass were 50.1, 47.4 and 15.9 mmol/kg for formic, acetic and propionic acid, respectively.

Table 9. EC_{50} values of pure formic, acetic and propionic acids and their mixtures obtained in subchronic assays for cress and ryegrass. The endpoints were shoot biomass (dry weight) and germination after 7d and 21d exposure time. EC_{10} - EC_{90} range is presented in parenthesis.

Acid	Germination 7d	Germination 21d	Biomass (dw)
	Cress EC_{50} (mmol/kg)		
Formic	38.2 (29.3 - 49.9)	36.9 (25.8 - 52.7)	35.6 (25.1 - 50.6)
Acetic	31.6 (19.5 - 51.1)	28.8 (18.6 - 44.5)	24.1 (13.8 - 42.1)
Propionic	25.3 (22.5 - 28.4)	16.8 (9.9 - 28.3)	11.4 (4.8 - 27.2)
	Ryegrass EC_{50} (mmol/kg)		
Formic	64.4 (43.7 - 94.9)	67.8 (45.2 - 102)	50.1 (24.1 - 104)
Acetic	78.4 (56.8 - 108)	51.5 (22.4 - 118)	47.4 (23.9 - 94.1)
Propionic	33.5 (26.1 - 43.0)	25.0 (14.7 - 42.7)	15.9 (6.0 - 41.7)

The dose response curves for cress and ryegrass biomass showed (figure 4) the trend with increasing toxicity with increase in carbon chain is most pronounced in ryegrass. The dose response curve for propionic acid is clearly shallower than formic and acetic, which might have indicated toxic effect in a wider range or problems fitting data to model.

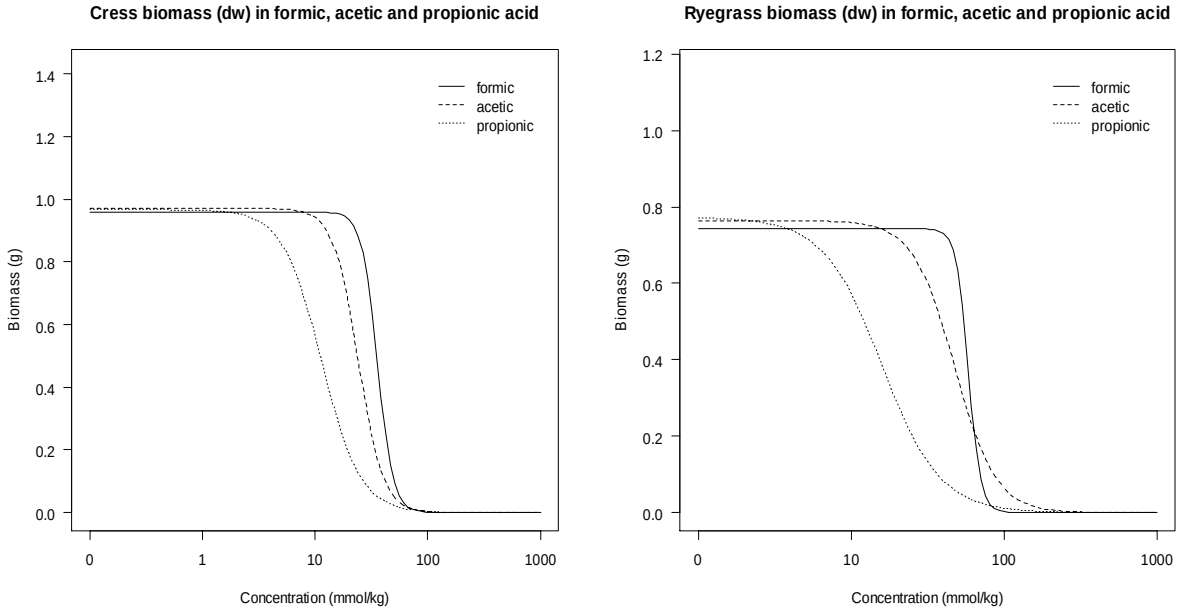

Figure 4. Subchronic dose response curves for cress and ryegrass biomass (dry weight) in formic, acetic and propionic-spiked soils. Log logistic model (LL.3).

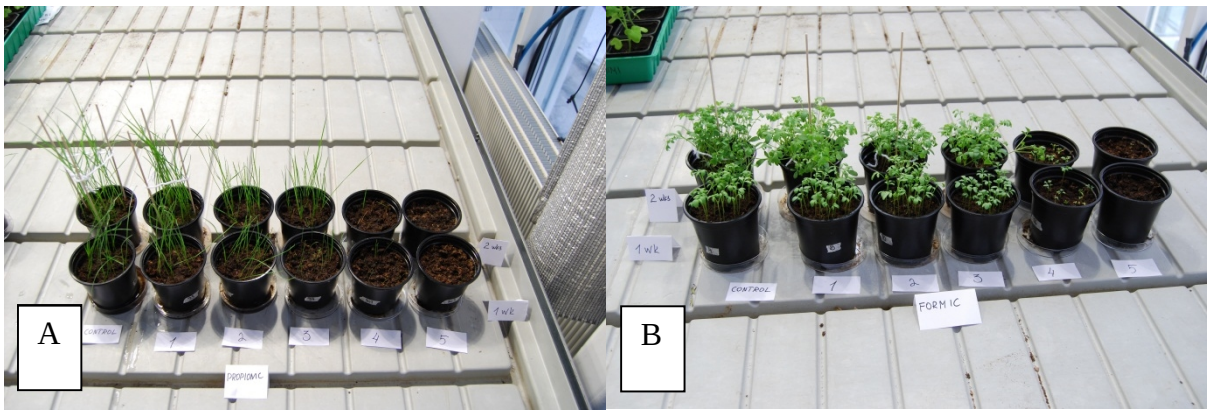

Figure 5. Plant growth at 7d (first row) and 14d (second row) in subchronic assay. A) Cress with formic acid. B) Ryegrass with propionic acid.

The dose spesific effects can also be clearly seen on the photographs taken during the subchronic bioassays that are presented in figure 5. The increasing inhibition in plant growth is evident as concentration grows from left to right.

The germination of cress and ryegrass seeds in substrate with formic acid is represented in figure 6. A slight hormesis phenomena can be observed by cress germination in all three LWCA. The phenomena is more varied on ryegrass. In figure 6, the effect of plant

mortality during the experiment can be seen on cress seeds as lowering of the amount of germinated seeds between exposure time of 7 and 21 days in concentrations of 5 and 40 mmol/kg in formic, 40 mmol/kg on acetic and 2 and 10 mmol/kg on propionic acid.

