**Master of Science thesis**

# Acute and subchronic phytotoxicity of volatile fatty acids (VFAs)

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## ABSTRACT

Volatile fatty acids (VFAs) are aliphatic short chain carboxylic acids produced during fermentative organic matter decomposition. They are widespread among organisms and environments in the world, and provide a substantial carbon and energy supply for many organisms. As a part of soil ʾs organic acid pool they play a role in plant nutrient solubilization and transportation. Phytotoxic effects of VFAs have been known for years. Especially composts can provide a source of VFAs. Raw, unmaturated, composts, exhibiting phytotoxic effects, have been shown to correlate with their VFA content. Composts are used as ameliorants, however if still active, they can pose adverse effects on arable plants. Lack of information on consistent and comparative phytotoxic assessment with statistical analysis of data has led into contradictive conclusions of VFAs ʾ different phytotoxic potentials.

The purpose of this study was to evaluate acute and subchronic toxic response of VFAs on dicotyledon garden cress (*Lepidium sativum*) and monocotyledon Italian ryegrass (*Lolium multiflorum*). Eight different VFAs (formic, acetic, propionic, *iso*-butyric, butyric, *iso*-valeric, valeric and caproic acids) were tested and dose-response relationships were modeled for each acid. In acute tests comparisons between toxicities of VFAs were made with radicle length at concentration 1.2 mmol/l. In subchronic growth experiments comparison was done using EC50 values for seedling emergence and shoot dry weight that were calculated from dose-response models.

A positive relationship between length of carbon skeleton of studied VFAs and increased toxicity was observed in subchronic growth experiments. Difference in response sensitivity between species tested was significant. In acute germination tests garden cress did not exhibit any germination inhibition up to concentration 9.6 mmol/l. Germination of Italian ryegrass was more sensitive to toxicity posed by VFAs and concentrations 2.4 - 4.8 mmol/l showed substantial decrease in germination. The species sensitivity to VFAs was reversed in subchronic growth experiments, where garden cress showed more sensitive response compared to Italian ryegrass. In spite of high variability in results by different response parameters formic and acetic acids were always the least toxic VFAs at both tested exposure levels. Thus, the importance of C3 - C6 VFAs in compost phytotoxicity assessment should be stressed.

Increased electrical conductivity or acidic pH were not considered as principal toxic factors of inhibitive response of VFAs.

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## TIIVISTELMÄ

Haihtuvat rasvahapot ovat alifaattisia lyhytketjuisia karboksyylihappoja, joita muodostuu orgaanisen materian hajoamisen tuotteina käymisprosessissa. Niillä on maailmanlaajuista merkitystä eri ympäristöissä ja organismeissa laajan esiintymiskentän vuoksi. Ne ovat tärkeä hiili- ja energialähde monille organismeille. Osana maaperän orgaanisia happoja niillä on myös merkitystä maaperän kasviravinteiden ja mineraalien liukenemis- ja kulkeutumisprosesseissa. Haihtuvien rasvahappojen fytotoksiset ominaisuudet on tunnettu jo vuosia. Erityisesti kompostit voivat olla haihtuvien rasvahappojen lähteitä. Raakojen kompostien fytotoksisuuden on todettu korreloivan kompostien haihtuvien rasvahappopitoisuuksien kanssa. Maanparannusaineina kompostit voivat raakoina haitata peltokasvien kasvua. Johdonmukaista, tilastollisesti merkitsevää arviointia eri haihtuvien rasvahappojen fytotoksisuuden välillä ei ole tehty.

Tämän tutkimuksen tarkoituksena oli arvioida haihtuvien rasvahappojen akuuttia ja subkroonista vastetta vihanneskrassilla (*Lepidium sativum*) ja Italian raiheinällä (*Lolium multiflorum*). Kahdeksan eri haihtuvaa rasvahappoa (muurahaishappo, etikkahappo, propionihappo, *iso*-voihappo, voihappo, *iso*-valeriaanahappo, valeriaanahappo, kapronihappo) testattiin ja annos-vaste suhde pyrittiin mallintamaan jokaiselle hapolle erikseen. Happojen välistä vertailua varten subkroonisissa testeissä määritettiin EC50 arvot annos-vaste mallista verson kuivapainolle ja taimen nousulle maasta. Akuuteissa tutkimuksissa käytettiin haihtuvien rasvahappojen myrkyllisyyden vertailuun sirkkajuuren pituuden vastetta happopitoisuudessa 1,2 mmol/l.

Subkroonisissa kasvutesteissä havaittiin positiivinen suhde haihtuvien rasvahappojen hiiliketjun pituuden ja lisääntyneen myrkyllisyyden välillä. Muurahaishappo ja etikkahappo olivat vähiten fytotoksisia molemmilla altistustasoilla, vaikka tulokset eri vastemuuttujilla, kasvilajeilla ja altistusajoilla eivät aina olleet johdonmukaisia. Tämä tulos painottaa C3 - C6 rasvahappojen merkitystä kompostin fytotoksuuden arvioinnissa. Tutkimuksessa käytettyjen kasvilajien välillä oli merkitseviä eroja. Akuutissa altistuksessa haihtuvien rasvahappojen ei havaittu vaikuttavan krassin itävyyteen negatiivisesti edes konsentraatiossa 9,6 mmol/l, kun taas pitoisuudet 2,4 - 4,8 mmol/l vähensivät merkittävästi Italian raiheinän itävyyttä. Subkroonisissa altistuskokeissa herkkyys haihtuville rasvahapoille kasvilajien välillä havaittiin käänteiseksi.

Lisääntyneen sähkönjohtokyvyn tai happamuuden vaikutusta ei voitu pitää tutkimuksen perusteella haihtuvien rasvahappojen inhibitoristen kasvivasteiden ensisijaisena syynä.

# Content

# 1 INTRODUCTION

## 1.1 Characteristics of volatile fatty acids (VFAs)

Volatile fatty acids (VFAs) are usually considered to contain carbon skeleton of no more than six carbon atoms in length (Cherrington et al., 1991; Hobson & Wheatley, 1993). Volatile fatty acids are in nature organic, aliphatic and saturated weak monocarboxylic acids and in literature one can encounter them under variety of other names and abbreviations. For example low-weight carboxylic acids (LWCAs) (Himanen et al., 2006), short chain fatty acids (SCFAs) (Cherrington et al., 1991), low molecular weight aliphatic acids (LMWAs) or volatile aliphatic acids (VAAs) (Baziramakenga & Simard, 1998) and still the usage of as broad description as volatile organic acids (VOAs) (Brinton, 1998) is also common.

VFAs are, as the name itself implies, volatile by nature. This is of course a relative term. By comparing vapor pressures of different volatile fatty acids in 25 °C one can get an idea of their volatility (Table 1). Only formic acid possesses higher volatility than water. All other VFAs have lower vapor pressures and six carbon caproic acid could be described as semivolatile (Mukund et al., 1995). The volatility on average decreases as the parent carbon chain of a particular VFA prolongs. Still, volatility does not represent all important aspects of odor control of these volatile organic compounds (VOC) as VFAs are components of malodorous compounds found in e.g. animal excrements (O ' Neill & Phillips, 1992). As an important aspect of odor nuisance in animal farming or composting may be the fact that although volatility decreases as length of the VFA carbon skeleton prolongs, the threshold concentration for detection becomes also smaller (Table 2). The threshold detection limit of some VFAs is of same order of magnitude or higher as that for, generally known, malodorous sulphur containing compounds, like $H_2S$.

Table 1. Vapor pressures (VP) of selected volatile fatty acids, water and ethanol at 25 °C (Lide, 1996), their boiling points (BP) at outside pressure of 760 mmHg and their melting points (MP) (Lide, 2004).

| VFAs | VP (KPa) | BP (°C) | MP (°C) | VFAs + other | VP (KPa) | BP (°C) | MP (°C) |
|---|---|---|---|---|---|---|---|
| Formic | 5.75 | 101 | 8.3 | *Iso*-valeric | 0.067 | 176.5 | - 29.3 |
| Acetic | 2.07 | 117.9 | 16.6 | Valeric | 0.024 | 186.1 | - 33.6 |
| Propionic | 0.553 | 141.5 | - 20.5 | Caproic | 0.005 | 205.2 | - 3 |
| *Iso*-butyric | | 154.5 | - 46 | Water | 3.169 | 100.0 | 0.0 |
| Butyric | 0.221 | 163.8 | - 5.1 | Ethanol | 7.87 | 78.3 | - 114.1 |

Table 2. Odor detection threshold ($mg/m^3$) for humans of various VFAs identified in livestock wastes (Gemert et al., 1977). A known malodorous compound, hydrogen suphide ($H_2S$), is given as a reference value[1].

| Formic | Acetic | Propionic | *Iso*-butyric | Butyric | *Iso*-valeric | Valeric | Caproic | $H_2S$ |
|---|---|---|---|---|---|---|---|---|
| 2 - 640 | 0.025 - 10 | 0.003 - 0.89 | 0.005 - 0.33 | 0.0004 - 42 | 0.0002 - 0.0069 | 0.0008 - 0.12 | 0.02 - 0.52 | 0.0001 - 0.27 |

[1] Hydrogen sulphide is also found in livestock wastes (O ' Neill & Phillips, 1992).

The only chemically relevant functional group of VFAs, the carboxylic group, makes these compounds acidic in nature. This functional group dissociates in water solutions followingly (Harris, 2003):

$$\underset{\text{molecular form of VFA}}{R\text{-}COOH} + H_2O \rightleftharpoons \underset{\text{anionic form of VFA}}{R\text{-}COO^-} + H_3O^+,$$

where R - represents H in formic acid or aliphatic hydrocarbon skeleton in other VFAs. But no relevant differences in acidities between different VFAs can be observed. Comparing acids strengths (Table 3) only the shortest carbon chain VFA, formic acid, stands really out with its stronger acidity, $pK_a = 3.75$ , which is about 1 $pK_a$-lower compared to other VFAs.

Table 3. Values of $pK_a$ at different ionic strengths (IS, in moles) of various volatile fatty acids (VFAs) at 25 °C, (Martell & Smith, 1979)[1].

| IS | Formic | Acetic | Propionic | *Iso*-butyric | Butyric | *Iso*-valeric | Valeric | Caproic |
|---|---|---|---|---|---|---|---|---|
| 0 | 3.745 | 4.757 | 4.874 | 4.849 | 4.819 | 4.781 | 4.843 | 4.857 |
| 0.1 | 3.53 | 4.57 | 4.67 | 4.64 | 4.62 | 4.56 | 4.62 | 4.64 |
| 1 | 3.55 | 4.56 | 4.57 | 4.63 | 4.63 | 4.58 | 4.64 | 4.63 |

[1] As Martell and Smith (1979) state, $pK_a$-values of infinite dilution (IS = 0) are the values usually used in theoretical discussions.

