Low-weight carboxylic acids as potential risk in phytotoxicity of processed biomasses

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ABSTRACT
Being intermediates of organic matter degradation, low-weight carboxylic acids (LWCA) can occur at phytotoxic concentrations in bioactive soils or in the processed organic material applied to soil. While toxicity potentials and mixture effects of LWCA are still poorly known, the potential phytotoxicity of the biomaterials is difficult to assess. In the study effective concentrations (EC) of formic, acetic and propionic acids at non-adjusted pH were obtained in short-term germination assays (48 h for cress, *Lepidium sativum*, and 120h for ryegrass, *Lolium multiflorum*) and subchronic growth assays (21d). Based on the EC values, LWCA inhibited more strongly plant growth than seed germination. In mixtures each acid acted separately without interaction other than additivity. Toxicity of LWCA increased with increase in the carbon chain length, being true also in mixtures. Nontoxic concentration (EC10) for equimolar mixture of formic, acetic and propionic acid was calculated to be below 1000 mg/kg dm.

Key words: mixture phytotoxicity, low-weight carboxylic acids, cress, ryegrass, digestate, compost

1 INTRODUCTION

Low-weight carboxylic acids (LWCA) are intermediates of the natural degradation of organic matter. Although being a pool of transition products, they may accumulate in high concentrations in bioactive natural soils (Stevenson, 1967), the green manures applied to soil due to water logging (Chandrasekaran and Yoshida, 1973) or during processing of biowaste. Analysis of 626 compost samples of different ages showed concentrations of LWCA ranging from 75 to 51474 mg kg^{-1} (Brinton, 1998). In another study concentration range of LWCA in 899 composts was from 72 to 88737 mg kg^{-1} (Brinton and Tränker, 1999). In digestate from organic fraction of municipal solid waste concentration was 18.9 ± 0.5 g acetic acid kg TS^{-1} (Tambone et al., 2009) Concentrations of LWCA in anaerobic effluent from dairy manure ranged from 200 to 800 mg/L depending on the duration of biogas production phase (Rico et al., 2011). Amounts of LWCA in stillage from bioethanol production ranged from 180 to 370 mg/L (Kaparaju et al., 2009). If the processed biomaterial with high concentrations of LWCA is applied for plant production it may exert phytotoxicity, impairing growth of crops (Schuman and McCalla, 1976; Lynch, 1977; Rao and Mikkelsen, 1977; Manois et al., 1987). Thus, although phytotoxicity of LWCA is a known phenomenon, individual effective concentrations (EC) of LWCA are known only to some extent (Himanen et al., 2012). EC values of pure LWCA can be useful in assessment of the phytotoxic potential of treated biowaste, however in the plant growth substrates the acids usually exhibit as a mixture. In a mixture, toxic effect may be predicted by an additive model, where each compound contributes to the toxicity in proportion to its dose. Deviation from additivity may reveal statistically stronger (synergistic) or weaker (antagonistic) outcome (Nielsen et al., 2008). Studies on the mixture of LWCA conducted so far have not been fully systematic. For example, Armstrong and Armstrong (2001) studied effect of two cocktails of LWCA on young common reed *Phragmites*: cocktail 1 (acetic, propionic, n-butyric, iso-butyric, and caproic) and cocktail 2 (formic, acetic, propionic, n-butyric, iso-butyric, valeric and caproic) with concentration of each acid being one mmol/L and pH adjusted to 6. Each cocktail decreased root growth and induced premature shoot senescence. Schuman and McCalla (1976) reported a 70% decrease in root length of wheat and sorghum in a mixture of acetic, propionic and butyric acids over a 40% decrease of individual acids. Although these data are useful in understanding mechanism of LWCA phytotoxicity, however they are not enough to conclude on the type of mixture effect of the acids.

The aim of the present study was to obtain EC values for short-term and subchronic phytotoxicity of formic, acetic and propionic acids and evaluate type of their mixture toxicity using the principle of concentration addition.

2 MATERIALS AND METHODS

Individual and mixture phytotoxicity of formic (F), acetic (A) and propionic (P) acids were studied using short-term (2.2) and subchronic (2.3) assays. Monocotyledonous ryegrass *Lolium multiflorum* and dicotyledonous cress *Lepidium stivum* were used as test plants.

2.1 Concentrations of acids in assays

For individual toxicity a series of pure acids with five concentrations and control were used (Table 1). Choice of the concentrations was based on the preliminary range-finding experiments. Phytotoxicity of binary (F+A, F+P, A+P) and ternary (F+A+P) mixtures of LWCA was studied using the principle of concentration addition. Binary and ternary mixtures were prepared using proportions of the respective EC50-values (=1 toxic unit (TU)) obtained in the preliminary individual toxicity assays. Due to high variability in seed germination, EC50 values for seedling or biomass growth were chosen as basis for the TU concentrations. Summed concentrations of the binary mixtures in short-term and subchronic assays were: ∑ 0.1 TU, ∑ 0.25 TU, ∑ 0.5 TU, ∑ 1 TU, ∑ 2 TU, ∑ 4 TU and in the ternary mixtures: ∑ 1/12 TU, ∑ 1/6 TU, ∑ 1/3 TU, ∑ 1½ TU, ∑ 3 TU. Procedures for conducting the short-term and subchronic assays are described in ch. 2.2 and 2.3.