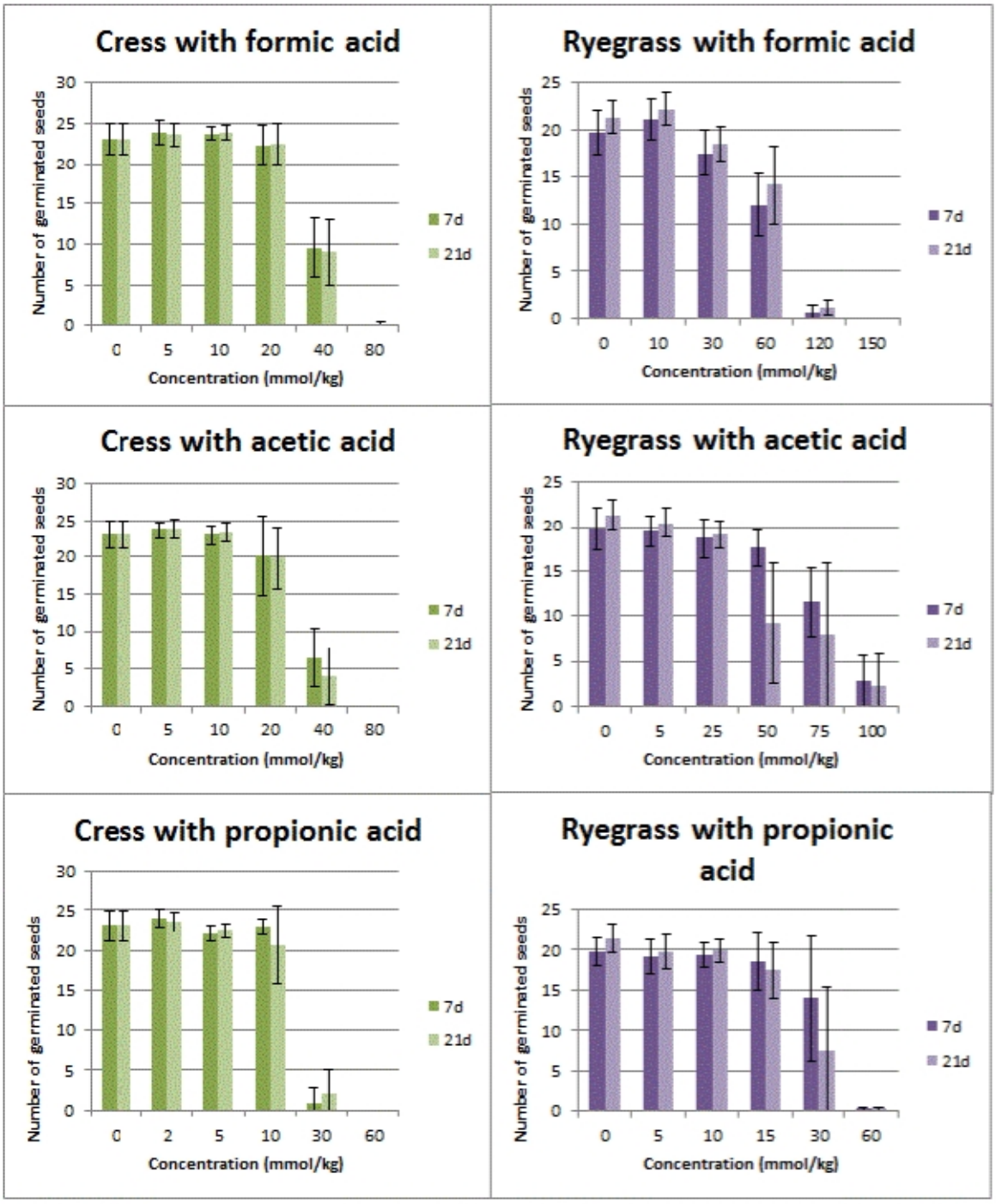

Figure 6. The germination of cress and ryegrass seeds (total 25 seeds sewn) in substrates with added formic, acetic and propionic acids after exposure time of 7 and 21 days. Bars represent standard deviation.

3.2.2 Binary mixtures

The germination of cress and ryegrass seeds after exposure time of 7 and 21 days during subchronic growth assays are shown in figure 8. Similar to figure 6, mortality during assay is evident, especially in mixtures containing acetic and propionic acid. Noteworthy is also the variance in 1 TU concentration, which is larger than in any other mixture for both cress and ryegrass.

The biomass of cress and ryegrass in binary mixtures followed similar trend as shoot length (figure 7). The mixtures containing propionic acid were more toxic than mixture containing only formic and acetic acid, once again evidencing increased toxicity as the carbon chain lengthens.

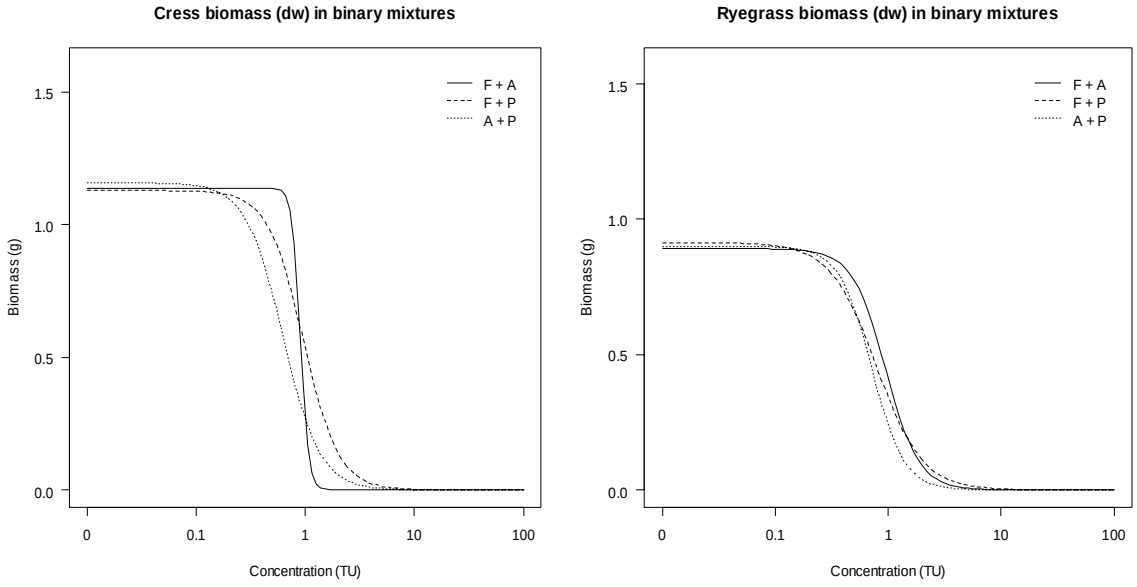

Figure 7. Dose response curves for cress and ryegrass biomass (dry weight) grown in substrates with the binary acid mixtures obtained using log-logistic model (LL.3). F = formic acid, A = acetic acid, P = propionic acid.

EC_{50} values of the mixtures derived from the toxic units are listed in table 10. For formic acid the EC_{50} mixture values ranged from 31.4 to 72.7 mmol/kg for germination and from 18.5 to 47.4 mmol/kg for biomass. The corresponding ranges for acetic acid were 20.9 – 53.4 mmol/kg and 10.5 – 39.8 mmol/kg, and for propionic acid 9.8 – 23.3 mmol/kg and 6.9 – 15.0 mmol/kg, respectively.

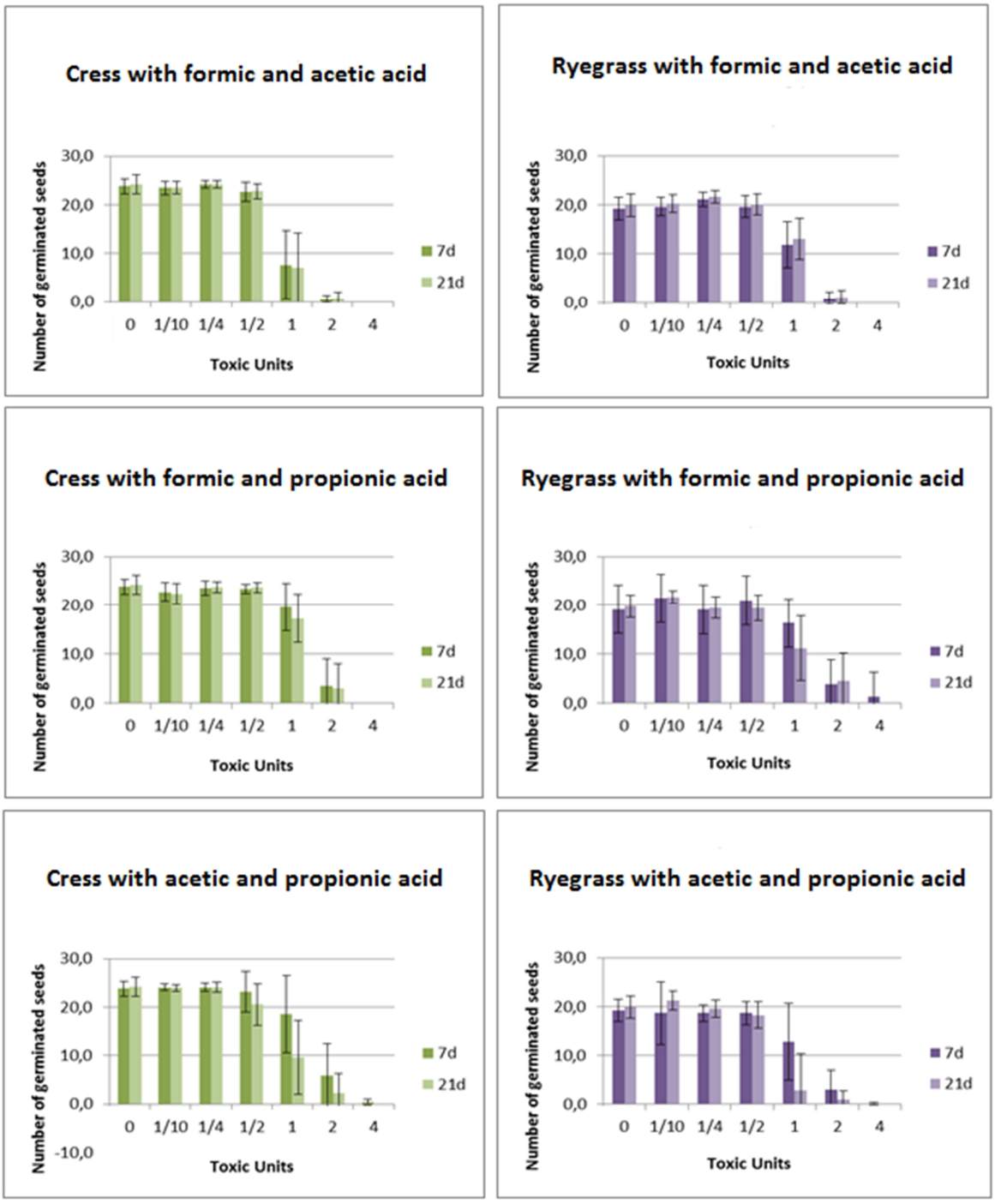

Figure 8. The germination of cress and ryegrass seeds (total 25 seeds sewn) in substrates with added binary acid mixtures containing formic, acetic and propionic acid after exposure time of 7 and 21 days. Bars represent standard deviation.