As is apparent from the dissociation of VFAs, their concentration has also an effect on the pH (concentration of $H^+$) (Harris, 2003) and electrical conductivity (EC) of their particular occurrence environment as the number of ions increases (CWT, 2004). Stronger acids (smaller $pK_a$-values) also increase the electrical conductivity and $H^+$ concentration to the greatest extend, when comparing different VFAs of same concentration (in moles). The relationship between pH (concentration of $H^+$) and dissociation proportion of monocarboxylic VFAs is described by Henderson-Hasselbalch equation (Formula 1).

$$pH = pK_a + \log_{10} [A^-] / [HA] \qquad [1]$$

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

**1.2 Volatile fatty acids: origin and occurrence in soils and composts**

VFAs are currently understood as being products of fermentative decomposition of organic matter, namely anaerobic in nature (Schuman & McCalla, 1976; Rao & Mikkelsen, 1977; Hobson & Wheatley, 1993; Madigan et al., 2003). The production reactions of straight chain VFAs may involve fermentation of carbohydrates and breaking down long chain fatty acids, but fermentation of carbohydrates and deamination of amino acids may also results in branched-chain VFAs (Hobson & Wheatley, 1993).

Volatile fatty acids are very common and widespread in the nature and among living organisms. To get an idea of their agrological and natural importance in the world one should consider that VFAs have been shown to built up in soils of anaerobic nature (Stevenson, 1967); waterlogged soils used for rice cultivation (Rao & Mikkelsen, 1977) or with forest vegetation (Sanderson & Armstrong, 1980). Green manure plough in to soils has shown to enhance their VFA production (Takijima et al., 1960; Chandrasekaran & Yoshida, 1973). Lynch (1977) also demonstrated that VFAs (especially acetic acid) can be produced also from wheat straw in phytotoxic concentrations, when subjected to anaerobic

decomposition conditions. These kinds of conditions could be present e.g., when after harvesting direct drilling is employed. And indeed it is known, as is stated by Zucconi et al. (1981a): " *..introduction of agricultural residues in soil causes toxic responses which may at times spoil the crop. This is particularly so when plowing in fresh plant material (including green manuring); yet it may occur with the decomposition of straw - mulch as well*."

Although composting is defined as an aerobic process by its nature (Kirchmann & Widén, 1994) especially high concentrations of VFAs in raw composts and in raw compost materials have been found and demonstrated to show inhibitory, phytotoxic effect on plants (Schuman & McCalla, 1976; DeVleeshauwer et al., 1981). The production of VFAs in compost has been widely reported also elsewhere (Chanyasak et al., 1982; Kirchmann & Widén, 1994; Brinton, 1998; Wiles et al., 2000; Himanen et al., 2006). There have been considerable differences in the reported volatile fatty acid contents. One contribution to this might involve the laboratory analytical technique used for identification of different VFAs, variations in detection limits (Himanen et al., 2006) and different extraction methods mentioned in literature (DeVleeschauwer et al., 1981; Baziramakenga & Simard, 1998; Himanen et al., 2006). The other reason is of more vigorous nature, since already at the beginning of the composting process its different raw materials can have and lead to observed diverse VFA concentrations (Chanyasak et al., 1982). And yet one has to mention the different composting conditions and inoculated microbial populations that can change the proportions of different VFAs produced (Brinton, 1998). Brinton (1998) mentions *Streptococcus fecalis* bacteria producing among other compounds 15.4 µmol of formic acid from 100 µmol of glucose at pH 5 and at pH 9 its production is 53 µmol.

As composting is in nature a process, where one can detect e.g. different pH and temperature changes throughout the process (García et al., 1991; Kirchmann & Widén, 1994), the difference in VFAs production is obvious. Still, one could detect a pattern in an overall change of VFA content in composts. When combining the work of e.g. DeVleeshauwer et al. (1981), Chanyasak et al. (1982), Saviozzi et al. (1987), Kirchmann & Widén (1994), Brinton (1998), Wiles et al. (2000), the composting process can be divided at least into three phases on behalf of VFA occurrence. Phase one is a short initial phase, where production of VFAs is increasing and is exceeding the decomposition or evaporization of VFAs. Second phase is equilibrium of high VFA production and decomposition; at this stage the highest VFA concentrations are obtained. This is followed

by third phase, where VFA production is smaller than its decomposition or evaporization and this decreases the content of VFAs in compost, as maturation of compost proceeds, to virtually zero. Final levels of zero, found in literature, can be questionable. They may be obscured by detection limits, since small concentrations of VFAs have been reported also from older composts samples (Schuman & McCalla, 1976; Baziramakenga & Simard, 1998). Several studies have pointed out that the peak formation of VFAs is associated with the termophilic phase of composting (Kirchmann & Widén, 1994; Wiles et al., 2000).

VFAs are a sign of fermentative anaerobic degradation, but also a sign of hypoxic, oxygen depleted, conditions in aerobic composting. So, VFAs are products of normal composting process (Brinton, 1998). It is interesting that although experiments with increased aeration of compost have been made, composts have still produced VFAs (DeVleeschauwer et al., 1981; Wiles et al., 2000). Wiles et al. (2000) suggested, according to their studies, that to reduce odor problems it may be important to concentrate on setting optimal conditions for quick launch of termophilic phase and indeed use low aeration, conditions set for rapid decomposition of VFAs and reduced evaporation. And indeed composting definition revision has been suggested by Kirchmann and Widén (1994) to "*..auto - heated, aerobic - anaerobic decomposition allowing a succession of mesophilic and thermophilic microbial populations.*"

### 1.3 Effects of VFAs on different organisms

Effects of volatile fatty acids have tremendous importance in the world. One could also point to the essential role of these acids in maintaining human welfare (agrological, food importance) and health aspects. Merely considering the impact of VFAs on energy supply to world organisms gives insight in to their importance. Volatile fatty acids are the main end products of microbial anaerobic cellulose (a carbohydrate) degradation in ruminants (Dehority, 1991). They represent an easy absorbable, high energy, nutritive substance for ruminants, but also for micro-organisms (Cherrington et al., 1991). Interestingly, it has also been estimated that even among people in modern society with western eating habits up to 10% of foods energy could be derived from cellulose by microbial fermentation products, especially from large intestine (Turunen, 1995). As part of soil organic acid pool (all organic acids in soil at a particular time), VFAs play also role in soil nutrient chemistry as organic acids have a significant role in solubilization, mobilization and transportation of soil mineral matter (Stevenson, 1967; Jones et al., 2003) and for that matter plant and human welfare. In world of plants there has been observed also positive effects on seed

germination. VFAs have been demonstrated to induce the process of dormancy breaking in rice seeds (Cohn et al., 1987).

Some special inhibitory effects of different VFAs are well known and documented and utilized in universal human use. Everyone has some experience in household use of vinegar (acetic acid) in pickling or has unknowingly smelled the malodors of VFAs in some dairy products, e.g. in aged, smelly, cheese. Two VFAs (acetic and propionic acid) are listed and characterized by Finnish Food Safety Authority, Evira, in it's food additive manual (Evira, 2006) as preservatives, namely E 280 and E 260, with expressing propionic acid as an effective agent against molds. Although Evira (2006) states that inhibitory effect of acetic acid on microbial growth is mostly caused by its decreasing effect on pH, according to the review by Cherrington et al. (1991) mineral acids (strong acids, expressed by their very low $pK_a$-values) have not been able to show such an antimicrobial effect compared to organic acids. It seems that, although as stated earlier considering VFAs as a source of energy for microbes, it is merely a question of concentration for these organic acids to become inhibitory in their effects (Cherrington et al., 1991). Interestingly, though showing inhibitory effects on microbes, these substances, E 208 and E 260, are considered so safe that ADI values (acceptable daily intake) are not considered necessary (Evira, 2006).

Inhibitory effects of VFAs are widely utilized also in agriculture. By 31.12.2006 Evira had two biocides listed in its registry, where the active substance was VFA (Evira, 2007). In herbicides, two product names, Ecoval No. 1 mot ogras, by Durga Technologies ApS and Bio Neko Rikkatorjunta, by Neko Oy Ab, where in the registry. Acetic acid, as an acting agent, is used on green foliage in final application concentration of 62 - 120 g /l. The other VFA, formic acid, is used as a pesticide in honey bee farming. Another inhibitory effect of VFAs of agrological importance is the usage VFAs in silage making, where especially formic acid has been commonly utilized (Pursiainen et al., 2004; Saarisalo & Jaakkola, 2006). The increasing understanding of VFAs has revealed recently also some important plant disease control aspects connected to usage of animal manure compared to synthetic fertilizers. E.g. VFAs, found in liquid swine manure, have been shown to exhibit the ability of killing microsclerotia of *Verticillium dahliae* (known fungal plant disease) in same concentrations as found in raw manure (Tenuta et al., 2002). Sole butyric acid (solutions and its fumes) has been demonstrated to provide effective protection in laboratory scale

experiments against many soil fungal and nematode plant pathogens by killing them (Browning et al., 2006).

## 1.4 Phytotoxic mechanisms of volatile fatty acids

Toxicity of VFA is rather complex phenomenon. Data found in literature contains contradictory conclusions. In the following several different aspects of the phytotoxicity mechanisms of VFAs are addressed.

### 1.4.1 Source of conductivity and acidity

There are two important aspects to be considered, when describing phytotoxic aspects of VFAs. As was shown in chapter 1.1 the dissociation of VFAs will increase the electrical conductivity and increase the concentration of $H^+$ ions (decrease pH) of a growth medium. It should be realized that this positive correlation between concentration of VFA and electrical conductivity or acidity could be found in all phytotoxic studies (unless buffered). These factors may themselves pose adverse effects on plants and can lead to misinterpretation of the primary phytotoxic cause of VFAs. But, if concentrations rise enough, in addition to toxicities by parent VFA molecules there can be expected toxicity also by higher dissolved ion concentrations.

Increased electrical conductivity means increase in dissolved ions (electrolytes) that are electric current conductors in water solutions (CWT, 2004) and rise in ionic strength of the medium (Harris, 2003). Higher concentration of dissolved ions decrease free water content, water potential (Bewley & Black, 1994; Taiz & Zeiger, 1998). The water potential difference between soil water and water inside plant is especially crucial in seed germination stages. As Bewley and Black (1994) state: "*Germination begins with water uptake by seed (imbibition)…*" The imbibition is most effective, when the water potential difference is the highest between water potentials of soil and seeds. Pure water holds highest water potential and high electrical conductivity means more electrolytes in water and thus lowering the soil water potential (Bewley & Black, 1994). If the water potential difference between environment and plant decreases, it causes several problems, which can be put in three categories: osmotic, ionic and habitat effects (Fitter & Hay, 1987). In osmotic effects plant has to overcome the water potential of surrounding medium for water uptake. When water potential in soils is very low it can decrease below osmotic potential of a plant, which causes e.g. wilting, because the turgor pressure can not be anymore maintained (Taiz & Zeiger, 1998). In ion effects the high concentration of electrolytes can

be itself toxic. Due to high ionic content, in habitat effects, the soil aggregation can be different and results in disadvantageous soil structure.

Acidicity posed by VFAs can also negatively affect plants. It has been demonstrated that at pH below 5 the availability of soil nutrients to plants is usually lowered (Taiz & Zeiger, 1998). But as the pH controls solubilities of mineral cations, including toxic metals, they may become also enriched into plant toxic concentrations in acidic soils. The increase in solubilities of heavy metals can lead to toxic concentrations in plants due to lowered soil pH (Fitter & Hay, 1987). These are indirect adverse effects of high $H^+$ ion content in plant growth media. It should be realized that for the most plants $H^+$ ions become toxic *per se* at concentrations above 1 mM, that is pH below 3 (Fitter & Hay, 1987). Enzymes have different pH optima and can also be affected by low pH. For example, during germination of barley seed starch reserves mobilized by α-amylase has an optimum of pH 5.5 - 6.0 (Bewley & Black, 1994).