Table 1: Concentrations of formic, acetic and propionic acids applied in short-term and subchronic assays for individual phytotoxicity studies.

		Cress					Ryegrass				
Short-term assay (mmol/L)											
Formic	Control[a]	0.5	1	2	4	12	0.4	0.8	1.6	3.2	9.6
Acetic	Control	0.5	1	1.5	3	12	0.4	0.8	1.2	2.4	9.6
Propionic	Control	0.3	0.6	1.2	2.4	9.6	0.3	0.6	1.2	2.4	9.6
Subchronic assay (mmol/kg dm)											
Formic	Control[b]	5	10	20	40	80	10	30	60	120	150
Acetic	Control	5	10	20	40	80	5	25	50	75	100
Propionic	Control	2	5	10	30	60	5	10	15	30	60

[a] de-ionized water

[b] de-ionized water added to inorganic growth substrate

2.2 Short-term phytotoxicity assays

For short-term assays Petri dishes (∅ 90 mm) were lined with the filter paper (Whatman no.1, ∅ 70 mm) and moisturized with 10 mL of pure acid solution, mixture of LWCA or control (de - ionized water) (see ch. 2.1). Each plate was seeded with 20 seeds of garden cress *L. sativum* (seed supplier Habitec Inc., Finland) or ryegrass *L. multiflorum v. fabio* (seed supplier Tilasiemen OY, Finland) and covered with the lid. Petri dishes were incubated in the darkness at 24–26 °C for 48 (garden cress) or 120 hours (ryegrass). After the incubation, number of the germinated seeds was counted and total length of the seedlings (root + shoot) for garden cress or the longest root for ryegrass was measured with the accuracy of 1 mm. The seed was considered to be germinated when radicle was over 1 mm long. One set of the experiment consisted of three Petri dishes for each concentration and six Petri dishes for control repeated three times. An experiment was considered successful when germination of controls of cress was over 95% and that of ryegrass over 85%.

2.3 Subchronic phytotoxicity assay

The subchronic assays were conducted according to the modified standard method ISO 11269-2 (ISO, 2008) with garden cress *L. sativum* and ryegrass *L. multiflorum v. fabio.* Five dilutions were prepared for each pure acid or LWCA mixture (see ch. 2.1). The inorganic growth substrate was made of the coarse-grained sand (particle size 0.5–1.2 mm; Maxit Inc., Finland) and the quartz sand (particle size 0.2–0.005 mm; NFQ Nilsiän kvartsi, SP Minerals Inc., Finland) in proportion 6+1 (v/v). The contamination of the growth substrate was made by adding 100 ml of pure acid, LWCA mixture solution, or control (de-ionized water) to 1.6 L of the growth substrate. The plastic pot (∅ = 10 cm, height = 8 cm) was filled as following: approx. one cm height of peat-based growing media (Kekkilä Kasvuturve B2, Kekkilä Inc., Finland) on the bottom, 300 g of the inorganic substrate, 25 seeds, and approx. one cm of the peat-based growing media on the top. Peat-based layers were used to prevent leaching of the substrate from the pot during watering and to decrease stripping of the acids from the substrate. The pots were incubated in a climate room at 23–27 °C with light/dark regime 16/8 hours, the light being 13 000 lx, 185 µmol m^{-2} s^{-1}, with the color temperature of 6500 K (Biolux, Osram, Germany). To avoid drying of the substrate, the plants were watered with general fertilizing solution (NPK 12–6–9, Kekkilä Kukkaravinne, Kekkilä Inc., Finland) every or every second day. Incubation time was 21 days, after which the number of germinated plants was counted, the plants were cut next to the substrate surface, dried at 70 °C over night and weighted. Each experiment consisted of six control replicates and three replicates for each acid or their mixture concentration, repeated three times.

2.4 Modeling dose–response relationships

Modeling of dose–response relationships for each endpoint (germination, early seedling growth and shoot biomass) was done using added concentrations of LWCA as actual concentrations in the substrates were not analyzed. Modeling and calculations of the EC-values and statistical parameters were done for each acid and LWCA mixture using R program (version 2.10.1) drc-package. Different models were tested and the three-parameter log-logistic model was used for the final data extrapolation. The model is applicable when the response pattern follows a non–linear curve, it is symmetric around EC50 point, and the lower limit is equal to zero. The model is described by equation 1.

$$f(x, (b, d, e)) = d / (1 + \exp\{b (\log (x) - \log (e))\}) \quad (1)$$

The parameter d is the lower limit, b is the relative slope around e, and e is the logarithm of the inflection point (Ritz and Streibig, 2005).

2.5 Toxicity analysis of LWCA mixtures

Toxicity analysis of the mixtures was made using the interaction index according to Marking (1985). For that, the sum of toxic action (S) and the additive index (AI) were calculated using equations 2–4.

$$S = (A_m/A_i) + (B_m/B_i) + (C_m/C_i) \quad (2)$$

S = sum of toxic action; A_m, B_m, C_m = EC50 for compounds A, B and C in mixtures; A_i, B_i, C_i = EC50 for compounds A, B and C individually. S values were used for calculation of the AI.

$$If\ S \leq 1.0,\ AI = (1/S) - 1.0 \quad (3)$$
$$If\ S \geq 1.0,\ AI = S\ (-1) + 1 \quad (4)$$