Table 10. Subchronic EC_{50} values for phytotoxicity of LWCA. Endpoints germination after exposure time of 7d and 21d and biomass (dry weight) of cress and ryegrass in substrates with the added acid pairs containing formic, acetic and propionic acid. EC_{10} and EC_{90} in parenthesis. F = formic acid, A = acetic acid, P = propionic acid.

Acid / Mixture	**Germination 7d**			**Germination 21d**			**Biomass (dw)**		
	F + A	**F + P**	**A + P**	**F + A**	**F + P**	**A + P**	**F + A**	**F + P**	**A + P**
	Cress - EC_{50} (mmol/kg)								
Formic	31.4	50.3	-	30.7	45.7	-	27.7	18.5	-
	(20.2 - 48.7)	(32.5 - 77.8)	-	(19.3 - 48.7)	(28.1 - 74.1)	-	(17.5 - 44.0)	(15.1 - 78.5)	-
Acetic	20.9	-	34.2	20.5	-	21.3	34.5	-	10.5
	(13.5 - 32.5)	-	(18.6 - 63.0)	(12.9 - 32.5)	-	(10.6 - 42.9)	(11.7 - 29.3)	-	(6.3 - 35.6)
Propionic	-	15.4	15.7	-	13.9	9.8	-	15.0	6.90
	-	(9.9 - 23.8)	(8.5 - 28.9)	-	(8.6 - 22.7)	(4.8 - 19.7)	-	(4.6 - 24.0)	(2.9 - 16.3)
	Ryegrass - EC_{50} (mmol/kg)								
Formic	54.3	72.7	-	63.6	57.7	-	47.4	39.4	-
	(35.4 - 83.2)	(37.9 - 139)	-	(41.1 - 98.3)	(27.4 - 121)	-	(22.4 - 100)	(14.0 - 111)	-
Acetic	45.6	-	51.9	53.4	-	30.5	39.8	-	30.1
	(29.7 - 69.9)	-	(28.8 - 93.6)	(34.5 - 82.6)	-	(18.0 - 51.6)	(18.9 - 84.1)	-	(14.5 - 62.2)
Propionic	-	23.3	19.8	-	18.4	11.6	-	12.6	11.5
	-	(12.1 - 44.6)	(11.0 - 35.7)	-	(8.8 - 38.8)	(6.9 - 19.7)	-	(4.5 - 35.4)	(5.5 - 23.7)

To evaluate the presumed additive toxic action of the acids in binary mixtures, the additive indices (*AI*) together with their ranges were calculated and are summarized in table 11. As described earlier, the mixture effect is determined rather by the range (95 % confidence interval) than the *AI* value itself. If this range overlaps zero, effect is judged to be additive. Ranges in negative values point to less than additive effect while range in positive values to greater than additive effect.

Table 11. Sums of toxic action (S) and additive indices (AI) of trinary mixture for cress and ryegrass biomass (dry weight) and germination. Range in parenthesis. F = formic acid, A = acetic acid, P = propionic acid. Type of toxic outcome: Ad = additive, GtAd = greater than additive, LsAd = less than additive.

Mixture	Factor	Germinadion 7d	Germination 21d	Biomass
			Cress	
F + A	**S**	1.48	1.55	1.55
	AI	-0.48	-0.55	-0.55
	95% conf in. of AI	(-0.92 - 0.94)	(0.92 - 0.93)	(-1.02 - -0.19)
	Toxicity	Ad	Ad	LsAd
F + P	**S**	1.93	2.07	1.89
	AI	-0.93	-1.07	-0.89
	95% conf in. of AI	(NA - 0.94)	(0.85 - 0.89)	(-1.81 - -0.26)
	Toxicity	Ad	GtAd	LsAd
A + P	**S**	1.71	1.32	1.23
	AI	-0.7	-0.32	-0.23
	95% conf in. of AI	(NA - 0.94)	(0.89 - 0.92)	(-1.04 - -0.14)
	Toxicity	Ad	Ad	LsAd
			Ryegrass	
F + A	**S**	2.16	1.97	1.79
	AI	-1.16	-0.97	-0.79
	95% conf in. of AI	(0.94 - 0.95)	(0.91 - 0.93)	(-1.56 - -0.23)
	Toxicity	GtAd	GtAd	LsAd
F + P	**S**	2.81	1.59	1.59
	AI	-1.81	-0.59	-0.59
	95% conf in. of AI	(0.90 - 0.92)	(0.90 - 0.93)	(-1.39 - -0.02)
	Toxicity	GtAd	GtAd	LsAd
A + P	**S**	1.25	1.05	1.37
	AI	-0.25	-0.05	-0.37
	95% conf in. of AI	(0.91 - 0.93)	(0.92 - 0.94)	(-1.09 - 0.47)
	Toxicity	GtAd	GtAd	Ad

NA = value could not be generated

The calculation of *AI* range for germination values proved difficult, perhaps due to mortality of the seedlings, so the additivity was judged by available values on these cases. The *S* and *AI* values for binary mixtures suggested additive or greater than additive toxicity for germination on both species. However, biomass as an endpoint suggested less than additive toxicity, mainly.

3.2.3 Trinary mixtures

The dose response curves for cress and ryegrass biomass in trinary mixture are presented in figure 9, and support the observations for binary mixtures where cress was more sensitive to LWCA than ryegrass. However, as cress and ryegrass produce different biomasses in control environment, relationships are not comparable.

Unlike germination in individual acids (figure 6) and binary mixture (figure 8), germination in trinary mixture (figure 10) did not exhibit notable mortality during the assay but showed that amount of the germinated seeds was higher after exposure time of 21 than of 7 days.

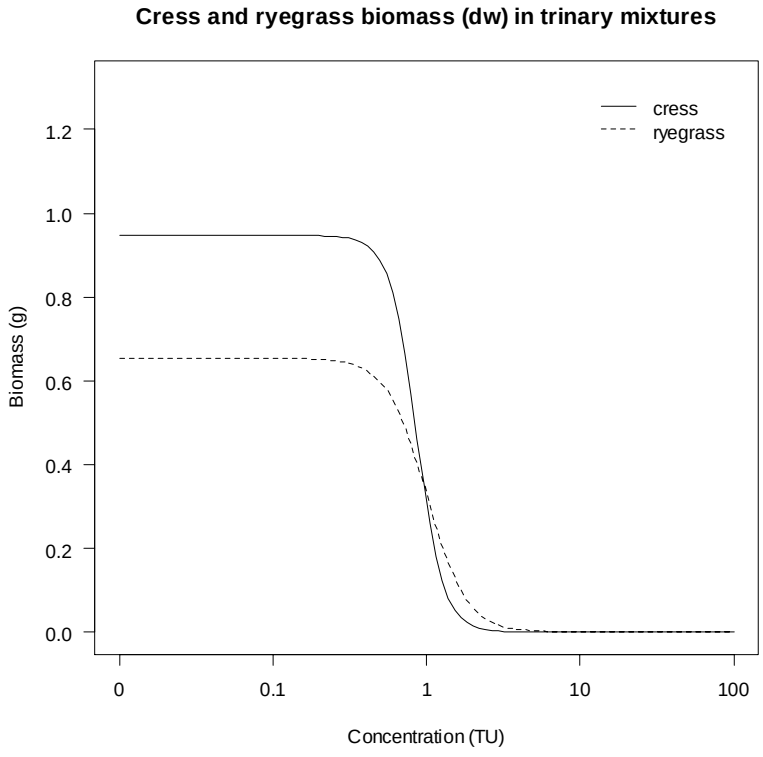

Figure 9. Dose response curves for cress and ryegrass biomass (dry weight) in the substrates with the added trinary acid mixtures. The curves were obtained using log logistic model (LL.3).