### 1.4.2 Lipophilicity and phytotoxicity

In spite of fact that as adverse effects of VFAs posed by higher VFA content of a growth medium go hand in hand with increased electrolyte concentration and acidity, data found in literature (Prill et al., 1949; Takijima, 1964; Buller et al., 1976; Lee, 1977; Marambe et al., 1993) support the hypothesis that there is a tendency towards higher phytotoxicity as the length of the VFA carbon chain increases or, in other words, lipophilic features become more pronounced (Table 4).

Table 4. Lipophilic nature of selected VFAs as expressed by octanol / water partition coefficient, log $P_{ow}$ at 25 °C, according to Lide (2004), *iso*-butyric acid (ICSC, 2002) and *iso*-valeric acid (estimate by Suzuki et al., 2000). For comparisons values for same carbon skeleton possessing alcohols, known fat soluble compounds, are included.

| VFAs | log $P_{ow}$ | VFAs | log $P_{ow}$ | Alcohols | log $P_{ow}$ | Alcohols | log $P_{ow}$ |
|---|---|---|---|---|---|---|---|
| Formic | - 0.54 | Butyric | 0.79 | Methanol | - 0.74 | 1-Butanol | 0.84 |
| Acetic | - 0.17 | *Iso*-valeric | 1.16 | Ethanol | - 0.30 | *Iso*-pentanol | 1.28 |
| Propionic | 0.33 | Valeric | 1.39 | 1-Propanol | 0.25 | 1-Pentanol | 1.51 |
| *Iso*-butyric | 0.88 | Caproic | 1.92 | *Iso*-butanol | 0.74 | 1-Heksanol | 2.03 |

Lipophilicity and the interferences with lipid membranes have been in the core of discussions by many authors, when phytotoxic action elicited by different VFAs has been in the focus. As early as 1949 Prill with coauthors published their work on different

organic acids including five VFAs. Measuring acute response of wheat (*Triticum* sp.) primary root elongation in pure culture solutions, they used VFA containing growth solution that was always buffered to pH 4.3. Although no statistical testing was performed, on average, the toxic potential increased with the carbon skeleton length. Prill et al. (1949) found just one exception, formic acid, which seemed to be inhibiting wheat primary root elongation more than acetic acid. Suggested explanation for this exception was aldehydic nature of formic acid. Prill et al. (1949) tend to think that different surface activity would be the cause for observed difference in toxicities, as was already suggested by Traube and Rosenstein in 1919. Prill et al. (1949) showed with studied VFAs that depression in wheat primary root elongation followed the sequence of acetic < formic < propionic < butyric < caproic < capric acids. It is interesting to notice, how low concentrations could really inhibit the root growth. For example concentration of acetic acid 0.05 mmol/l inhibited already the root elongation to around 50% of controls value. These finding were supported by studies on rice growth by Takijima (1964). The inhibitory effect of VFAs on rice growth was growing in a row formic < acetic < propionic < butyric. Both shoots and roots of rice were affected (Takijima, 1964).

The distortion of membrane function by VFAs has been described by several studies, especially in plant roots, which have been shown to be sensitive to VFAs. Rao & Mikkelsen (1977) observed with acetic, propionic and butyric acids that VFAs decreased elongations of rice (*Oryza sativa*) seedling roots and inhibited new root initiation. Phosphor and potassium uptake by roots was reduced. Lee (1977) found that barley (*Hordeum vulgare*) roots treated with short chain fatty acids (acetic, butyric, caproic, caprylic acids) caused in acute exposure experiments loss of potassium, nitrate and UV-absorbing material to the external medium. UV-absorbing material was suggested to contain nucleotides or aromatic compounds. The increased cell membrane permeability of intact roots of barley and rice to ions under study was suspected in these studies to be behind the effects of VFAs. Interestingly, known fat-soluble substances, such as diethyl ether or chloroform, did not cause leakage of studied electrolytes from barley root (Lee, 1977). But Lee (1977) reported the observations about the suspected important role of VFA ’s lipophilic nature. Radioactive labeling did indeed indicate 3.4 times faster absorption of *n*-octanoic acid (log P ≈ 2.9; $pK_a$ ≈ 4.9) by barley root system compared to acetic acid (log P ≈ - 0.17; $pK_a$ ≈ 4.76) in the first 30 seconds of immersion!

Since the works of Prill et al. (1949), Takijima (1964), Rao and Mikkelsen (1977) and Lee (1977) are ultimately culminated to a question of VFAs being able to penetrate or incorporate into cell lipid membrane and distort it ʼs and cell ʼs functions, the findings support the idea that the longer the carbon chain of VFAs or the more lipophilic is their nature, the smaller concentrations are needed for equivalent response effect to be observed. In fact, the study by Marambe et al. (1993) on germination events of sorghum (*Sorghum bicolor*) revealed that the long chain fatty acids could be more inhibitory in their effects than short or medium chain fatty acids.

There are also other inhibitive actions of VFAs. Acetic and propionic acids have been demonstrated to inhibit the photosynthetic electron transport between phototosystem II and photosystem I in lyophilized chloroplast thylakoids (Helfrich et al. 1998). Buller et al. (1976) reported VFAs as being powerful inhibitors of gibberellin induced amylolysis (inhibiting hydrolase production) with increased inhibition hand in hand with longer parent carbon chain.

### 1.4.3 Phytotoxicity of dissociated versus undissociated forms

The studies by Jackson and Taylor (1970), Rao and Mikkelsen (1977) and Lee (1977) address important aspects of VFAs toxicity. Even if the toxic effect correlates with pH (more toxic with lower pH) these studies have shown that it is in fact the quantity of undissociated acid (HA) that counts most. This is the form of VFAs that is lipophilic and most phytotoxic. There have been suggestions also in literature for the antimicrobial activity of VFAs to be associated with the undissociated forms VFA molecules (Cherrington et al., 1991). And, according to Henderson-Hasselbalch equation (Formula 1, chapter 1.1), the proportions of VFAs in the undissociated forms (HA) are dependent on pH, thus, the apparent misleading correlation of toxic effect of decreasing pH and toxicity by VFA that go hand in hand can be understood. The lower the pH the higher is the proportion of phytotoxic protonated form (HA) of VFA in the growth medium. Studies made by Lee (1977) and Rao and Mikkelsen (1977) reported also the notion that the higher was the pH the lower or absent were the adverse effects elicited by VFAs of the same concentration.

A question thus arises, how to distinct the adverse effects posed by increased acidity and ionic strength from toxicities of VFAs? On these problems studies have been made by Rao and Mikkelsen (1977), Lee (1977) and Shiralipour and McConnell (1997). They tested different VFAs with adjusted pH and compared response with reference compounds of

similar or dissimilar chemical/physical properties that of VFAs. According to these studies, lowered pH or increase in ionic strength are not the main reasons for observed adverse effects.

### 1.4.4 Contradictions

Not all results correlated well with the general conclusions and suggestions stated above. As can be deduced from work by Rao and Mikkelsen (1977) different parts of rice seedling can have different responses. Especially measurements of parameters from shoots (dry weight and potassium content) did not always correlate well with VFA toxicity as was the case with roots. In fact, there were instances were VFAs showed signs of none effect or even induction of growth response parameter, especially, when VFAs were added to higher pH solution than would be otherwise observed by pure VFAs.

Further exceptions to discussion about the VFA toxicities are presented by Jackson and Taylor (1970). They observed that uptake and retention of ions $Ca^{2+}$, $Cl^{-}$, $Na^{+}$, $K^{+}$ in barley (*H. vulgare*) root differed upon exposure to C1 - C3 VFAs and attributed this also to increased root cell membrane permeability to given ions due to entry of lipophilic undissociated forms of VFAs. But their findings suggested that the lighter was the molecular weight of VFA the more toxic it was.

Ulbright et al. (1982a, 1982b) findings are also partly contrary to observations reported previously. Shoots of lettuce (*Lactuca sativa*) and radicle emergence were influenced by the length of VFA carbon chain, but some response parameters (e.g. root growth) did not correlate well with assumptions of a longer parent carbon chain VFA eliciting more sensitive response. Although, as stated previously, roots have been demonstrated to be sensitive on exposure to VFAs (also acknowledged by Ulbright et al. (1982a)), in work by Ulbright et al. (1982a) root growth was not affected by the length of VFA carbon chain (C3 - C8). They concluded controversially that, in inhibition, permeability and membrane perturbation were not the principal factors of toxic mechanisms at least in lettuce root growth. Ulbright et al. (1982a) suggested that the toxic effects of the shortest VFAs, observed by other authors, might have been underestimated due to significant evaporative loss of lighter VFAs compared to longer carbon chain VFAs. Also Ulbright et al. (1982a) did not support the idea of the importance in understanding and modeling the toxicities of VFAs by their protonated and dissociated form ratios. But it should be stressed that although volatility of shorter carbon chain VFAs may have affected results of different VFA phytotoxicity studies the notation made by Lee (1977) of quicker C8 (octanoic) fatty

acid absorption into the barley roots compared to acetic acid (C2) could indeed mean, as Lee (1977) states, that longer chain VFAs could influence the cells to quicker and greater extend, even if the toxic mechanisms of different VFAs were the same.

Cohn et al. (1989) came with a very interesting notion. Their study evaluated the seed (red rice, *O. sativa*) dormancy breaking activity of different compounds (including VFAs) and came to the same conclusions as is suggested for the inhibitory action of different VFAs. There was a correlation between VFAs lipophilicity and dormancy breaking concentration (the more lipophilic its nature the smaller was the concentration needed for 50 % germination to occur). In previous studies at different pH, Cohn et al. suggested already in 1987 that dissociated forms of VFAs were not responsible for dormancy breaking success and that possible acidification of seed inner environment could initiate the dormancy breaking response. This hypothesis was somewhat halted by findings of Cohn et al. (1989) that alcohols and other lipophilic compounds could also break the dormancy, although in higher concentrations than carboxylic acids (measured as 50% germination response). But it needs to be pointed out that in Cohn et al. (1989) work the alcohols were tested in buffered acidic solutions, which could have increased the effects of non-acidic lipid soluble substances.

Lowered plant metabolic rate (respiration) caused by VFAs (C1 - C3) has also been reported by Jackson and Taylor (1970) as a secondary symptom of VFAs toxicity. Ulbright et al (1982a) could not detect respiration inhibition with C7 fatty acid.

### 1.4.6 Growth regulatory role

It is very important to note, that although as stated in chapter 1.1 that VFAs are currently mainly understood as by-products of fermentative microbial degradation of organic matter, different fatty acids, also VFAs, have been found in many plants, especially in dormant plant organs (Berrie et al., 1976; Berrie et al., 1979; Ulbright, 1980). As energy rich compounds they can act as food reserves in seeds (Ulbright, 1980), but their role in regulating dormancy or germination has been suggested. The study of Berrie et al. (1979) concluded that the concentrations of C6 - C10 fatty acids, found endogenously in oat (*Avena fatua*) seeds, correlated well with the dormancy status of the seeds. The notation was also made that the germination inhibition potential of different fatty acids varied and their relative abundances differed in separate phases of seed maturation (preharvest) or storage (postharvest). The inhibition by VFAs can occur rather abruptly (threshold phenomenon) and can lack graded phenomenon common for e.g. inhibition by known

dormancy regulating abscisic acid, which can act on a concentration range over 10 000 fold (Buller et al., 1976). Ulbright et al. (1982a) have shown in lettuce seeds (*L. sativa*) in acute exposures that almost total inhibition of germination and none effect take place within concentration range from 0.5 - 6 mmol/l, when treated exogenously with of C5 - C7 fatty acids solutions.