The AI significantly less than zero indicates antagonistic toxicity and greater than zero synergistic toxicity. The significance of deviation from zero was evaluated by generating confidence interval (CI) values for AI by substituting the EC50 values in equation 1 with the corresponding CI limit values of EC50 according to equations 5 and 6.

$$AI_{m(low)} = (CI_{A\ m(up)}/CI_{A\ i\ (low)}) + (CI_{B\ m(up)}/CI_{B\ i\ (low)}) + CI_{C\ m(up)}/CI_{C\ m(low)}) \quad (5)$$
$$AI_{m(up)} = (CI_{A\ m(low)}/CI_{A\ i(up)}) + (CI_{B\ m(low)}/CI_{B\ i(up)}) + CI_{C\ m(low)}/CI_{C\ m(up)}) \quad (6)$$

where $AI_{m(low)}$ *and* $AI_{m(up)}$ = lower and upper limits of the AI interval, $CI_{A/B/C\ m(up)}$ *and* $CI_{A/B/C\ m(low)}$ = upper and lower limits of EC50 confidential intervals obtained for the

compounds A, B, and C in mixture assays; $CI_{A/B/C\ i(up)}$ *and* $CI_{A/B/C\ i(low)}$ = upper and lower limits of EC50 confidential intervals obtained for the compounds A, B, and C in individual assays. If AI confidential interval overlaps zero, mixture can be judged to have additive toxicity (Marking, 1985).

3 RESULTS AND DISCUSSION

3.1 Toxicity of individual acids

Dose–response relationships of formic, acetic and propionic acids for seed germination, early seedling growth, and plant growth were modeled (Figs. 1 and 2) and the models were used to calculate effective concentration values such as EC10, EC50, and EC90 for each LWCA (Table 2).

Results showed ryegrass being more sensitive to LWCA than cress. For seed germination obtained in short-term assays, EC50 values for formic, acetic and propionic acids were between 2.9 and 4.4 mmol/L for cress and between 1.8 and 3.6 mmol/L for ryegrass. In subchronic assays EC50 values for delayed germination of cress were between 25 and 37 mmol/kg dm, and of ryegrass 35 and 78 mmol/kg dm. Regarding to the early seedling growth EC50 values for cress were between 1.1 and 2.0 mmol/L and for ryegrass between 1.8 and 2.4 mmol/L. EC50 values for plant biomass of cress were between 12 and 36 mmol/kg dm, and of ryegrass between 16 and 50 mmol/kg dm. Variability of data was similar in both tests, relative standard error (RSE) was between 3 and 13%.

Considering EC10–EC90 intervals, the transition from non-toxic to inhibitory levels of LWCA occurred more abruptly in short-term than in subchronic assays being narrower for cress than for ryegrass. In short-term assays the interval for all endpoints for cress ranged from 2 to 4 mmol/L and for ryegrass from 3 to 7 mmol/L. In subchronic assays this interval ranged from 23 to 33 mmol/kg dm for cress and from 22 to almost 80 mmol/kg dm for ryegrass. Thus, in subchronic assays, cress was somewhat more sensitive to LWCA than ryegrass, EC50 values of cress were by 30 to 75 % lower than of ryegrass. However, in short-term assays the difference between species was not significant.

EC values obtained in the short-term assays of this study were on the same level as found by Reynolds (1975) on lettuce and Himanen et al. (2012) on cress and ryegrass. However, current values were about ten times higher than the values reported by Prill et al. (1949) on wheat or hundred times higher reported by Shiralipour and McConnel (1997) on cucumber. Though, the EC values on barley presented by Lynch (1977 and 1980) were three to five times lower than the values in this research. In subchronic assays, the EC values in this research were by 15–40% lower than obtained by Himanen et al. (2012) on ryegrass and cress.

Phytotoxicity of LWCA increased with the length of the carbon chain that was more clearly observed in subchronic assays than in short-term assays, which is compatible with the earlier observations (Prill et al., 1949; Ulbright et al., 1982; Himanen et al., 2012).