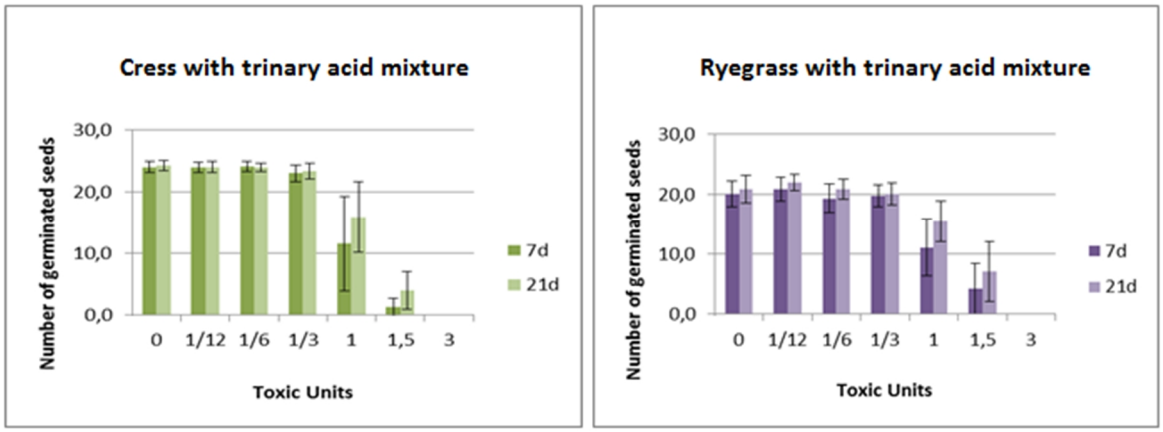

Figure 10. The germination of cress and ryegrass seeds in the substrates with added acid mixture.

It can be observed that biomass is more sensitive to toxicity of trinary mixture than germination (table 12). In both species for both endpoints (germination and biomass), toxicity of the acid increased as the carbon chain lengthens.

Table 12. Individual EC_{50} values for biomass (dry weight) and germination (7d and 21d) of cress and ryegrassa in the substrates with the added acid mixture of formic, acetic and propionic acid. EC_{10} and EC_{90} in parenthesis. F = formic acid, A = acetic acid, P = propionic acid.

Acid / Mixture	**EC_{50} (mmol/kg)**					
	Cress			**Ryegrass**		
	Germination 7d	**Germination 21d**	**Biomass (dw)**	**Germination 7d**	**Germination 21d**	**Biomass (dw)**
	F + A + P	**F + A + P**	**F + A + P**	**F + A + P**	**F + A + P**	**F + A + P**
Formic	35.7 (26.1 - 48.7)	40.6 (27.8 - 59.2)	31.0 (19.9 - 48.3)	53.5 (32.6 - 87.8)	56.0 (36.3 - 86.5)	50.1 (26.0 - 96.3)
Acetic	23.8 (17.4 - 32.5)	27.0 (18.5 - 39.5)	20.6 (13.2 - 32.2)	44.9 (27.4 - 73.7)	47.1 (30.5 - 72.7)	42.1 (21.9 - 80.9)
Propionic	10.9 (8.0 - 14.9)	12.4 (8.50 - 18.5)	9.50 (6.10 - 14.7)	17.1 (10.4 - 28.1)	17.9 (11.6 - 27.7)	16.0 (8.30 - 30.8)

The *S* and *AI* values for trinary mixtures are represented in table 13. The values all suggest less than additive toxicity for both cress and ryegrass in germination and biomass. Like in binary mixtures, calculation of *AI* range for germination values was difficult so the additivity was judged by *AI* alone.

Table 13. Sums of toxic action (S) and additive indices (AI) of trinary mixture for cress and ryegrass biomass (dry weigth) and germination. Range in parenthesis. F = formic acid, A = acetic acid, P = propionic acid. Type of toxic outcome: Ad = additive, GtAd = greater than additive, LsAd = less than additive.

Species	Response	S	AI	95% conf in. of AI	Toxicity
Cress	**Germination 7d**	2.12	-1.12	(0.92 - 0.91)	LsAd
	Germination 21d	2.78	-1.78	(-0.83 - 0.84)	LsAd
	Biomass	2.56	-1.56	(-1.36 - -1.69)	LsAd
Rye	**Germination 7d**	1.91	-0.91	(0.9 - 0.9)	LsAd
	Germination 21d	2.46	-1.46	(0.87 - 0.87)	LsAd
	Biomass	2.92	-1.92	(-1.83 - -1.94)	LsAd

3.2.4 pH of subchronic assay soils

The mean pH-values measured from test soils before and after the subchronic assays are summarized in appendix 2. Unfortunately the samples from the ending, after exposure time of 21 days, of first replica for trinary mixture were lost and thus the mean for trinary mixture was calculated using only two replicates.

The pH measurement data contained some irregularities which are listed on table 14. Usually pH-value of the soils was from 5 to 7 at the end of the assays but irregularities were found in some occasions.

Table 14. pH values measured from substrate samples obtained in the beginning of and after 21 days of exposure time in subchronic assays.

Mixture	Species	Concentration (TU)	pH at 0d	pH at 21d
F + P	Ryegrass	4	2.2	3.2
F + P	Ryegrass	4	2.2	3.5
A + P	Ryegrass	4	2.9	3.5
F + P	Cress	4	2.7	2.4
F + P	Cress	4	4.1	3.8
F + A + P	Ryegrass	3	2.5	3
F + A + P	Ryegrass	3	2.5	3.8

3.3 Summary

EC_{50} values obtained in either individual or mixture assays were mostly in the same range (table 15). Although most EC values conformed to assumed trend of increasing toxicity as the carbon chain lengthens, this was not always the case. Ryegrass was found to be less sensitive in subchronic assays, but this effect was not visible in short-term assays. Germination as an endpoint was less sensitive in both species than plant growth in either seedling length or biomass.

Table 15. The range of EC_{50} values obtained in individual, binary and trinary mixture assay settings.

Short-term	**Cress**			**Ryegrass**		
	Germination	**Shoot length**		**Germination**	**Shoot length**	
Formic	2.4 – 2.9	0.6 - 1.8		1.6 - 5.2	0.8 - 1.7	
Acetic	2.0 – 3.2	0.5 - 1.5		2.3 - 7.1	1.1 - 3.3	
Propionic	1.5 – 3.3	0.4 - 1.0		1.4 - 2.9	0.7 - 1.3	
Subchronic	**Germination (7d)**	**Germination (21d)**	**Biomass (dw)**	**Germination (7d)**	**Germination (21d)**	**Biomass (dw)**
Formic	31.4 - 50.3	30.7 - 45.7	18.5 - 35.6	54.3 - 72.7	56.0 - 67.8	39.4 - 50.1
Acetic	20.9 - 34.2	20.5 - 28.8	10.5 - 34.5	44.9 - 78.4	30.5 - 53.4	30.1 - 47.4
Propionic	10.9 - 25.3	9.80 - 16.8	6.90 - 15.0	17.1 - 33.5	11.6 - 25.0	11.5 - 16.0

4. DISCUSSION

4.1 Problematics of dose-response modeling

The modeling of the results proved to be somewhat problematic in some cases, especially with the germination of cress in short-term bioassays. Modeling of these results produced large standard errors, large EC_{10} to EC_{90} range and extreme confidence intervals that in two cases were negative in value. The difficulty in modeling was evident also in the dose response relationship (figure 1) where the dose response curves for acetic and propionic acid are not very steep and it becomes more probable that the model cannot fit the data properly. The question rises whether this particular model was the best choice for this bioassay including germination responses. Recent studies suggest that using a model not incorporating low-level stimulation effects can cause unreliability to the dose response modeling (Schabenberger & Birch 2001, Belz & Piepho 2012). Models based on poorly fitted data caused difficulties in calculating *S* and *AI* values creating uncertainty in determining the nature of mixture toxicity.

While some uncertainty can rise from the model, some of the problems might lie in the data itself. As the experiments started with the individual acid assays, it was probable that results of the assays contain errors in judging whether the seeds in the strongest concentration were germinated or not. As a cress seed germinates, it typically generates a mucous coating around the seed as shown in figure 11 and the seed itself swells during imbibition. This is followed by cracking of the seed coat and partial unfurling of the radicle beneath the coat as the radicle begins to grow (Bewley & Black 1994).