Although the action of fatty acids in function of growth regulation may not be finely "tuned" by wide concentration range, there have been demonstrated other mechanisms regulating the effects of fatty acids on e.g. radicle emergence. There is evidence that light may effectively reverse the inhibition. Studies by Ulbright et al. (1982a) indicate that short irradiation with red light or with appropriate combination of far red and red light after seed exposure to vapors of valeric acid (C5) can resume radicle emergence in lettuce seeds close to control values (otherwise inhibiting radicle emergence by 70 %). Berrie et al. (1976) findings support this phenomenon. There is some controversy about whether plant hormones could reverse the inhibition of fatty acids. Berrie et al. (1976) found that gibberellins ($GA_3$) could very successfully reverse the germination inhibition posed by C6 - C10 fatty acids, but Ulbright et al. (1982a) study on C5 - C7 fatty acids could not support these finding. Nor did, in Ulbright et al. (1982a) study, the common auxin, indole-3-acetic acid (IAA) reverse inhibition at studied concentration ranges 0.001 - 1 mmol/l and 0.001 - 0.1 µmol/l. Berrie et al. (1976) also reported that kinetin could reverse the inhibition by fatty acids C6 - C10 well, although at studied concentrations (0.1 and 1 mmol/l) the germination success was not resumed entirely up to levels of the control.

### 1.4.7 Importance of functional group

The importance of hydrophilic group in VFAs on the appropriate function of VFAs has been given credence. Ulbright et al. (1982a) reported that short chain alcohols (C3 - C8) could also inhibit radicle emergence and root growth in lettuce (*L. sativa*) with increasing toxic effect hand in hand with longer carbon chain, although alcohols were observed as less effective inhibitors compared to C3 - C8 fatty acids studied. Findings of higher concentration of alcohols needed to observe same effects encountered from exposures to VFAs can be also supported by Cohn et al. (1989), albeit the study objective was concentrating on dormancy breaking activity, not inhibition. Lack of hydrophilic group in a compound can be determinant factor for it to act similarly to VFAs as alkanes (C5 - C7) did not have any effect on lettuce radicle emergence or seedling growth at studied concentration (3 mmol/l) (Ulbright et al., 1982a). These finding can be at least partly

supported by Lee (1977) with barley root ion loss studies, which indicated no or very small loss of ions after exposure to lipophilic substances like chloroform, diethyl ether, as previously mentioned.

But it should be stressed, although, that as hydrophilic part of VFAs is essential for elucidating effects observed, too hydrophilic nature of organic acids makes them less phytotoxic. For example, di- or tri-basic acids have been demonstrated to elicit less toxic effects compared to aliphatic monobasic acids (Prill et al., 1949; Takijima et al., 1960). In fact, according to Takijima et al. (1960), rice root elongation followed sequence of inhibition: " *aromatic acids (benzoic, cinnamic and salicylic)* > *aliphatic mono - basic acids (formic - capric)* ⩾ *aliphatic di- and tri-basic acids (oxalic etc.) or oxy acids (lactic etc.).*" It should be noted that many important metabolic acids, common in organism, are much more hydrophilic di- or tri-basic or oxy acids, such as citric, malic, succinic or lactic acids, and this fact could stress the particular phytotoxic importance of VFAs in soils.

**1.5 Goals**

The inhibitory effects of VFAs have been known for years. Consistent and comparative (to the extent of statistical significance) assessment of their toxic potentials at different exposure timeframes and with all carbon skeleton lengths (C1 - C6) VFAs has not been made. This study reports the phytotoxic effects and the different toxicities of eight, C1 - C6, VFAs on early stages of plant development at acute and subchronic levels. Present study is part of research on compost phytotoxicity.

The main objectives of present study were:

1. to model dose-response relationships for eight (C1 - C6) VFAs and calculate EC50 for each acid

2. to rank VFAs according to their phytotoxic potential

3. to compare phytotoxicity of VFAs of acute and subchronic exposure

4. to compare sensitivity of two plant species, dicotyledon *Lepidium sativum* (garden cress) and monocotyledon *Lolium multiflorum* (Italian ryegrass), to VFAs

# 2 MATERIALS AND METHODS

This study was conducted on eight volatile fatty acids of carbon (C) chain lengths from one to six. Studied acids (VFAs) were: formic (C1), acetic (C2), propionic (C3), *iso*-butyric (C4), butyric (C4), *iso*-valeric (C5), valeric (C5) and caproic (C6).

Tested plant species included monocotyledon Italian ryegrass (*Lolium multiflorum*) and dicotyledon garden cress (*Lepidium sativum*). Information on chemicals and tested plant seeds is provided in Appendix 1.

"Preliminary Experiments" will go in detail through preparations and search for suitable conditions and performance of experiments. Second part of "Materials and Methods" contains information on actual experiments performed to obtain answers according to the objectives stated in chapter 1.5. In the last part of this chapter experiments with growth substratum will be presented, which will describe some tests performed in a search for understanding of pH variation of growth substratum in subchronic growth experiments.

## 2.1 Preliminary experiments

### 2.1.1 Preliminary acute experiments

Tests were made to select volatile fatty acid concentration range correctly for actual experiments and to obtain preliminary data on test design. Series of acid concentrations was the same for every single tested VFA, with the exception of caproic acid. The acid dilution series consisted of five concentrations with a nearly hundredfold range and a constant multiplication factor of three used between every concentration. Concentrations were 0.5, 1.5, 4.5, 13.5, 40.5 mmol/l. For caproic acid the concentrations were 0.2, 0.6, 1.8, 5.4, 16.2 mmol/l. Deionized water was used for diluting. In the preliminary experiments original acids were assumed to have 100 % purity (analytical grade chemicals, see Appendix 1) and dilutions were calculated and prepared according to this approximation. Solutions of VFAs were prepared into 100 ml or 50 ml volumetric flasks. VFAs were weighted to the flasks on analytical balance (d = 0.0001 g) to the closest 0.002 g. Weights for every single VFAs were calculated according to the Formula 2.

$$m_l = n * M \qquad [ 2 ]$$

$m_l$ = mass of a compound in 1 liter solution or 1 kg growth substratum, gram
n = amount of substance, mole
M = molecular weight of a compound (g/mol)

Solutions of VFAs were made only once for the whole series of preliminary acute toxicity experiments and were stored between the experiments in enclosed volumetric flasks sealed with film (Parafilm® M) in dark, refrigerated conditions (4°C). According to Nykänen J. (verbal notice, 2006) VFAs are very stable in water solutions and may be preserved for several months in such conditions (dark and cold) without any notice of change in chemical composition by gas chromatography and mass spectrometry measurements.

Each plastic Petri dish (Ø = 9 cm) was lined with filter paper (Whatman No. 1, Ø = 7 cm) and 20 seeds of a particular tested plant species were added. Moisture was provided by 10 ml of tested VFA solution. The dishes were closed with lids and incubated (Appendix 2, Table 1) in dark at 25 ± 1°C. Duration of the preliminary acute phytotoxicity tests for garden cress (*L. sativum*) was 72 hours and for Italian ryegrass (*L. multiflorum*) 120 hours. One preliminary, whole dilution series, experiment consisted of 1 replicate, i.e. one Petri dish, of each VFA concentration. Altogether three separate whole dilution series experiments were made with garden cress and two with Italian ryegrass. In every experiment there were five replicates of a control (deionized water).

Tests were terminated by inserting all Petri dishes in to refrigerator (4 °C) and dark. Germination success and length of the seedling (radicle length, measured to a nearest millimeter) of every seed were recorded on all Petri dishes in the following 10 - 12 hours. To describe toxicity of acute testing, germination index by Zucconi et al. (1981a) was calculated (Formula 3).

$$\text{Germination Index} = \frac{\text{Seed germination} * \text{radicle length of a treatment} * 100}{\text{Seed germination} * \text{radicle length of control}} \qquad [\ 3\ ]$$

Seed germination = number of germinated seeds
Radicle length = average radical length (mm)

2.1.2 Preliminary subchronic experiments

2.1.2.1 Peat as a growth substrate

Limed and fertilized light *Sphagnum* peat (Kekkilä Kasvuturve B2, Kekkilä OYj, Finland) was considered suitable for subchronic tests. Dry and compressed peat from a bale was moistured with deionized water to a level were squeezing gently in hand drop few drops of water. This was done in advance. The moisturized peat was stored in room temperature under lid and plastic film cover. Two days before spiking the peat with VFAs the bulk density and dry weight (dw) of the moisturized peat was determined according to standard CEN 13040 (CEN, 1999a).

Each volatile fatty acid had a dilution series of 5 concentrations, same as in acute preliminary experiments: 0.5, 1.5, 4.5, 13.5, 40.5 mmol/kg dw, for caproic acid 0.2, 0.6, 1.8, 5.4, 16.2 mmol/kg dw. VFA solutions were prepared on volume basis from analytical grade pure originals in precision of 1 µl. Purity of original VFAs was taken into account in calculations (the lowest given purity was considered, Appendix 1). The volume of acid was calculated with Formulas 2, 4, and 5.

$$m_2 = m_1 * (a * b) / c * 100 \quad [4]$$

$m_2$ = mass of VFA in a bucket, gram
$m_1$ = mass of VFA in 1 kg (dry weight) of growth substratum, gram
a = mass of moisturized substratum (peat = 0.5807 kg; sand mixture = 1.0360 kg)
b = coefficient of dry weight for moisturized substratum (peat = 0.21; sand mixture = 1)
c = purity of original VFAs, %

$$V = m_2 / \rho \quad [5]$$

V = volume of VFA, ml
$m_2$ = mass of VFA in a bucket, gram
ρ = density of original analytical grade VFA, g / ml

The day before starting the test, 580.7 g (≈ 1.6 liters) of moisturized peat was weighted into each five liter plastic buckets, equipped with tight lids. One hundred milliliters of VFA solution was added into each bucket and the mixture was thoroughly mixed. In control treatment pure deionized water (100 ml) was used. All enclosed buckets were stored for overnight in dark at 4 °C.

On the next day, tests started by transferring spiked peat substrata into plastic pots (Ø = 10 cm). Every pot was filled with 400 ml of spiked peat substratum or, in a case of a control, moisturized peat. Seeds were sown at a quantity of 25 seeds per pot. For each VFA concentration there was one replicate and eight control replicates of both garden cress (*L. sativum*) and Italian ryegrass (*L. multiflorum*). Seeds were covered by approximately 1 cm of pure moisturized peat. Each pot was watered with deionized water to the moisture content, when few drops of water appeared on a saucer. Each pot was weighted and weight recorded. Leftovers of moisturized peat were stored at 4 °C, darkness, for further measurements of pH and EC (electrical conductivity). Subsequent day, samples of peat were extracted for determination of pH and electrical conductivity (EC) as described in chapter 2.1.2.3. For these determinations (pH and EC) a new bulk density of the moisturized peat was determined.