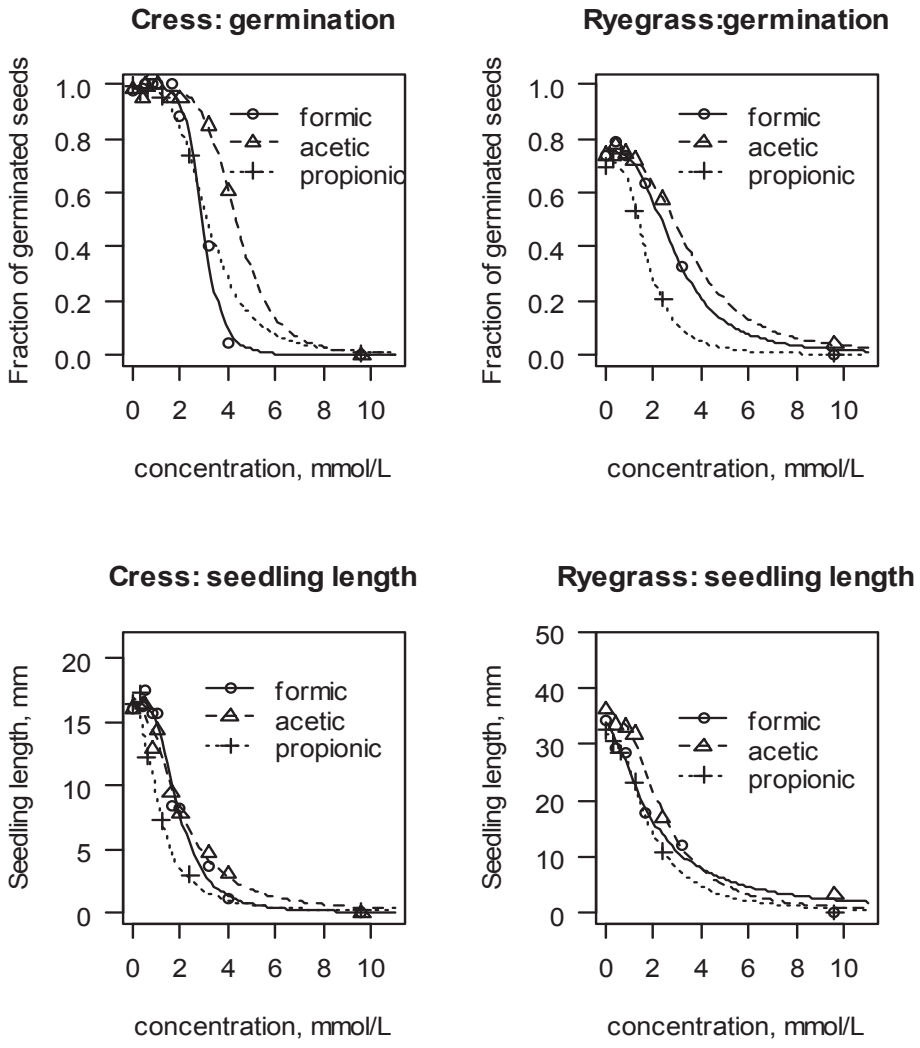

Figure 1: Dose-response curves of individual formic, acetic and propionic acids for germination and seedling growth of cress and ryegrass obtained in short-term assays.

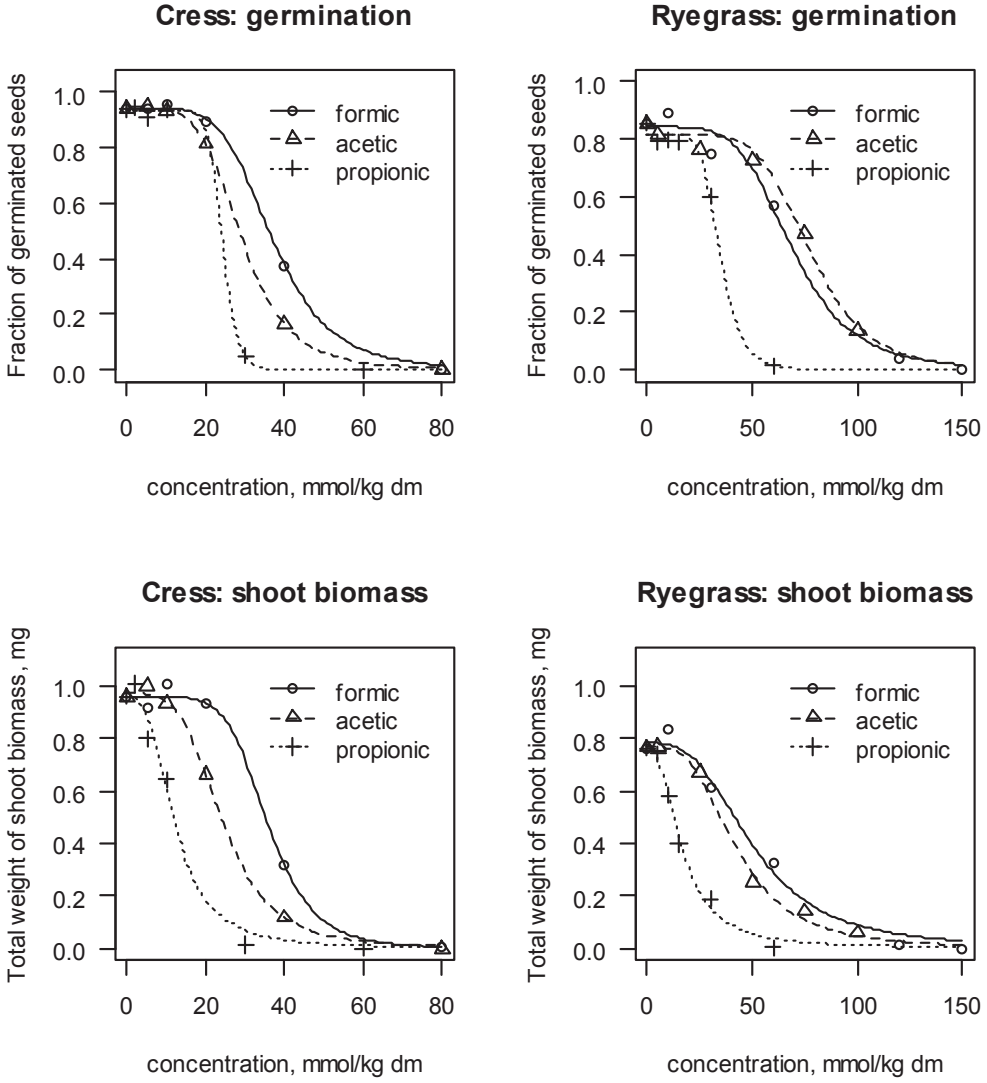

Figure 2: Dose-response curves of individual formic, acetic and propionic acids for germination and seedling growth of cress and ryegrass obtained in subchronic assays.