However, at the stage where imbibition has occurred and radicle uncurled from the coat it was sometimes difficult to judge whether the seed had germinated a little or it was the case of swelling and unfurling of the radicle. In later assays, the requirement to seed to count as germinated was decided at the radicle being elongated from the seed coat at least one mm. After initially modeling the cress germination results, it became clear that the model was still not fitting the data to the model optimally. In the last stage the prerequisite of cress seed being germinated was assigned as two mm.

Figure 11. Mucous coating surrounding germinating cress seed.

4.2 Individual phytotoxicity of low weight carboxylic acids

Unfortunately there is not a lot of literature available with the EC_{50} values for LWCA so the comparisons have to rely on the work of Himanen et al. (2012) which presents EC_{50} values for cress and ryegrass obtained in similar test conditions (table 16). In addition to these values, only one other source citing an EC_{50} value of 7,8 mmol/kg for germination of cress in acetic acid (DeVleeschauwer et al. 1982). Owerall conclusion is that the EC_{50} values obtained in this study are very much in the same range.

Table 16. Acute EC_{50} (EC_{10}-EC_{90}) values for cress and ryegrass germination and early growth (Himanen et al. 2012).

Short-term	**Cress**		**Ryegrass**	
	Germination	**Shoot length**	**Germination**	**Shoot length**
Formic acid	3.6 (2.5 - 4.5)	2.3 (2.1 - 2.5)	3.7 (2.3 - 5.1)	1.8 (0.9 - 2.7)
Acetic acid	4.2 (2.5 - 5.8)	2.0 (1.3 - 2.6)	6.4 (3.8 - 9.0)	2.7 (0.9 - 5.5)
Propionic acid	3.8 (2.2 - 5.2)	1.3 (0.5 - 2.2)	2.2 (1.3 - 3.0)	0.9 (0.4 - 1.4)
Subchronic	**Germination**	**Biomass (dw)**	**Germination**	**Biomass (dw)**
Formic acid	45.9 (17.1 - 86.2)	39.5 (13.6 - 77.7)	127 (58.6 - 201)	93.2 (24.1 - 221)
Acetic acid	42.8 (23.5 - 62.8)	14.9 (2.20 - 50.2)	90.4 (55.1 - 124)	31.2 (2.80 - 45.9)
Propionic acid	41 (19.3 - 66.3)	11.2 (1.60 - 38.2)	56.6 (18.0 - 117)	16.8 (1.60 - 74.1)

All in all, the results showed that cress is more sensitive for LWCA toxicity and plant development as growth was more sensitive than germination as a response for LWCA toxicity. Although Schuman & McGalla (1975) suggested that toxicity values of LWCA are different for germination and plant development, results obtained in this study showed that the toxicity values for these responses did not differ greatly.

EC_{50} values in short-term assays for shoot length of cress were 1.8, 1.5 and 1.0 mmol/l in formic, acetic and propionic acid, respectively. These results are in conforming to previous observations by Himanen et al. (2012) on growing toxicity as the LWCA chain lengthens. It is also apparent that cress was more sensitive than ryegrass. However, the EC_{50} values for cress germination and ryegrass shoot length and germination vary from this perception. EC_{50} values obtained for germination for cress were 2.9, 3.2 and 3.3 mmol/l, and for ryegrass 5.16, 7.12 and 2.92 mmol/l for formic, acetic and propionic acid, respectively. EC_{50} values for shoot length of ryegrass 1.7, 3.3 and 1.3 mmol/l for formic, acetic and propionic acid, respectively (Himanen et al. 2012).

Table 17. Extrapolated EC_{50} values for LWCA with several plant species and endpoints. Adapted from Himanen et al. (2012).

Acid	Endpoint	Extrapolated EC50 value (mmol/l)	Species	Reference
Formic	Germination	4.4	Lettuce	Reynolds (1975)
	Seedling growth	0.24	Wheat	Prill et al. (1949)
Acetic	Germination	0.1	Cucumber	Shiralipour & McConnell (1997)
	Germination	3.5	Lettuce	Reynolds (1975)
	Germination	7.8[a]	Cress	DeVleeschauwer et al. (1982)
	Germination	15	Barley	Lynch (1977)
	Seedling growth	0.018	Cucumber	Shiralipour & McConnell (1997)
	Seedling growth	1.03	Wheat	Prill et al. (1949)
	Seedling growth	8.2	Lettuce	Manois et al. (1987)
	Seedling growth	10	Barley	Lynch (1977)
Propionic	Germination	1.7	Lettuce	Reynolds (1975)
	Germination	5	Barley	Lynch (1977)
	Root elongation	0.05	Wheat	Prill et al. (1949)

[a] Concentration expressed as mmol/kg

These results can also be compared to extrapolated EC values obtained by Himanen et al. (2012) by reweaving data from earlier studies that did not report actual EC values (table 17). These studies offered information on the LWCA phytotoxicity in different species, such as lettuce (*Lactuca sativa*), wheat (*Triticum sp.),* cucumber (*Cucumis sativus*), cress (*L. sativum*) and barley (*Hordeum vulgare*). The resulting EC values vary in a magnitude of hundred between endpoints and assayed species.

Results of the subchronic assays showed similar increase in toxicity with increase in carbon chain length. Germination of cress and ryegrass seeds, after exposure time of 7d and 21d generally followed this trend, with the sole exception of 7d germination of ryegrass where EC_{50} value for acetic acid was higher than for formic acid, 78.4 and 64.4 mmol/kg, respectively. For cress biomass, the EC_{50} values were 35.6, 24.2 and 11.4 mmol/kg for formic, acetic and propionic acid, respectively. Corresponding values for ryegrass biomass were 50.1, 47.4 and 15.9 mmol/kg for formic, acetic and propionic acid, respectively. These values differ from ones reported by Himanen et al. from 1.8 to 46 %. While cress biomass with propionic acid was only slightly higher in this study, the ryegrass biomass with formic acid was nearly halved.

The study of Himanen et al. (2012) supports the observation of general increase in toxicity as carbon chain lengthens but shows that while this might be a general trend, the difference in the toxicity ranking is not always straightforward. Himanen et al. (2012) offers the order of toxicity ranking for the LWCA as described in table 18. The order of the LWCA isn't always F < A < P, similar to the general trend. However, the order of F < A < P applied to most cases in this study, for example shoot length of cress in short-term bioassay. Only few cases dispute this order and instead imply toxicity rank of A < F < P. These cases are found in ryegrass short-term bioassay and also in early germination of the subchronic bioassay.

Table 18. Toxicity order of LWCA in short-term and subchronic (21d) bioassays. F = formic, A = acetic and P = propionic acid (Himanen et al. 2012).

Response	**Cress**	**Ryegrass**
Short-term seed germination	A < P < F	A < F < P
Short-term plant development	F = A < P	A < F < P
Subchronic germination	F < A = P	F < A < P
Subchronic plant development	F < A < P	F < A < P

4.3 Mixture phytotoxicity of low weight carboxylic acids

As can be seen in table 15, the EC_{50} values obtained in different test settings result in varying values but the ranges are not considerably large. This fact suggests that mixture toxicity of the three LWCA is somewhat less than additive toxicity. However, while compiling these ranges to tabular format, an interesting observation arose from the subchronic assays with ryegrass. After exposure of both 7 and 21 days, results showed greater than additive action for every acid, while for biomass the toxic action was lesser than additive toxicity again for every acid. This could support suggestion that germination is more sensitive endpoint and that greater effort is required to overcome toxicity in germination phase than actual seedling growth phase. It is also noteworthy that EC values obtained in the individual acid assays are slightly higher than those obtained from mixture assays.

4.4 Withering and plant mortality in subchronic assays

As the germinated seeds were counted on two occasions – after exposure time of 7 days and at the end of the assay, after exposure time of 21 days – it can be assumed that if the acid slowed down but not completely inhibited the germination process the amount of germinated seeds after 21 days would be higher than after 7 days. This effect stems from the method of determining the germination at the end of the experiment when all the plants were counted and cutted from the pot for weighing. At this time it is possible that some of the plants have been miscounted, especially in the case of ryegrass, since closely growing shoots were difficult to separate from each other but it was not the whole reason. Nevertheless, while also withered stems were counted and gathered at the end of the experiments it is curious that their number was still visibly less than the amount of stems counted after 7 days of exposure. It is also possible that exposure to LWCA in the trinary mixture has slowed down the germination process. Variability in germination of control was smaller than concentrations of 1 and 1.5 TU. As the concentration of mixtures increased, more time was needed for the seeds to overcome the inhibiting effect of LWCA.