Pots were put randomly into phytotron with light/dark regime 16/8 hours. Lightning was 13 000 lx and 185 µmol $m^{-2}$ $s^{-1}$, color light 6500 K (Biolux, Osram, Germany). The temperature was 25 ± 4 °C. Humidity varied between 80 - 100 %. Due to smaller light intensity around the phytotron walls, on every watering occasion order of pots was mixed. Watering with deionized water was performed on demand, according to the weight of a pot.

Duration of the test was 14 days. On day seven, number of seedlings was reduced to 15 per pot. The test was terminated by cutting shoots close to substrate surface. Fresh weight of the shoots was recorded (d = 0,001 g). Dry weight was measured (d = 0,0001 g) after drying shoots in 70 °C in accordance with OECD test guidelines 208 (OECD, 1984). As with acute toxicity testing also in subchronic tests results were expressed as index. Growth index was calculated according to Formula 6.

$$\text{Growth index} = \frac{\text{dw of shoot in treatment} * \text{seedling emergence in treatment} * 100}{\text{dw of shoot in control} * \text{seedling emergence in control}} \quad [6]$$

dw = dry weight
Seedling emergence = number of emerged seedlings

Results of this preliminary experiment were not conclusive. Dose-response relationship could not be modeled from the results for average dry weight of plants versus acid concentration (perhaps except for garden cress and caproic acid). Utilized, limed, peat substratum apparently prohibited some adverse effects posed by VFAs. The ability of the limed, peat substrate to prohibit adverse affects posed by VFAs, even at 4 - 5 times higher concentrations than applied in acute experiments, led to change of a growth medium.

2.1.2.2 Sand as a growth substrate

After no clear results from peat experiments, the decision was made to move towards more inert growth media. Pure sand (Ø = 0.5 - 1.2 mm) (Maxit puhallushiekka, Maxit Oy Ab, Helsinki, Finland) and quartz sand (Ø = 0.2 - 0.05 mm) (NFQ Nilsiän kvartsi, SP Minerals Oy Ab, Halluna, Finland) were chosen for this purpose. The concentrations of different VFAs remained the same as in peat experiments. Mixing ratio of quartz sand and sand was determined to be 1:6 (v / v). For this purpose the bulk density and dry weight of sand and quartz sand was determined by principals of the CEN 13040 standard (CEN, 1999a). Dry weight was > 99.5 % of the mass. It was decided to approximate the dry weight of both sand and quartz in calculations to equal 100 %.

Spiked sand mixture growth media were made by weighting 877.4 g (± 0.5 g) of air dry sand and 158.6 g (± 0.5 g) of quartz sand into five liter buckets with tight lids. Formulas 2, 4 and 5 were used to calculate the needed volume of VFAs. VFAs were diluted with deionized water into 50 or 100 ml volumetric flasks. Acid solutions were poured on to sand mixture and volumetric flasks were rinsed with 25 ml of deionized water (twice for a 100 ml volumetric flask and four times for 50 ml volumetric flasks). This was very important, since e.g. caproic acid did not dissolve in the highest concentration anymore. The smaller volumetric flasks were used for the low soluble, C5 - C6 carbon chain VFAs, for better rinsing. VFAs were mixed to the sand mixture in the buckets with stainless steel spoon. As a control, 150 ml of deionized water was used to moisturize sand mixture in control buckets. Buckets were stored at 4 °C in dark over night, contact time of spiking being app. 14 hours.

Subsequent day, the bottoms of plastic pots, diameter of 9,5 cm (V = 0,39 l), were lined with approximately one centimeter layer of compressed moisturized peat (same peat used in all experiments, see chapter 2.1.2.1) to restrain sand from escaping the pots through bottom openings. In each pot 400 g (± 1 g) of spiked or control sand mixture was weighted. Twenty five seeds were sowed with tweezers on sand mixture and covered loosely with approximately one centimeter of the same moisturized peat as used in the coverage of the bottom openings of pots. Pots were doused with deionized water until few drops appeared on the saucer. Pots with saucers were weight and put to the phytotron to same conditions as in previous peat experiment (2.1.2.1). No thinning was performed and all seeds were let to grow till the end of the experiment.

Since there were only minor layers of fertilized, limed peat, additional fertilization was necessary. Therefore, plants were watered with fertilizing solution, NPK (12-6-9) fertilizer with micronutrients (Kekkilä Kukkaravinne, Kekkilä Oyj, Eurajoki, Finland). As the fertilizer was dissolved into deionized water, the pH of the solution was balanced with 1 mol/l KOH from 3.7 to 5.5 (ca. the pH of deionized water), to avoid acidity of the fertilizer solution influencing results. Watering was performed on demand by weight as needed.

The duration of preliminary experiments was seven days for garden cress and 20 days for Italian ryegrass. Emerged seedlings were counted on a day seven and at the end of the experiments. The experiments were terminated by cutting down shoots. Fresh (d = 0.001 g) and dry weights (d = 0.0001 g) of the shoots were determined according to procedures

described in chapter 2.1.2.1. These experiments were found to be successful and therefore sand-quartz mixture was selected as a growth medium for the actual experiments.

Electrical conductivity (EC) and pH were determined according methods described in the chapter 2.1.2.3. It needs to be specified that the EC and pH measurements were performed always the day after start of the experiment and the day after termination of the experiment.

*Managing malodors*

Aspect of malodors from VFAs under study was important to be considered since after starting to use sand mixtures the odor nuisance was substantially greater than with small amounts of VFA in light weight peat. The used growth chamber was not air tight (Appendix 3, Fig. 1 - 5) and had also condensation water outlet.

The odorant problem was overcome by installing air pumps inside the phytotron and pumping the out coming air through NaOH solution (Appendix 3). There were installed inner and outer VFA-absorbing NaOH solutions. The operational time for these inner NaOH-containing bottles and pumps was 14 days. This was, because after two weeks the malodorous aspect of tests was almost entirely gone and all the space was needed for the growing plants. The photographs of purification apparatus are provided in Appendix 3, Fig. 1 - 5.

One could speculate about the quantity of $CO_2$ concentrations in the growth chamber air. Although these concentrations were not measured inside the growth chamber, used basic solution apparently did not have any effect on $CO_2$-concentrations. This was managed by 2,5 times higher pumping capacity of the outlet air versus circulation capacity of inner air pumps. Difference in pumping capacities ensured enough fresh air in the phytotron. Secondly, the pumps were in operation usually around two days before tests started, for performance check up. This period supposedly also gave time for $CO_2$ to dissolve at least near to equilibrium concentrations in to the basic solutions.

2.1.2.3 Determination of pH and electrical conductivity (EC)

Determinations of pH and electrical conductivity (EC) were performed according to standards CEN 13037 and CEN 13038 (CEN, 1999b; CEN, 1999c). If extraction procedure was needed for pH and EC determinations, as was the case with every growth substratum, it was also done in accordance to standards CEN 13037 and CEN 13038 (CEN, 1999b; CEN, 1999c), with modification that extracted solution was filtered through pure, natural

cotton and pH and EC measurements were done from the same filtered extraction solution. In subchronic growth experiments pH and EC were determined from the sand mixture layer, unless otherwise reported. Differences in the procedures are itemized below.

2.1.2.1 Peat as a growth substrate:

> the extracted solution of peat layer was stored in 4 °C (dark) in brown glass flasks for one to four days until pH and EC measurements

2.1.2.2 Sand as a growth substrate, 2.2.2 Subchronic growth experiments and 2.3 Dynamics of pH (experiments A - E):

> as the bulk density of sand mixture at the end of the experiments could not be determined and could be different in each pot (due to different amounts of roots, water etc.) the measurement of 60 ml of sand mixture, for pH and EC determinations, was performed not on weight basis, but on volume basis in a glass beaker mixing the content of the sand layers of two replicates in a bucket and taking one composite sample for pH and EC determination, except in experiment E, in which pH and EC were determined separately from each pot.

All measurements of pH and electrical conductivity (EC) were performed with following apparatus: pH (MeterLab® PHM220, with electrode “Red Rod” Ag/AgCl Combined pH electrodes, Radiometer Analytical, France) and electrical conductivity (EC) (MeterLab® CDM210 with conductivity cell CDC 642T, Radiometer Analytical, France).

## 2.2 Experiments

2.2.1 Acute toxicity experiments

Results from the preliminary experiments were extrapolated and new series of volatile fatty acid (VFA) concentrations was set. Same dilution series was considered to suite both tested species, the garden cress and the Italian ryegrass. Dilution concentrations were the same for every tested VFA, with exception of butyric acid. Seven concentrations for each acid were 0.10, 0.30, 0.60, 1.20, 2.40, 4.80, 9.60 mmol/l and for butyric acid 0.10, 0.30, 0.61, 1.21, 2.42, 4.84, 9.68 mmol/l. Pure VFAs were measured volumetrically to the nearest 1 µl. Dilution series were made into volumetric flasks. In the calculations, lowest reported purity of original VFA was considered. Formulas 2, 4 and 5 (chapters 2.1.1 and 2.1.2.1) were used to calculate the needed volume of the acid. VFA solutions were prepared one

day prior to start of the toxicity tests. For every single experiment new VFA dilutions were made, except for the days, where second experiment started already the next day. After start of the test, pH and EC of the VFA dilution solutions were determined according methods described in chapter 2.1.2.3).

Petri dishes (Ø 9 cm) were lined with filter paper Whatman No. 1 (Ø = 7 cm) moisturized with 10 ml of particular VFA concentration. Twenty seeds per Petri dish were used. Petri dishes were covered with lids and incubated at 25 ± 1 °C, 72 hours in garden cress and 120 hours in Italian ryegrass experiments. Incubation was performed in three different incubators, for information on incubators see Appendix 2. One experiment on one plant species consisted of one replicate (one Petri dish) of each VFA concentration and eight controls (deionized water). With Italian ryegrass the experiments were repeated for 12 times and with garden cress seven times. Termination and response measurements were done as mention in chapter 2.1.1.

### 2.2.2 Subchronic experiments

Results from preliminary growth experiments in sand mixture were extrapolated and new VFA concentrations were selected. For garden cress (*L. sativum*) and Italian ryegrass (*L. multiflorum*) selected concentrations are presented in Table 5.

Preparation of VFA solutions and sand mixture growth media, filling of the pots and growth conditions were kept the same as in preliminary subchronic experiments described in chapter 2.1.2.2. All experiments were 21 days long. For both tested species the whole experiment was repeated three times with two replicates, one replicate standing for one pot with 25 seeds. Special attention was given to the watering, since fertilization was provided only with each watering occasion. Irrigation was performed according to the change in the pot weight (chapter 2.1.2.2). For the first 10 days watering was performed every second day to the top of the pot with the fertilizer solution (1 g/l). For the next 10 days fertilization was nearly doubled (fertilizer solution 1.8 g/l) and irrigation was done from the bottom to diminish leaching of VFAs. Watering continued after $10^{th}$ day for every 36 hours in experiments with garden cress and every 48 hours with Italian ryegrass until the end incubation period. At the beginning of the experiment and the evening before termination of experiments watering was performed with deionized water. Pots were randomized after every irrigation occasion.