Table 2: Effective concentrations (EC) of formic, acetic and propionic acids, assayed individually, for reduction of seed germination, seedling growth, and shoot biomass obtained in short-term and subchronic assays. Test species were cress *Lepidium sativum* and ryegrass *Lolium multoflorum*. EC values are expressed as means with standard errors in parenthesis.

	Short-term assay (mmol/L)		Subchronic assay (mmol/kg dm)	
	Cress	Ryegrass	Cress	Ryegrass
	EC50	EC50	EC50	EC50
	EC10 - EC90	EC10 - EC90	EC10 - EC90	EC10 - EC90
			Germination	
Formic	2.9 (0.1)	2.9 (0.2)	37 (1.2)	69 (2.3)
	2.2 (0.1) - 4.0 (0.1)	1.4 (0.2) - 6.0 (0.1)	24 (2.8) - 57 (4.5)	44 (4.0) - 108 (11.4)
Acetic	4.4 (0.1)	3.6 (0.5)	29 (1.1)	78 (2.1)
	3.0 (0.2) - 6.4 (0.7)	1.7 (0.3) - 7.5 (2.1)	19 (1.2) - 46 (2.4)	53 (4.3) - 114 (7.1)
Propionic	3.2 (0.2)	1.8 (0.1)	25 (NA*)	35 (2.3)
	1.8 (0.1) - 5.6 (1.0)	0.9 (0.1) - 3.5 (0.5)	21 (NA) - 30 (NA)	25 2.2) - 47 (9.3)
	Seedling growth		Shoot biomass	
Formic	1.9 (0.1)	1.9 (0.2)	36 (2.9)	50 (4.6)
	1.0 (0.1) - 3.7 (0.3)	0.5 (0.1) - 7.5 (1.9)	25 (7.0) - 51 (7.4)	24 (4.7) - 104 (14.5)
Acetic	2.0 (0.1)	2.4 (0.2)	24 (1.7)	42 (3.3)
	0.8 (0.1) - 5.2 (0.5)	1.0 (0.3) - 5.8 (2.1)	14 (1.9) - 42 (6.3)	21 (3.8) - 87 (11.6)
Propionic	1.1 (0.1)	1.8 (0.2)	12 (1.3)	16 (1.6)
	0.4 (0.0) - 3.0 (0.3)	0.7 (0.2) - 4.9 (1.3)	5 (1.2) - 28 (6.0)	6 (1.3) - 43 (8.4)

*NA - Data could not be generated due to lack of data between 100% germination (10 mmol/kg dm) and no germination (30 mmol/kg dm), that defined almost vertical slope around EC50 value.

3.2 Mixture toxicity of LWCA

In binary mixture assays for all endpoints (germination, early seedling growth and biomass production) EC50 values were around 1 TU, the means ranging from 0.8 to 1.4

TU for F+A mixture and from 0.6 to 1.2 TU for F+P and A+P mixtures (Table 3). In ternary mixture EC50 in short term assays was slightly lower (range between 0.4 and 1.0 TU) than in subchronic assays (range between 0.9 and 1.3 TU).

Table 3: EC50 values and 95% confidential intervals (in parenthesis) generated from the data obtained in short-term and subchronic assays for binary and ternary mixtures of formic (F), acetic (A) and propionic (P) acids. The values are expressed as toxic units and were extrapolated from three-parametric log-logistic model.

	Short-term assay		Subchronic assay	
	Cress	Ryegrass	Cress	Ryegrass
	Germination		Germination	
F + A	1.2	1.4	0.9	1.1
	(1.2–1.3)	(1.2–1.5)	(0.8–0.9)	(1.0–1.2)
F + P	1.2	1.0	1.3	1.1
	(1.2–1.2)	(1.0–1.2)	(1.2–1.4)	(1.0–1.3)
A + P	1.2	1.0	0.9	0.7
	(1.1–1.3)	(1.0–1.1)	(0.8–1.0)	(0.6–0.8)
F+A+P	1.0	0.7	1.1	1.3
	(1.0–1.0)	(0.6–0.9)	(1.1–1.2)	(1.2–1.4)
	Seedling growth		Shoot biomass	
F + A	0.8	1.5	0.9	0.8
	(0.7–0.9)	(1.2–1.7)	(NA*–NA)	(0.6–1.0)
F + P	0.6	0.8	1.0	0.9
	(0.5–0.6)	(0.7–1.0)	(0.8–1.1)	(0.6–1.2)
A + P	0.6	0.1	0.6	0.6
	(0.5–0.6)	(0.8–1.2)	(0.5–0.7)	(0.5–0.8)
F+A+P	0.4	0.7	0.9	1.0
	(0.4–0.5)	(0.5–0.9)	(0.7–1.1)	(0.8–1.2)

*NA – the values of the confidential interval could not be generated.