As can be seen from the results of temperature monitoring, during some of the assays, temperature soared very high in the experimental greenhouse making irrigation challenging

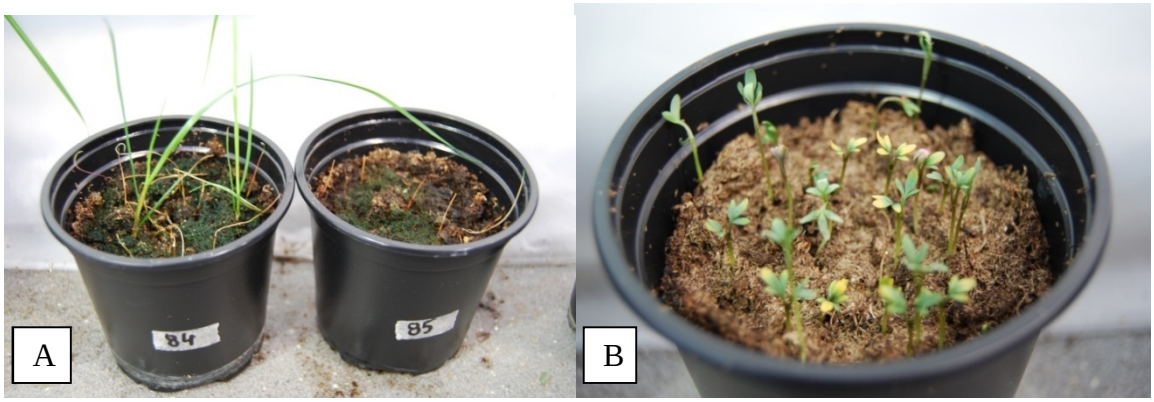

Figure 12. Plant damage during 21-day subchronic bioassay. A) Ryegrass with acetic acid, at concentrations 50 and 75 mmol/kg. B) Cress with propionic acid, concentration 10 mmol/kg.

(Appendix 2). It is possible that toxic effect of the carboxylic acid was combined with some heat stress, causing mortality in the test plants and several cases of withering and mortality was observed during the experiments. As the effect can be seen only in treatments and not in the control it can be assumed that the toxicity of the carboxylic acids had some part in the phenomenon. Also when the last assays with mixtures were carried out the heat as an additional stressor was eliminated, resulting in no observed withering effect.The damage affecting the plants were also seen during the assay and are depicted in figure 12. For cress the usual damage was withering and yellowing of leaves. For ryegrass, typical damage was withering and mortality of young stalks. These effects were mentioned also by Himanen et al. (2012).

4.5 Role and adjustment of soil pH

By reviewing the data given in appendices 3, 4 and 5, it is evident, that pH of the tested soils rose during the subchronic assays. This can result from a variety of reasons, including leaching of the acids from the substrate with the irrigation water, their vaporization and microbial degradation in the substrate. However, it is interesting to note that in some cases the pH in the strongest concentration remained very low to the end of the assay as other soil pH-values rose to a steady level around from 5 to 7. Even more interesting is that all these low pH-values were observed in mixtures containing propionic acid. These occurrences are found in table 14.

This phenomenon suggests that microbial activity in these concentrations was inhibited and biodegradation of LWCA was reduced. According to the observations, this microbe

inhibiting effects are most likely to occur in mixtures containing propionic acid, suggesting once again increased toxicity as carbon chain lengthens. Propionic acid has been recognized as a microbial growth inhibiting agent (Cherrington et al. 1991). This suggested that although acidity itself is not a major factor in LWCA toxic action, soil pH could be relevant in LWCA reactions in the soil and thus have indirect effect on LWCA phytotoxicity.

4.6. Further research

Further study interests could include modeling the data sets obtained in this study with other means, possibly with a model incorporating stimulating effects and different model family. One way to further assess the data would be to calculate the concentrations of dissociated adics in the assays and model the results with these concentrations to see whether they differ from original results.

Investigating the mode of action with lipophility and lengthening carbon chain could also prove interesting and help in further research with the question of acidity and the dissociation of the acids in soil. If suitable buffering method could be found, the effects of the acid chain alone would be possible to determine.

Finding the mode of action would help in preventing the land masses with phytotoxic LWCA content from hindering growth, making the utilization of these masses easier. As it is, this study was able to provide some applicable EC values to use in evaluating whether any given land mass, either composted or digested, is usable in horticulture.

5. CONCLUSIONS

According to the data obtained in this research, is not possible to judge with certainty the nature of LWCA mixture toxicity, but it is possible to give an estimation. Reviewing of the sums of toxic action (*S)* and additive indices (*AI)* and their ranges showed values ranging from greater than additive to less than additive toxicity, but most of the values indicated less than additive toxicity. Therefore is safe to assume that according to this data, the toxicity of the LWCA is less than additive. Considering the similarity of the LWCA structure and functional group, the larger would suggest similar mode of action. This fact would also support the estimation that the toxicity is less than or additive. The EC values obtained in this research were in similar vein to previous studies, and mixture EC values were similar to individual EC values. Soil pH data suggested that although acidity itself is not a major factor in LWCA toxic action, soil pH could be relevant in LWCA reactions in the soil and thus have indirect effect on LWCA phytotoxicity.

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APPENDICES

APPENDIX 1. EC_{10}, EC_{50} and EC_{90} with standard error values for pure formic (F), acetic (A) and propionic (P) acids and their mixtures obtained in short-term assays for cress and ryegrass. 5 % confidence intervals presented for EC_{50} values. The endpoints were seed germination and shoot length. EC values for the pure acids are expressed as mmol/l, and for binary and trinary mixture values in toxic units (TU).

Species	Acid	Response	EC_{10}	EC_{50}	EC_{90}	EC_{50} 5 % conf. int.
Cress	F	Shoot length	1,04 ± 0,08	1,84 ± 0,06	3,27 ± 0,23	1,72 1,96
Cress	A	Shoot length	0,75 ± 0,06	1,54 ± 0,06	3,17 ± 0,31	1,42 1,67
Cress	P	Shoot length	0,37 ± 0,04	1,02 ± 0,05	2,82 ± 0,26	0,93 1,12
Cress	F + A	Shoot length	*0,31 ± 0,03*	*0,73 ± 0,03*	*1,73 ± 0,14*	*0,67 0,08*
Cress	F + P	Shoot length	*0,75 ± 0,06*	*1,54 ± 0,06*	*3,17 ± 0,31*	*1,42 1,67*
Cress	A + P	Shoot length	*0,28 ± 0,02*	*0,56 ± 0,02*	*1,12 ± 0,10*	*0,52 0,60*
Cress	F + A + P	Shoot length	*0,18 ± 0,01*	*0,40 ± 0,02*	*0,87 ± 0,09*	*0,36 0,43*
Cress	F	Germination	2,19 ± 0,13	2,94 ± 0,08	3,96 ± 0,10	2,79 3,10
Cress	A	Germination	2,26 ± 0,14	3,26 ± 0,09	4,68 ± 0,49	3,08 3,43
Cress	P	Germination	1,83 ± 0,13	3,16 ± 0,21	5,45 ± 1,04	2,74 3,59
Cress	F + A	Germination	*0,88 ± 0,02*	*1,24 ± 0,03*	*1,76 ± 0,10*	*1,18 1,31*
Cress	F + P	Germination	*0,81 ± 0,02*	*1,20 ± 0,02*	*1,77 ± 0,08*	*1,16 1,24*
Cress	A + P	Germination	*0,88 ± 0,02*	*1,19 ± 0,03*	*1,62 ± 0,11*	*1,13 1,26*
Cress	F + A + P	Germination	*0,76 ± 0,05*	*0,98 ± 0,01*	*1,26 ± 0,07*	*0,96 1,00*
Ryegrass	F	Shoot length	0,55 ± 0,17	1,69 ± 0,21	5,15 ± 1,24	1,26 2,11
Ryegrass	A	Shoot length	1,76 ± 0,47	3,30 ± 0,28	6,21 ± 1,45	2,74 3,87
Ryegrass	P	Shoot length	0,68 ± 0,10	1,30 ± 0,08	2,47 ± 0,36	1,13 1,46
Ryegrass	F + A	Shoot length	*0,56 ± 0,09*	*1,05 ± 0,07*	*1,97 ± 0,30*	*0,91 1,19*
Ryegrass	F + P	Shoot length	*0,38 ± 0,04*	*0,70 ± 0,05*	*1,28 ± 0,14*	*0,61 0,79*
Ryegrass	A + P	Shoot length	*0,45 ± 0,06*	*0,75 ± 0,05*	*1,25 ± 0,14*	*0,65 0,86*
Ryegrass	F + A + P	Shoot length	*0,31 ± 0,06*	*0,52 ± 0,09*	*0,86 ± 0,26*	*0,33 0,70*
Ryegrass	F	Germination	3,14 ± 0,25	5,16 ± 0,28	8,50 ± 0,67	4,61 5,72
Ryegrass	A	Germination	4,90 ± 1,27	7,12 ± 0,99	10,36 ± 0,55	5,19 9,06
Ryegrass	P	Germination	2,19 ± 0,25	2,92 ± 0,61	3,89 ± 2,03	1,71 4,12
Ryegrass	F + A	Germination	*1,28 ± 0,16*	*1,98 ± 0,09*	*3,05 ± 0,22*	*1,81 2,15*
Ryegrass	F + P	Germination	*0,95 ± 0,05*	*1,30 ± 0,05*	*1,77 ± 0,08*	*1,20 1,40*
Ryegrass	A + P	Germination	*0,87 ± 0,07*	*1,44 ± 0,06*	*2,38 ± 0,12*	*1,32 1,56*
Ryegrass	F + A + P	Germination	*0,80 ± 0,03*	*1,04 ± 0,02*	*1,36 ± 0,05*	*1,00 1,08*