Table 5. VFA concentrations (mmol/l) in subchronic experiments.

| Plant species - VFA | Conc.1 | Conc. 2 | Conc. 3 | Conc. 4 | Conc. 5 |
|---|---|---|---|---|---|
| Cress - Formic | 10.0 | 20.0 | 40.0 | 60.0 | 80.0 |
| Cress - Acetic | 10.0 | 20.0 | 40.0 | 50.0 | 60.0 |
| Cress - Propionic | 1.5 | 4.5 | 13.5 | 40.5 | 60.0 |
| Cress - *Iso*-butyric | 0.5 | 1.5 | 4.5 | 13.5 | 40.5 |
| Cress - Butyric | 1.5 | 4.5 | 13.5 | 25.0 | 40.5 |
| Cress - *Iso*-valeric | 0.5 | 1.5 | 3.0 | 15.0 | 30.0 |
| Cress - Valeric | 0.5 | 1.5 | 3.0 | 15.0 | 30.0 |
| Cress - Caproic | 0.2[A]<br>0.5[B] | 0.6[A]<br>1.5[B] | 1.8[A]<br>3.0[B] | 5.4[A]<br>15.0[B] | 16.2[A]<br>30.0[B] |
| Ryegrass - Formic | 10.0[C]<br>10.0[D] | 30.0[C]<br>30.0[D] | 70.0[C]<br>70.0[D] | 90.0[C]<br>90.0[D] | 120.0[C]<br>200.0[D] |
| Ryegrass - Acetic | 5.0 | 25.0 | 50.0 | 75.0 | 100.0 |
| Ryegrass - Propionic | 5.0 | 10.0 | 20.0 | 35.0 | 50.0 |
| Ryegrass - *Iso*-butyric | 1.5 | 5.0 | 15.0 | 30.0 | 45.0 |
| Ryegrass - Butyric | 2.5 | 10.0 | 25.0 | 40.0 | 60.0 |
| Ryegrass - *Iso*-valeric | 1.5 | 7.5 | 13.5 | 25.0 | 40.0 |
| Ryegrass - Valeric | 2.5 | 10.0 | 20.0 | 30.0 | 40.5 |
| Ryegrass - Caproic | 0.6 | 6.0 | 12.5 | 17.0 | 22.5 |

[A] = experiment replicates 1 & 3
[B] = experiment replicate 2
[C] = experiment replicate 1
[D] = experiment replicates 2 & 3

On every seventh day the seedling emergence was counted. Termination was done as described in chapter 2.1.2.1.

Garden cress showed tendency toward the end of the tests to lean out from the pots. In order to avoid leaning a thin cord was loosely bound around the plants.

Due to malfunction of the growth chamber in Italian ryegrass experiment replicate 3 the pots were transferred from the phytotron for remaining last 5 days of the experiment to a greenhouse. Lighting was somewhat lower, about 10 000 lx and photosynthetically active radiation only about 130 μmol $m^{-2}$ $s^{-1}$. Temperature range was about equal to the growth chamber range, 25 ± 4 °C.

In every experiment pH and EC were determined as stated in chapter 2.1.2.3.

## 2.3 Dynamics of pH under subchronic exposures

The idea of conducting further experiments with pure growth media was to receive information on the pH behavior of the growth media during the subchronic experiments. It was apparent from the results of preliminary experiments made on sand mixture growth media that pH raised strangely towards the end of the experiments. The end pH values were usually the higher, the higher were the applied starting VFA concentrations. Therefore, five small scale experiments were performed to get an idea, what was behind this phenomenon. These experiments were performed without any plant seeds, to get at the same time an idea of how plants change, through metabolic and physical activity of their roots, the pH of the sand mixture compared to plantless pots.

*Experiment A*

In experiment A the influence of peat layers on a pot sand mixture pH of subchronic experiments was tested. As an only representative of VFAs, formic acid, the most acidic and volatile acid under study, was chosen for testing. Pots with different growth substrate amendments, without any seeds, were prepared and incubated in room temperature, outside phytotron, for 21 days. Experiment consisted of six treatments (Table 6) with variations in the peat and VFA content or fertilization procedures compared to actual growth test. Each treatment had two replicates. As a difference to actual subchronic experiments, peat, the same as used in previous growth studies (2.1.2.1), was heavily moisturized with deionized water to drop water, when lifted in the air. Sand mixtures were prepared as described in chapter 2.1.2.2, with the exception of total liquid volume used to moisturize sand mixtures in buckets, which was 200 ml. This was done to obtain directly wanted moisture content of pots and not to need to water the pots anymore before the start of the test. To have exactly the same amount of growth media, peat (two layers, bottom and top, each of 42 g ± 0.5 g) and moisturized sand mixture (417 g ± 0.5 g) were weight.

Watering was performed every second day (as with actual exposure tests with Italian ryegrass), with the same method as describe in chapter 2.2.2. EC and pH were determined at the beginning and at the end of the experiment period according to procedure mentioned in chapter 2.1.2.3.

Table 6. Treatments of experiment A to study the effect of formic acid on substrate pH during subchronic incubations without plants. Where indicated, Whatman No. 1 filter paper was used to cover bottom opening of pots. Formic acid was used at sand mixture concentration of 80 mmol/kg dw.

| Treatment | Substrate | Acid | Irrigation |
|---|---|---|---|
| 1 | peat + sand mixture | yes | fertilizer solution |
| 2 | peat + sand mixture | yes | deionized water |
| 3 | sand mixture + Whatman No. 1 filter paper | yes | deionized water |
| 4 | sand mixture + Whatman No. 1 filter paper | yes | fertilizer solution |
| 5 (control) | peat + sand mixture | no | fertilizer solution |
| 6 (control) | peat + sand mixture | no | deionized water |

*Experiment B*

Experiment B was performed to acquire information on neutralizing capability of peat-sand mixture, if the evaporation of VFA would be prohibited. The content of the growth substratum in one pot, used in the actual subchronic growth experiments, was mixed together and sealed into tightly closed glass flask to prevent excessive evaporation of VFA. As an only representative of VFAs, formic acid, as the most acidic and volatile acid of VFAs under study, was tested. Experiment B consisted of two treatments with two replicates. Treatments were:

1. peat + sand mixture + 300 ml of deionized water
2. peat + sand mixture + 300 ml deionized water, with 1.8 g/l fertilizer concentration

Flasks were regularly mixed without opening the flasks. The estimated amount of moisturized (to the limit until few drops drip off, when gently squeezing in hand), limed and fertilized peat, as used in actual exposure test pots (chapter 2.1.2.1), was weighted (55 g) into flasks. Each flask received the pot weight 400 g (± 0.5 g) of sand mixture contaminated with 80 mmol/kg dw formic acid, as was the case in actual exposure experiments.

The quantities of deionized water and fertilizer were approximations of quantities of water and fertilizer received by pots in actual subchronic growth experiments, where plants were not detected to grow. At the beginning and at the end of the experiment B the pH and EC were determined. Also the pH and EC of pure peat and formic acid contaminated sand mixture were determined at the beginning as duplicates according to methods described in chapter 2.1.2.3.

*Experiment C*

Above mentioned experiments gave hints about the cause of pH fluctuations observed in pots of the subchronic growth experiments. It was decided to concentrate only on the effects of peat (see chapter 2.1.2.1) and the impact of the fertilizer on the results. The fertilizer used in present study was N-P-K fertilizer with micronutrients (chapter 2.1.2.2). Of those micronutrients manganese, zinc and copper were provided as EDTA-chelates and iron as HEDTA-chelate.

The experiment C was performed in open glass flasks to mimic the evaporation possibility of VFAs in actual subchronic growth experiments. Again, the influence of formic acid, as the only representative of all VFAs under study, on pH was investigated. Moisturized peat was weight (55 g) into each glass flasks according to the approximation of the quantity in one pot used in the subchronic growth experiments. The experiment consisted of 6 different treatments with two replicates in each. Peat was mixed in glass flasks with 300 ml of either of solutions (treatments):

1. deionized water
2. fertilizer solution
3. deionized water with EDTA
4. deionized water with formic acid (equivalent of 80 mmol/kg dw in a pot)
5. mixture of fertilizer solution and formic acid (equivalent of 80 mmol/kg dw in a pot)
6. mixture of deionized water, formic acid (equivalent of 80 mmol/kg dw in a pot) and EDTA

Opened flasks were incubated at room temperature for 21 days, mixing them occasionally. No light was provided.

Used liquid volume of 300 ml was the approximation of water quantity used to irrigate a pot, which was experiencing no plant growth in actual subchronic experiments. Fertilizer solution was pH adjusted with KOH (0.1 mol/l) to pH = 5.5. EDTA was given according to product info on fertilizer in quantities calculated to be present in 300 ml of fertilizer solution of applied higher (1.8 g/l) fertilizer concentration. The approximation of amount of EDTA applied in treatments three and six was done on weight percentage (total of 0.2 % by weight) of reported, afore mentioned, micronutrients present in fertilizer.

Only pH of the solutions was measured after one hour on a shaker. The pH was determined at the beginning of the experiment, on the $10^{th}$ day of the experiment and at the end (21

days) of the experiment. The weight of the glass flasks was determined and deionized water was added to match the weight of the flask (at the beginning of the experiment) before every pH determination event.

*Experiment D*

The purpose of this experiment was to acquire information how could the pH change in sand mixtures during 21 day incubation and how evaporation of acids would change the pH? This would also give indirect evidence, if some acid could have been still present after three weeks of exposure in an inert material. The experiment was performed in open buckets on sand mixtures. Four buckets of sand mixtures were prepared as described previously in chapter 2.1.2.2. Two buckets of sand mixture were moisturized with deionized water (200 ml) and two were spiked with 80 mmol/kg dw formic acid (in 200 ml deionized water solution). Buckets were weighted and irrigation was performed according to weight, every second day, only with deionized water. The experiment lasted for 21 days and was conducted at room temperature. Only pH was determined, in accordance with methods described in chapter 2.1.2.3. It needs to be stated that the sand layer was pressed to be evenly flat in the bucket and about five to seven centimeters thick. This represented somewhat thinner layer than in pots of actual subchronic growth experiments. Determinations of pH were done at the beginning, on a third and tenth day and at the end of the experiment.

*Experiment E*

This experiment was performed to receive information on how the pH and EC of growth medium used in control pots of subchronic growth experiments could change during the 21 day incubation period without an interference influence from plants. As in all above mentioned experiments A - D, this was also conducted without seeds. There were two treatments, in which peat and sand mixture was irrigated either with deionized water or fertilizer solution.

The growth media and the pots were prepared in the same manner as was done for the actual subchronic growth tests (chapter 2.2.2) with the difference that this time also the amount of moisturized peat for the coverage of the bottom openings of the pot and coverage of seeds was also weighted. Peat (55 g; 35 g on the bottom and 20 g on the top of the pot) was used in each pot. Each plastic pot was weighted and irrigation procedure was performed, as was done with the pots in the subchronic growth experiments on Italian

ryegrass (chapter 2.2.2). Same fertilization concentration and irrigation method was applied. Pots were incubated at room temperature (about 22 °C). At daytime, only normal room lightning was present in the room.

This experiment was repeated six times, with each treatment having altogether 18 replicates. EC and pH were determined according to procedures in chapter 2.1.2.3. It needs to be noted that as a difference compared to previous experiments with this experiment exactly the same procedure of test preparations and pH and EC determination methods were employed as with actual subchronic growth experiments (2.2.2). Especially one should note, that this included growth media storage in refrigerator (4 °C) prior to the test start and pH and EC determinations day after pots were prepared and incubation time started (this could meant up to 48 h storage of growth media before pH and EC determinations and up to 24 h delay in pH and EC determinations after 21 day incubation period ended).