For most endpoints interaction index of binary mixtures analysis showed additive value around one (Tables 4 and 5). The range of the index limit values was slightly negative or overlapping zero, meaning no other type of interactions like synergistic or antagonistic effects. So, toxic unit and interaction index analysis suggested simple dose addition mechanism of the LWCA in mixtures. This means that in a mixture each acid acts separately and no interactions occur between the acids. Therefore, phytotoxicity of the substrate depends on what LWCA are present in the substrate and is proportional to concentration of each acid. As LWCA belong to the same group of carboxylic acids they possibly act similarly on plants. Mechanisms of LWCA phytotoxicity is not clearly understood, yet. There is no common agreement whether dissociated or undissociated form of LWCA is phytotoxic. The toxicity of LWCA can be regarded as partly due to

H^+-ion and partly due to undissociated acid or dissociated anion (Stiles and Rees, 1935). One possible way of LWCA toxic action is disturbance of osmotic regulations of root cells. Once the molecules have entered the root cells, they change permeability of the membrane and cause hyperpolarization of transmembrane electrical potential. This leads to rapid loss of K^+, Ca^{2+}, and Cl^- ions into the external substrate and flow of Na^+ ions into the cell that was demonstrated in many experimental studies (Jackson and Taylor, 1970; Lee, 1977; Marrè et al., 1983).

Role of the carbon chain length becomes more important in mixture toxicity, i.e. mixture of acids with longer chain is more phytotoxic than mixture of acids with shorter chain. For example, in short-term responses for cress, EC50 value of acetic acid alone was 2.0 mmol/L (95% conf.int 1.8–2.2), which is higher than 1.0 mmol/L (1.0–1.1) in A+F, 0.8 mmol/L (0.7–0.8) in A+P and 0.6 mmol/L (0.5–0.6) in F+A+P. However, the trend was not so obvious in subchronic endpoints. As an example, graphical comparison of EC50 values from individual and mixture assays for cress from short-term and subchronic assays are presented in Figure 3. Increase of toxicity in mixture compared to the pure acids was observed for acetic, propionic and butryric acids by Shuman and McCalla (1976). In the study germination of wheat in the equimolar mixture was 51% compared to toxicity of pure acids that was 73-89%, for sorghum respective values were 49% and 53–67%. The trend may suggest that in a mixture each LWCA has its own relative potency (REP) value that increases with the increase of the carbon chain. Similar suggestion was made by Lynch (1977) stating that at equivalent concentrations, the phytotoxicity of propionic and butyric acids was greater than that of acetic acid by factors of about 2 and 3, respectively. If this hypothesis is true propionic and butyric acids still make a contribution to the phytotoxicity of compost, although they present in small concentrations. However, more research is needed following also phytotoxic effect of C4–C6 LWCA.

Table 4: EC50 values, confidential intervals (95% CI), the sums of toxic action (S) and the additive indices (AI) for individual and mixture toxicity of formic (F), acetic (A) and propionic (P) acids obtained in short-term germination assays for cress and ryegrass. Concentrations are expressed in mmol/L.

	Cress		Ryegrass	
	EC50	95% CI	EC50	95% CI
GERMINATION				
		Formic + Acetic		
Formic individual	2.9[a]	2.8 – 3.1	2.9	2.5 – 3.3
Formic in F + A	2.0[b]	1.9 – 2.1	2.0	1.8 – 2.2
Acetic individual	4.4	4.1 – 4.6	3.6	2.7 – 4.5
Acetic in F + A	1.6	1.5 – 1.7	3.0	2.7 – 3.3
S		1.1 (1.2; 1.0)[c]		1.5 (2.1; 1.2)
AI		-0.1 (-0.2; 0.1)[d]		-0.5 (-1.1; -0.2)
		Formic + Propionic		
Formic individual	2.9	2.8 – 3.1	2.9	2.5 – 3.3
Formic in F + P	1.9	1.8 – 2.0	1.5	1.3 – 1.7
Propionic individual	1.8	1.5 – 2.0	1.8	1.5 – 2.0
Propionic in F + P	1.2	1.2 – 1.2	1.3	1.1 – 1.5
S		1.0 (1.2; 0.9)		1.3 (1.7; 1.0)
AI		0.0 (-0.2; 0.1)		-0.3 (-0.7; 0.0)
		Acetic + Propionic		
Acetic individual	4.4	4.1 – 4.6	3.6	2.7 – 4.5
Acetic in A + P	1.6	1.5 – 1.6	2.2	2.1 – 2.4
Propionic individual	1.8	1.5 – 2.0	1.8	1.5 – 2.0
Propionic in A + P	1.2	1.1 – 1.3	1.3	1.2 – 1.4
S		0.7 (0.9; 0.6)		1.4 (1.8; 1.1)
AI		0.4 (0.2; 0.6)		-0.4 (-0.8; -0.1)
		Formic + Acetic + Propionic		
Formic in F+A+P	1.6	1.5 – 1.6	1.1	0.9 – 1.3
Acetic in F+A+P	1.3	1.2 – 1.3	1.6	1.3 – 1.9
Propionic in F+A+P	1.0	1.0 – 1.0	0.9	0.8 – 1.1
S		1.39 (1.6; 1.2)		1.4 (2.0; 0.9)
AI		-0.4 (-0.6; -0.2)		-0.4 (-1.0; 0.07)