APPENDIX 2. EC_{10}, EC_{50} and EC_{90} with standard error values for pure formic (F), acetic (A) and propionic (P) acids and their mixtures obtained in subacute assays for cress. 5 % confidence intervals presented for EC_{50} values. The endpoints were seed germination after 7 and 21 days and biomass (dw). EC values for the pure acids are expressed as mmol/kg, and for binary and trinary mixture values in toxic units (TU).

Species	Acid	Response	EC_{10}	EC_{50}	EC_{90}	EC_{50} 5 % conf. int.
Cress	F	Germination (7d)	29,28 ± 6,36	38,22 ± 1,65	49,88 ± 6,91	34,98 41,45
Cress	A	Germination (7d)	19,52 ± 1,27	31,58 ± 1,06	51,09 ± 2,21	29,50 33,66
Cress	P	Germination (7d)	22,49 ± 115,97	25,28 ± 77,43	28,42 ± 27,54	-126,47 117,04
Cress	F + A	Germination (7d)	*0,56 ± 0,03*	*0,87 ± 0,02*	*1,35 ± 0,05*	*0,82 0,92*
Cress	F + P	Germination (7d)	*0,90 ± 0,05*	*1,40 ± 0,04*	*2,16 ± 0,09*	*1,32 1,48*
Cress	A + P	Germination (7d)	*0,77 ± 0,05*	*1,42 ± 0,05*	*2,62 ± 0,13*	*1,33 1,52*
Cress	F + A + P	Germination (7d)	*0,73 ± 0,04*	*0,99 ± 0,02*	*1,35 ± 0,04*	*0,95 1,03*
Cress	F	Germination (21d)	25,79 ± 2,01	36,88 ± 1,06	52,74 ± 2,46	34,79 38,97
Cress	A	Germination (21d)	18,60 ± 0,99	28,76 ± 0,90	44,48 ± 1,78	27,00 30,52
Cress	P	Germination (21d)	9,95 ± 0,78	16,78 ± 0,85	28,30 ± 1,53	15,11 18,45
Cress	F + A	Germination (21d)	*0,54 ± 0,03*	*0,85 ± 0,02*	*1,35 ± 0,05*	*0,81 0,90*
Cress	F + P	Germination (21d)	*0,78 ± 0,04*	*1,27 ± 0,04*	*2,06 ± 0,09*	*1,19 1,34*
Cress	A + P	Germination (21d)	*0,44 ± 0,03*	*0,89 ± 0,03*	*1,79 ± 0,09*	*0,83 0,95*
Cress	F + A + P	Germination (21d)	*0,77 ± 0,04*	*1,13 ± 0,02*	*1,64 ± 0,06*	*1,08 1,17*
Cress	F	Biomass (dw)	25,12 ± 6,94	35,64 ± 2,88	50,57 ± 7,35	29,87 41,41
Cress	A	Biomass (dw)	13,85 ± 1,83	24,15 ± 1,61	42,11 ± 6,09	20,93 27,37
Cress	P	Biomass (dw)	4,77 ± 1,10	11,40 ± 1,21	27,24 ± 6,12	8,99 13,81
Cress	F + A	Biomass (dw)	*0,49 ± 0,06*	*0,77 ± 0,04*	*1,22 ± 0,10*	*0,68 0,86*
Cress	F + P	Biomass (dw)	*0,42 ± 0,10*	*0,96 ± 0,10*	*2,18 ± 0,44*	*0,76 1,16*
Cress	A + P	Biomass (dw)	*0,26 ± 0,06*	*0,63 ± 0,06*	*1,48 ± 0,27*	*0,68 0,86*
Cress	F + A + P	Biomass (dw)	*0,55 ± 0,19*	*0,86 ± 0,10*	*1,34 ± 0,21*	*0,66 1,06*

APPENDIX 3. EC_{10}, EC_{50} and EC_{90} with standard error values for pure formic (F), acetic (A) and propionic (P) acids and their mixtures obtained in subacute assays for ryegrass. 5 % confidence intervals presented for EC_{50} values. The endpoints were seed germination after 7 and 21 days and biomass (dw). EC values for the pure acids are expressed as mmol/kg, and for binary and trinary mixture values in toxic units (TU).

Species	Acid	Response	EC_{10}	EC_{50}	EC_{90}	EC_{50} 5 % conf. int.
Ryegrass	F	Germination (7d)	43,75 ± 2,68	64,43 ± 2,00	94,88 ± 4,37	60,50 68,35
Ryegrass	A	Germination (7d)	56,77 ± 3,57	78,45 ± 1,88	108,40 ± 4,40	74,77 82,13
Ryegrass	P	Germination (7d)	26,08 ± 1,00	33,49 ± 0,93	42,99 ± 2,60	31,66 35,31
Ryegrass	F + A	Germination (7d)	*0,71 ± 0,04*	*1,09 ± 0,03*	*1,66 ± 0,08*	*1,02 1,15*
Ryegrass	F + P	Germination (7d)	*0,76 ± 0,06*	*1,45 ± 0,06*	*2,79 ± 0,16*	*1,33 1,57*
Ryegrass	A + P	Germination (7d)	*0,69 ± 0,06*	*1,24 ± 0,05*	*2,23 ± 0,13*	*1,13 1,34*
Ryegrass	F + A + P	Germination (7d)	*0,65 ± 0,05*	*1,07 ± 0,04*	*1,76 ± 0,09*	*1,00 1,14*
Ryegrass	F	Germination (21d)	45,23 ± 2,64	67,75 ± 2,11	101,49 ± 4,33	63,62 71,89
Ryegrass	A	Germination (21d)	22,43 ± 2,34	51,54 ± 2,22	118,44 ± 8,18	47,18 55,90
Ryegrass	P	Germination (21d)	14,69 ± 1,53	25,05 ± 1,18	42,71 ± 2,30	22,73 27,36
Ryegrass	F + A	Germination (21d)	*0,82 ± 0,05*	*1,27 ± 0,03*	*1,97 ± 0,09*	*1,20 1,34*
Ryegrass	F + P	Germination (21d)	*0,55 ± 0,05*	*1,15 ± 0,05*	*2,43 ± 0,16*	*1,05 1,26*
Ryegrass	A + P	Germination (21d)	*0,43 ± 0,03*	*0,73 ± 0,03*	*1,23 ± 0,06*	*0,67 0,78*
Ryegrass	F + A + P	Germination (21d)	*0,73 ± 0,04*	*1,12 ± 0,04*	*1,73 ± 0,09*	*1,05 1,19*
Ryegrass	F	Biomass (dw)	24,06 ± 3,57	50,12 ± 3,53	104,41 ± 11,12	43,07 57,18
Ryegrass	A	Biomass (dw)	23,90 ± 5,65	47,43 ± 4,60	94,13 ± 14,95	38,24 56,62
Ryegrass	P	Biomass (dw)	6,04 ± 1,34	15,88 ± 1,71	41,74 ± 9,10	12,45 19,31
Ryegrass	F + A	Biomass (dw)	*0,45 ± 0,11*	*0,95 ± 0,09*	*2,00 ± 0,34*	*0,76 1,13*
Ryegrass	F + P	Biomass (dw)	*0,28 ± 0,08*	*0,79 ± 0,09*	*2,21 ± 0,47*	*0,60 0,98*
Ryegrass	A + P	Biomass (dw)	*0,35 ± 0,08*	*0,72 ± 0,08*	*1,48 ± 0,30*	*0,56 0,88*
Ryegrass	F + A + P	Biomass (dw)	*0,52 ± 0,16*	*1,00 ± 0,10*	*1,93 ± 0,41*	*0,80 1,20*

APPENDIX 4. Subchronic assay schedule and temperature variation during assays.