**2.4 Statistical analysis**

Statistical analysis was performed with software program SPSS 14.0. Where no actual statistical modeling or tests on the data were performed, statistical parameters (mean, ± standard deviation) were calculated with Microsoft Office Excel 2003.

The normal distribution of data and its qualification for parametric tests was checked by Shapiro-Wilk's test. In VFA comparisons either 95 % confidence limits of normally distributed data were compared or two independent samples t-test was applied. In two independent samples t-test equal variances were not assumed and obtained significance level ($p$) was approximated from two-tailed normal distribution. Due to inability to obtain or construct normally distributed data sets, in data sets of seedling emergence, germination, radicle length comparisons were consecutively statistically tested by non-parametric Mann-Whitney U-test (M-W U-test).

Modeling of dose-response curves for obtaining EC50-values was performed with probit analysis. Level of controls (natural response) was considered. Natural response was calculated from the control data, and obtained EC50-values with 95% confidence limits represent effective concentration of 50% response of the control average.

Since probit analysis in SPSS 14.0 is for ordinal scale variable only, ratio scale variable of plant dry weight was transformed to ordinal scale to represent accuracy of analytical balance (d = 0.0001 g). Iterations were raised until SPSS 14.0 found optimal solution. It

needs to be stated, that *p*-values of Pearson's Goodness of fit-test were for the data to be probit analyzed always under 0.19. This was accepted, since SPSS 14.0 uses a heterogeneity factor for calculating 95 % confidence limits, when Pearson's Goodness of Fit-test results with *p*-value under 0.15.

# 3 RESULTS

## 3.1 Results of toxicity tests

Modeling toxicities of volatile fatty acids (VFAs) is dependent upon response parameters measured. Each volatile fatty acid (C1 - C6) was tested with two plant species and at acute and subchronic levels. VFA exposure results are presented separately for each VFA and response parameter (chapters 3.1.1 - 3.1.8). As it is common to present results in e.g. compost phytotoxicity studies also as indecies, the relationship between dose and response will be presented thereafter by means of germination and growth indecies, in chapter 3.2.1. Indecies are omitted from statistical comparisons and comparisons made by means of original response parameters are introduced in chapters 3.2.2 and 3.2.3. Changes in growth media pH and EC of acute and subchronic tests, as well results of experiments A - E, which clarify reasons of pH changes in growth substrate of subchronic experiments, are presented at the end, in chapters 3.2.4 and 3.3.

### 3.1.1 Phytotoxicity of formic acid

Formic acid is the strongest volatile fatty acid (Table 3, chapter 1.1) and as such also increases acidity ($H^+$ content) of the growth media the most (Appendix 4, Tables 1 - 2). Thus, stress effect posed by decrease in pH of germination or growth media on studied plant species can be expected to be the highest in series of tested VFAs.

Formic acid had no effect on germination of garden cress (Figure 1) even at highest studied acid concentration (9.6 mmol/l) compared to control (Mann-Whitney U-test, $p = 0.073$). Italian ryegrass was much more sensitive in terms of germination. Concentration 4.8 mmol/l impacted germination strongly and, on average, only 12.7 % of Italian ryegrass seeds germinated at this concentration (M-W U-test, $p < 0.001$). At the highest concentration (9.6 mmol/l) average germination was 0.5 %. It is interesting that at concentrations 0.3 - 0.6 mmol/l germination of ryegrass was induced (hormesis) of about 5 % (M-W U-test, $p = 0.016$ - $0.038$) over control.

Radicle length was more sensitive parameter than the germination. As garden cress did not experience any germination reduction with any studied formic acid concentration, at 2.4 mmol/l concentration the average radicle length was reduced by almost 70 % (M-W U-test, $p = 0.001$). And at highest applied concentration (9.6 mmol/l) the average radicle length was already close to detection limit, 0.9 mm. Ryegrass showed a more sensitive response to formic acid as the average radicle length was reduced to the extend of statistical significance already at concentration 1.2 mmol/l by 20 % (M-W U-test, $p = 0.001$) (Figure 1) and at 2.4 mmol/l reduction of average length was almost 80 % of control value.

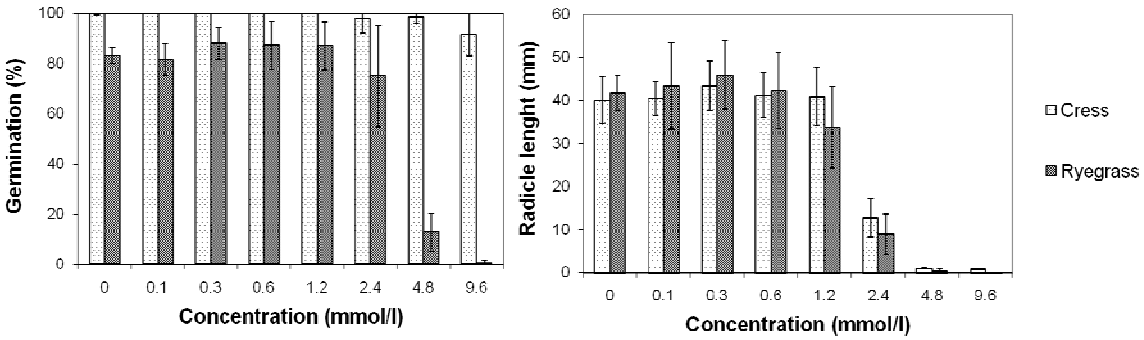



Figure 1. Germination (left) and radicle length (right) in acute exposures to formic acid performed on Italian ryegrass (120 h) and garden cress (72 h). Results represent means with standard deviation error bars.

In contrary to acute test, in subchronic growth the seedling emergence of both species was not affected by concentrations up to 10 - 20 mmol/kg dw (Figures 2 and 3). The seedling emergence was not inhibited to the statistically significant difference until 40 mmol/kg dw in cress and 90 mmol/kg dw in Italian ryegrass (respectively M-W U - test, cress $p < 0.001$, ryegrass $p < 0.001$). Statistically significant reduction in dry biomass production was observed in cress at 40 mmol/kg dw and in ryegrass at 30 mmol/kg dw (95 % confidence limits considered), although in subchronic experiments cress was on a whole much more sensitive to formic acid than ryegrass. This was apparent since cress seedling emergence was on average only approximately 33 % at 60 mmol/kg dw and at strongest concentration (80 mmol/kg, dw) no more than 16 %. On the contrary, Italian ryegrass showed on average over 60 % seedling emergence success still at 90 mmol/kg dw and growth of dry biomass was still about 57 % of control's growth, when garden cress experienced only 37 % growth compared to controls at already 40 mmol/kg dw.

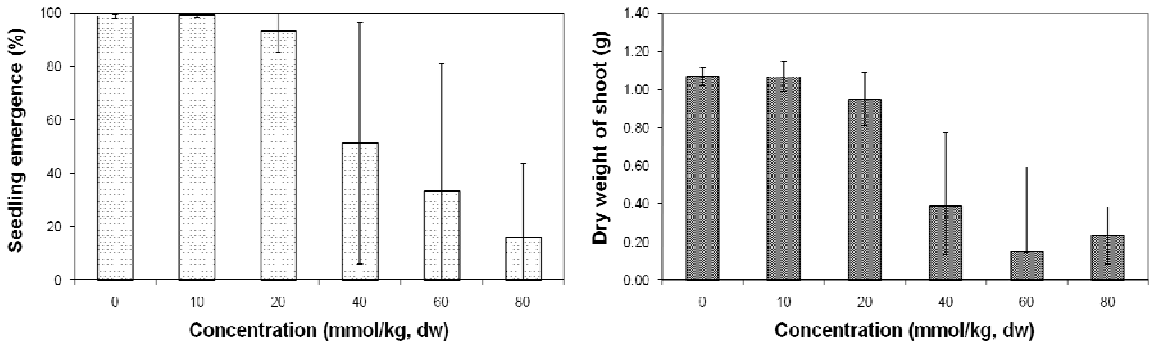



Figure 2. Results of 21-day growth of garden cress exposed to formic acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass with exception of 80 mmol/kg dw, were error bars represent standard deviation. Transformation of shoot weights was performed (square root) to obtain a normal distribution of the data. The shoot dry weight data of strongest acid concentration was not normally distributed, mean and SD are presented, because normality is still assumed to exist.

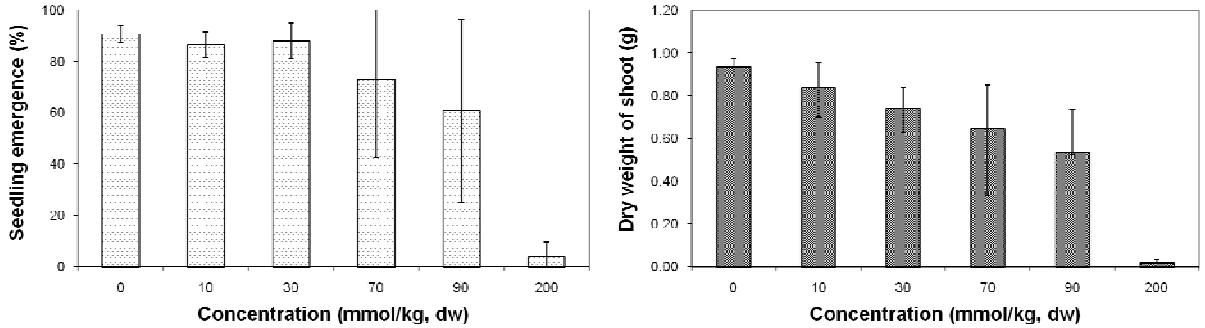



Figure 3. Results of 21-day growth of Italian ryegrass exposed to formic acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass. Transformation of shoot dry weight was performed (power of two) to obtain a normal distribution of the data.

### 3.1.2 Phytotoxicity of acetic acid

Acetic acid is the second strongest volatile fatty acid in studied C1 - C6 VFAs, although it's $pK_a$-value is only about 0.1 units lower than of other VFAs (except formic acid). As formic acid, garden cress did not experience any statistically significant inhibition of germination in acute testing. In Italian ryegrass the inhibiting effect on germination was statistically significant at the same concentration as with formic acid, 4.8 mmol/l (M-W U-test, $p = 0.004$), at which germination was on average 64 % (Figure 4).

Similarly to formic acid, radicle length was more sensitive parameter over germination. Inhibition of radicle elongation was observed at the same concentration as with formic acid (2.4 mmol/l, M-W U-test, $p = 0.001$) in garden cress (Figure 4). Statistically significant inhibition of radicle growth in Italian ryegrass was observed at concentration 2.4 mmol/l (M-W U-test, $p < 0.001$).