SEEDLING GROWTH				
	Formic + Acetic			
Formic individual	1.9	1.8 – 2.1	1.9	1.4 – 2.4
Acetic individual	2.0	1.8 – 2.2	2.4	1.9 – 2.9
Formic in F + A	1.3	1.2 – 1.4	2.4	2.0 – 2.8
Acetic in F + A	1.0	0.9 – 1.1	1.9	1.6 – 2.2
S		1.2 (1.4; 1.0)		2.0 (3.1; 1.4)
AI		-0.2 (-0.4; 0.0)		-1.0 (-2.1; -0.4)
	Formic + Propionic			
Formic individual	1.9	1.8 – 2.1	1.9	1.4 – 2.4
Formic in F + P	0.9	0.9 – 1.0	1.3	1.1 – 1.5
Propionic individual	1.1	1.0 – 1.2	1.8	1.4 – 2.2
Propionic in F + P	0.6	0.5 – 0.6	0.8	0.7 – 1.0
S		1.0 (1.2; 0.9)		1.2 (1.8; 0.8)
AI		0.0 (-0.2; 0.1)		-0.2 (-0.8; 0.2)
	Acetic + Propionic			
Acetic individual	2.0	1.8 – 2.2	2.4	1.9 – 2.9
Acetic in A + P	0.8	0.7 – 0.8	1.3	1.1 – 1.5
Propionic individual	1.1	1.0 – 1.2	1.8	1.4 – 2.2
Propionic in A + P	0.6	0.5 – 0.6	1.0	0.8 – 1.2
S		0.9 (1.1; 0.8)		1.1 (1.6; 0.7)
AI		0.1 (-0.1; 0.3)		0.0 (-0.6; 0.3)
	Formic + Acetic + Propionic			
Formic in F+A+P	0.7	0.6 – 0.8	1.0	0.7 – 1.4
Acetic in F+A+P	0.6	0.5 – 0.6	1.5	1.0 – 2.0
Propionic in F+A+P	0.4	0.4 – 0.5	0.9	0.6 – 1.2
S		0.6 (0.7; 0.5)		1.3 (0.7; 2.0)
AI		0.7 (0.4; 1.0)		-0.3 (-1.0; 0.4)

[a] calculated by extrapolation of the data from the assays on pure acid

[b] converted from TU values that were obtained by extrapolation of the data from mixture assays

[c] the sum of toxic action calculated according to eq. 2 with 95% confidential interval in parenthesis

[d] additive index, calculated according to eq. 3 or 4, with 95% confidential interval in parenthesis calculated according to eq. 5 and 6.

Table 5: EC50 values, confidential intervals (95% CI), the sums of toxic action (S) and the additive indices (AI) of individual and mixture toxicity of formic (F), acetic (A) and propionic (P) acids obtained in subchronic plant growth assays for cress and ryegrass. Concentrations are expressed in mmol/kg dm.

	Cress		Ryegrass	
	EC50	95% CI	EC50	95% CI
GERMINATION				
		Formic + Acetic		
Formic individual	37[a]	35 – 39	69	64 –74
Formic in F + A	31[b]	28 – 35	56	51 – 61
Acetic individual	29	35 – 39	78	73 – 82
Acetic in F + A	25	18 – 22	47	43 – 51
S		1.7 (1.8; 1.3)[c]		1.4 (1.7; 1.2)
AI		-0.7 (-0.8; -0.3)[d]		-0.4 (-0.7; -0.2)
		Formic + Propionic		
Formic individual	37	35 – 39	69	34 – 74
Formic in F + P	47	43 – 52	57	49 – 64
Propionic individual	25	27 – 31	35	30 – 39
Propionic in F + P	14	13 – 16	18	16 - 21
S		1.8 (2.1; 1.5)		1.4 (1.7; 1.1)
AI		-0.8 (-1.1; -0.5)		-0.4 (-0.7; -0.1)
		Acetic + Propionic		
Acetic individual	29	35 – 39	78	73 – 82
Acetic in A + P	32	29 –35	31	27 – 34
Propionic individual	25	27 – 31	35	30 – 39
Propionic in A + P	10	9 – 11	12	10 – 13
S		1.5 (1.7; 1.2)		0.7 (0.9; 0.6)
AI		-0.3 (-0.4; -0.2)		0.4 (0.1; 0.7)
		Formic + Acetic + Propionic		
Formic in F+A+P	41	39 – 43	64	59 – 69
Acetic in F+A+P	27	26 – 28	54	50 – 58
Propionic in F+A+P	12	12 – 13	20	19 – 22
S		2.5 (2.8; 2.2)		2.2 (2.6; 1.9)
AI		-1.5 (-1.8; -1.2)		-1.2 (-0.9; -1.6)

SHOOT BIOMASS				
		Formic + Acetic		
Formic individual	36	30 – 41	50	41 – 59
Acetic individual	32	NA – NA	40	31 – 48
Formic in F + A	24	21 – 27	42	36 – 49
Acetic in F + A	22	NA – NA	33	27 – 40
S		1.8 (NA;NA)		1.6 (2.3; 1.1)
AI		0.8 (NA; NA)		-0.6 (-1.3; -0.1)
		Formic + Propionic		
Formic individual	36	30 – 41	50	41 – 59
Formic in F + P	34	28 – 41	45	30 – 59
Propionic individual	12	10 – 15	16	13 – 19
Propionic in F + P	11	9 – 12	14	10 – 19
S		1.8 (2.6; 1.3)		1.8 (2.9; 1.0)
AI		-0.8 (-1.6; -0.3)		-0.8 (-1.9; 0.0)
		Acetic + Propionic		
Acetic individual	24	21 – 27	42	36 – 49
Acetic in A + P	15	12 – 18	27	22 – 32
Propionic individual	12	10 – 15	16	13 – 19
Propionic in A + P	7	6 – 8	10	8 – 12
S		1.2 (1.7; 0.8)		1.3 (1.8; 0.9)
AI		-0.2 (-0.7; 0.2)		-0.9 (-0.8; 0.1)
		Formic + Acetic + Propionic		
Formic in F+A+P	31	24 – 38	50	40 – 60
Acetic in F+A+P	21	16 – 25	42	34 – 51
Propionic in F+A+P	9	7 –12	16	13 – 19
S		2.5 (3.7; 1.6)		3.0 (4.3; 2.0)
AI		-1.5 (-0.6; -2.7)		-2.0 (-1.0; -3.3)