Assay	Started	Ended	Temperature °C	
			min	max
Individual 1	26.2.2010	19.3.2010	17	35
Individual 2	3.3.2010	24.3.2010	16	35
Individual 3	13.3.2010	3.4.2010	16	34
Binary mixture 1	21.4.2010	12.5.2010	17	34
Binary mixture 2	23.4.2010	14.5.2010	17	33
Binary mixture 3	7.5.2010	28.5.2010	17	40
Trinary mixture 1	13.5.2010	3.6.2010	18	29
Trinary mixture 2	21.5.2010	11.6.2010	16	27
Trinary mixture 3	2.6.2010	23.6.2010	17	29

APPENDIX 5. Mean pH-values before (0d), and after (21d) the exposure time in sub-chronic assays for individual acids. F = formic acid, A = acetic acid, P = propionic acid. Concentrations in mmol/kg.

Individual acids at 0d			Individual acids at 21d			
Acid	**Concentration**	**Mean**	**Acid**	**Concentration**	**Species**	**Mean**
Control	0	5,4	**Control**	0	Cress	5,2
F	5	4,0	**F**	5	Cress	5,4
F	10	3,7	**F**	10	Cress	5,1
F	20	3,4	**F**	20	Cress	5,5
F	30	3,2	**F**	40	Cress	5,7
F	40	3,2	**F**	80	Cress	5,8
F	60	3,1	**A**	5	Cress	5,3
F	80	3,0	**A**	10	Cress	5,5
F	120	2,8	**A**	20	Cress	5,6
F	150	2,7	**A**	40	Cress	6,2
A	5	4,4	**A**	80	Cress	6,4
A	10	4,3	**P**	2	Cress	5,6
A	20	4,0	**P**	5	Cress	5,8
A	25	3,9	**P**	10	Cress	5,9
A	40	3,7	**P**	30	Cress	6,4
A	50	3,6	**P**	60	Cress	6,7
A	75	3,4	**Control**	0	Ryegrass	4,9
A	80	3,4	**F**	10	Ryegrass	5,1
A	100	3,3	**F**	30	Ryegrass	5,7
P	2	4,6	**F**	60	Ryegrass	6,1
P	5	4,5	**F**	120	Ryegrass	6,6
P	10	4,3	**F**	150	Ryegrass	6,7
P	15	4,2	**A**	5	Ryegrass	5,0
P	30	3,9	**A**	25	Ryegrass	5,7
P	60	3,6	**A**	50	Ryegrass	6,5
			A	75	Ryegrass	6,6
			A	100	Ryegrass	6,7
			P	5	Ryegrass	5,5
			P	10	Ryegrass	5,4
			P	15	Ryegrass	5,9
			P	30	Ryegrass	6,6
			P	60	Ryegrass	6,7

APPENDIX 6. Mean pH-values before (0d), and after (21d) the exposure time in sub-chronic assays for binary mixtures. F = formic acid, A = acetic acid, P = propionic acid. Concentrations in mmol/kg.

Binary mixtures at 0 d				**Binary mixtures at 21d**			
Acid	**TU**	**Species**	**Mean**	**Acid**	**TU**	**Species**	**Mean**
Control	0	Cress	5,4	Control	0	Cress	5,4
F + A	1/10	Cress	4,2	F + A	1/10	Cress	4,2
F + A	1/4	Cress	3,7	F + A	1/4	Cress	3,7
F + A	1/2	Cress	3,3	F + A	1/2	Cress	3,3
F + A	1	Cress	3,0	F + A	1	Cress	3,0
F + A	2	Cress	2,7	F + A	2	Cress	2,7
F + A	4	Cress	2,4	F + A	4	Cress	2,4
F + P	1/10	Cress	4,0	F + P	1/10	Cress	4,0
F + P	1/4	Cress	3,7	F + P	1/4	Cress	3,7
F + P	1/2	Cress	3,3	F + P	1/2	Cress	3,3
F + P	1	Cress	3,1	F + P	1	Cress	3,1
F + P	2	Cress	2,8	F + P	2	Cress	2,8
F + P	4	Cress	2,5	F + P	4	Cress	2,5
A + P	1/10	Cress	4,5	A + P	1/10	Cress	4,5
A + P	1/4	Cress	4,2	A + P	1/4	Cress	4,2
A + P	1/2	Cress	4,0	A + P	1/2	Cress	4,0
A + P	1	Cress	3,7	A + P	1	Cress	3,7
A + P	2	Cress	3,4	A + P	2	Cress	3,4
A + P	4	Cress	3,1	A + P	4	Cress	3,1
Control	0	Ryegrass	5,4	Control	0	Ryegrass	4,5
F + A	1/10	Ryegrass	3,8	F + A	1/10	Ryegrass	4,9
F + A	1/4	Ryegrass	3,4	F + A	1/4	Ryegrass	5,2
F + A	1/2	Ryegrass	3,1	F + A	1/2	Ryegrass	5,8
F + A	1	Ryegrass	2,8	F + A	1	Ryegrass	6,5
F + A	2	Ryegrass	2,5	F + A	2	Ryegrass	6,7
F + A	4	Ryegrass	2,2	F + A	4	Ryegrass	6,8
F + P	1/10	Ryegrass	3,7	F + P	1/10	Ryegrass	4,8
F + P	1/4	Ryegrass	3,4	F + P	1/4	Ryegrass	5,2
F + P	1/2	Ryegrass	3,2	F + P	1/2	Ryegrass	5,8
F + P	1	Ryegrass	2,9	F + P	1	Ryegrass	6,5
F + P	2	Ryegrass	2,6	F + P	2	Ryegrass	6,4
F + P	4	Ryegrass	2,3	F + P	4	Ryegrass	4,1
A + P	1/10	Ryegrass	4,0	A + P	1/10	Ryegrass	4,9
A + P	1/4	Ryegrass	4,0	A + P	1/4	Ryegrass	5,1
A + P	1/2	Ryegrass	3,7	A + P	1/2	Ryegrass	5,7
A + P	1	Ryegrass	3,4	A + P	1	Ryegrass	6,4
A + P	2	Ryegrass	3,1	A + P	2	Ryegrass	6,9
A + P	4	Ryegrass	2,8	A + P	4	Ryegrass	5,5

APPENDIX 7. Mean pH-values before (0d), and after (21d) the exposure time in sub-chronic assays for trinary mixtures. F = formic acid, A = acetic acid, P = propionic acid. Concentrations in mmol/kg.

Tertiary mixture at 0d				**Tertiary mixture at 21d**			
Acid	**TU**	**Species**	**Mean pH**	**Acid**	**TU**	**Species**	**Mean pH**
Control	0	Cress	5,6	Control	0	Cress	5,0
F + A + P	1/12	Cress	4,4	F + A + P	1/12	Cress	5,2
F + A + P	1/6	Cress	4,1	F + A + P	1/6	Cress	5,5
F + A + P	1/3	Cress	3,8	F + A + P	1/3	Cress	5,7
F + A + P	1	Cress	3,3	F + A + P	1	Cress	6,6
F + A + P	1,5	Cress	3,1	F + A + P	1,5	Cress	7,0
F + A + P	3	Cress	2,8	F + A + P	3	Cress	5,6
Control	0	Ryegrass	5,6	Control	0	Ryegrass	4,9
F + A + P	1/12	Ryegrass	4,3	F + A + P	1/12	Ryegrass	5,3
F + A + P	1/6	Ryegrass	3,9	F + A + P	1/6	Ryegrass	6,0
F + A + P	1/3	Ryegrass	3,6	F + A + P	1/3	Ryegrass	6,1
F + A + P	1	Ryegrass	3,1	F + A + P	1	Ryegrass	6,9
F + A + P	1,5	Ryegrass	2,9	F + A + P	1,5	Ryegrass	6,6
F + A + P	3	Ryegrass	2,5	F + A + P	3	Ryegrass	3,4