[a] calculated by extrapolation of the data from the assays on pure acid

[b] converted from TU values that were obtained by extrapolation of the data from mixture assays

[c] the sum of toxic action calculated according to eq. 2 with 95% confidential interval in parenthesis

[d] additive index, calculated according to eq. 3 or 4, with 95% confidential interval in parenthesis calculated according to eq. 5 and 6.

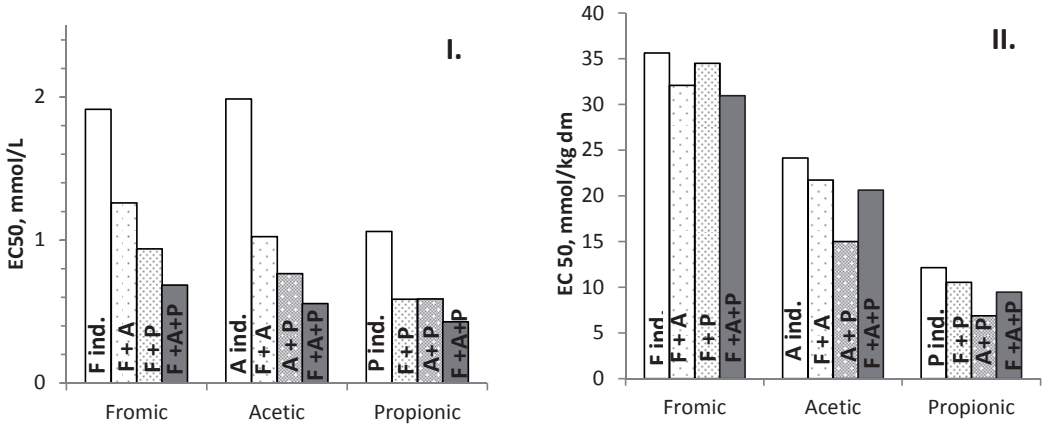

Figure 3: Comparative example of individual and mixture EC50 values of formic (F), acetic (A) and propionic (P) acids obtained in short-term (I) and subchronic (II) assays for growth of cress. EC50 values in I are for seedling length and in II for dry biomass.

3.3 Phytotoxic levels of LWCA in processed biomasses

For utilization of the results in evaluation of the toxicity potential of growth substrates, it might be more practical to use values of EC10 or EC20 instead of EC50. EC10 and EC20 indicate a 10% or 20% decrease in plant production compared to the experimental control and can be considered as near nonphytotoxic. For example, according to a national decree 1784/14/2011 on fertilizers issued by the Finnish Ministry of Forestry and Agriculture, a 20% decrease in germination index of cress is acceptable for soil improvers produced from treated organic material. Based on the present study, for formic, acetic and propionic acids, mixed in equal proportions, EC10 of LWCA mixture would be around 15 mmol/kg dm (900 mg/kg dm) for cress and 17 mmol/kg dm (1000 mg/kg dm) for ryegrass. Generalizing, we may suggest a nontoxic concentration of LWCA to be some less than 1000 mg/kg dm. The value is close to the concentration of 1250 mg/kg of total LWCA in compost-containing growth media suggested by Brinton and Tränkner (1999). Additionally it is important to mention, that in treated organic materials acetic acid is usually dominating over the other LWCA (DeVleeschauwer et al., 1982; Himanen and Hänninen, 2011). In such cases, EC values of the acetic acid would practically define phytotoxic level of the substrate. However, if REP values for LWCA can be estimated, their contribution to the phytotoxicity of substrate should be taken into consideration even if they present in low concentrations.

In order to decrease levels of LWCA in treated biomaterials and, thus, decrease phytotoxicity of the substrate, volatility and biodegradability features of the acids may be utilized. In practice it means that mixing of the mass and improved aeration will increase evaporation of LWCA from the mass and better oxygen supply enhances oxidative microbial degradation. However, stripping of LWCA from the mass will increase offensive odors, which may add to nuisance problems. By increasing duration of the maturation stage, slow degradation of LWCA may be achieved and thus decrease the phytotoxicity as well.

4 CONCLUSIONS

The research aimed to study individual and mixture phytotoxicity of formic, acetic and propionic acids by short-term and subchronic assays. According to the EC values, phytotoxicity of LWCA increases with the length of carbon chain, which is also true in mixtures. In mixtures LWCA act according to the dose addition mechanism, suggesting each acid acts separately and no other type of interaction occurs between the acids. Thus, knowing concentration of each acid in a plant growth substrate, total phytotoxicity can be evaluated, e.g. nontoxic concentration (EC10) for equimolar mixture of formic, acetic and propionic acid is below 1000 mg/kg dm.

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