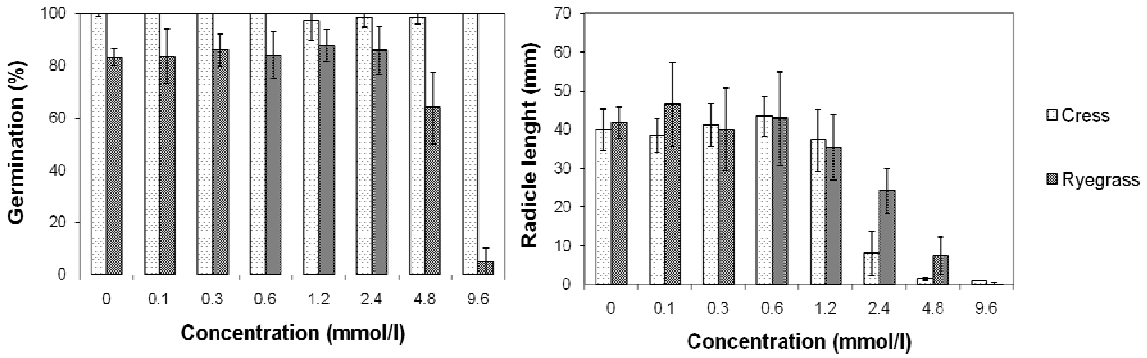



Figure 4. Germination (left) and radicle length (right) in acute exposures to acetic acid performed on Italian ryegrass (120 h) and garden cress (72 h). Results represent means with standard deviation error bars.

In subchronic exposures, ryegrass exhibited statistically significant reduction in seedling emergence at 50 mmol/kg dw (M-W U-test, $p < 0.001$), in which on average 85 % of seeds germinated (Figure 5). Garden cress exhibited more sensitive response and at 40 mmol/kg dw seedling emergence was on average 55 % (M-W U-test, $p < 0.001$) (Figure 6).

Average dry biomass (Figures 5 and 6) in subchronic experiments was lower than in control at all studied concentrations, but statistically significant decrease (95 % confidence limits considered) was not observed in ryegrass until 25 mmol/kg dw and 40 mmol/kg dw in garden cress. Cress was more sensitive to acetic acid compared to Italian ryegrass. For example, at 50 mmol/kg dw on average 27 % of cress seedlings emerged and biomass growth was 9.6 % of control, while in ryegrass seedling emergence was 85 % and growth of biomass 45 %.

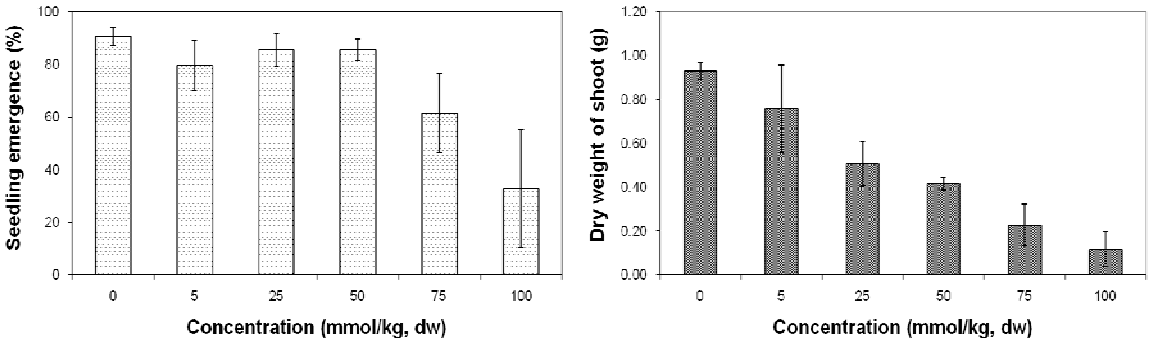



Figure 5. Results of 21-day growth of Italian ryegrass exposed to acetic acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass.

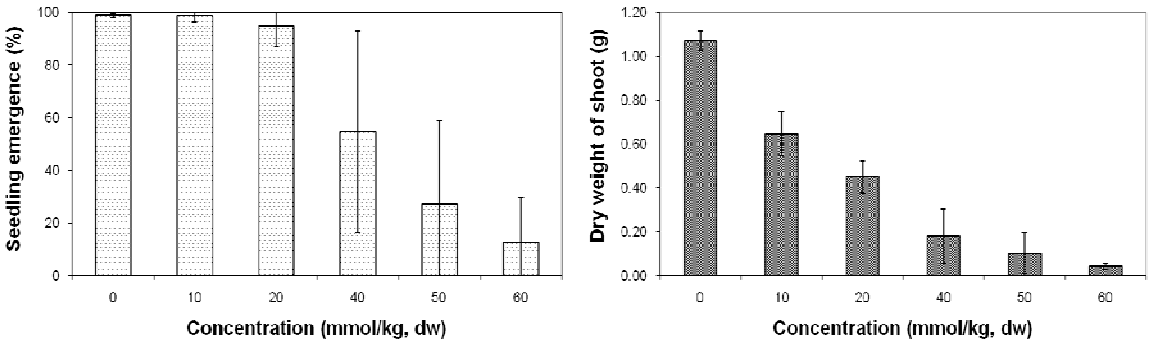



Figure 6. Results of 21-day growth of garden cress exposed to acetic acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass with exception of 60 mmol/kg dw, where error bars represent standard deviation. The shoot dry weight data of strongest acid concentration was not normally distributed, mean and SD are presented, because normality is still assumed to exist.

### 3.1.3 Phytotoxicity of propionic acid

Propionic acid was the shortest carbon chain VFA in studied row of C1 - C6 volatile fatty acids, where total inhibition of growth and germination of Italian ryegrass in acute experiments, in tested concentration series, was observed. Statistically significant inhibition of germination in Italian ryegrass was observed at 2.4 mmol/l (reduction of 4 %, M-W U-test, $p < 0.001$) (Figure 7). Garden cress did not experience any inhibition of germination at studied concentration range.

In acute experiments inhibition of radicle elongation was observed already at concentration 0.6 mmol/l (M-W U-test, $p = 0.004$) in Italian ryegrass and in garden cress at 1.2 mmol/l (M-W U-test, $p = 0.001$). Radicle elongation can be seen to be totally inhibited in both plant species by propionic acid at concentrations 4.8 and 9.6 mmol/l (Figure 7).

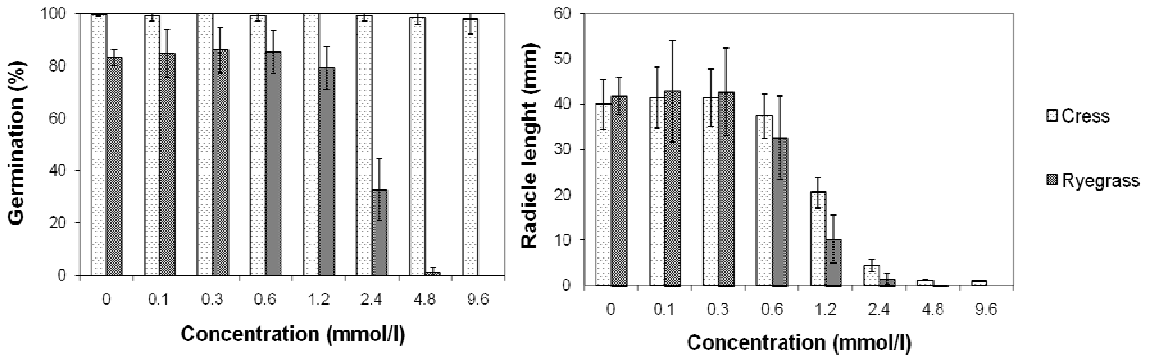



Figure 7. Germination (left) and radicle length (right) in acute exposures to propionic acid performed on Italian ryegrass (120 h) and garden cress (72 h). Results represent means with standard deviation error bars.

Decrease in growth was observed in subchronic experiments at 5 mmol/kg dw in Italian ryegrass and at 4.5 mmol/kg dw in garden cress (Figures 8 and 9) (95 % confidence limits considered). The seedling emergence was statistically significantly inhibitive at 20 mmol/kg dw in ryegrass and at 40.5 mmol/kg dw in cress (95 % confidence limits considered). But on average cress was more sensitive to propionic acid in terms of seedling emergence than ryegrass. At 40.5 mmol/kg 49 % of possible cress seedlings emerged and dry biomass was 10 % of control, while in ryegrass at 50 mmol/kg dw seedling emergence was 54 % and growth rate 17 % of control.

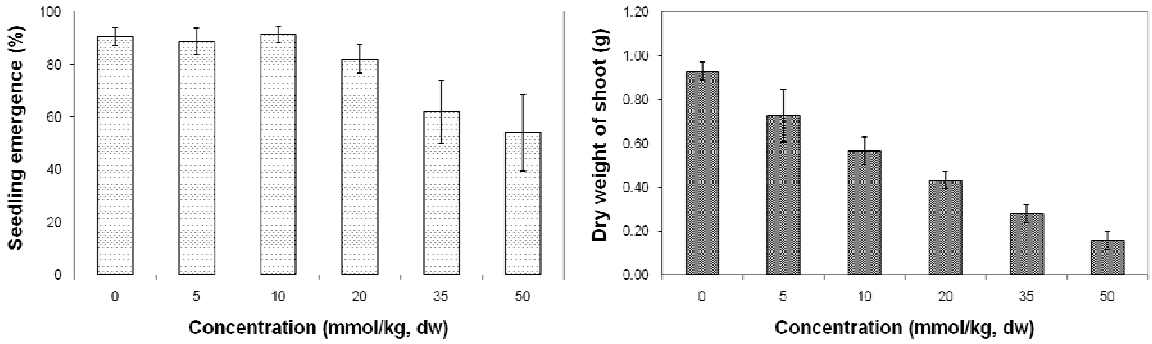



Figure 8. Results of 21-day growth of Italian ryegrass exposed to propionic acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass.

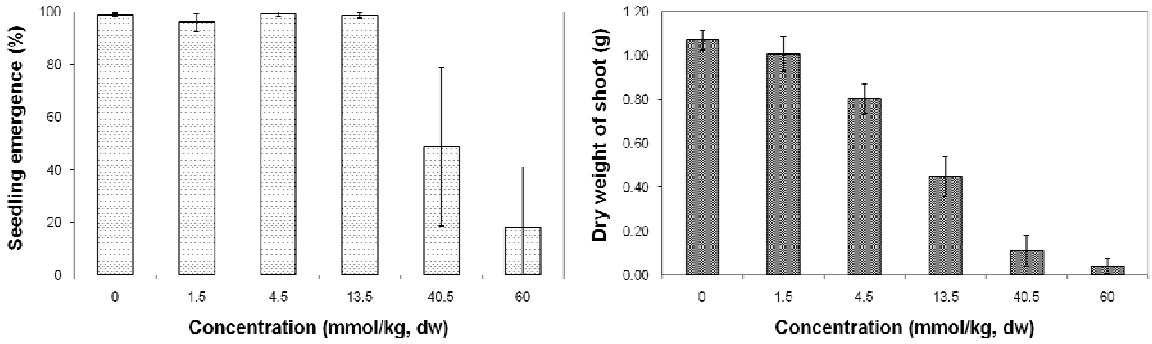



Figure 9. Results of 21-day growth of garden cress exposed to propionic acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass.

### 3.1.4 Phytotoxicity of *iso*-butyric acid

Results of acute toxicity experiments of *iso*-butyric acid are presented in Figure 10. The germination of Italian ryegrass and garden cress were affected also by *iso*-butyric acid differently. Germination of cress was not affected at studied concentration range, but germination of ryegrass was affected negatively already at concentration 2.4 mmol/l (M-W U-test, $p = 0.001$), as was the case with propionic acid. Inhibition of radicle elongation in

both species was observed at 1.2 mmol/l (M-W U-test, ryegrass $p < 0.001$; cress $p = 0.001$) (Figure 10). Interestingly, the observation of germination inhibition was not observed at 0.6 mmol/l in Italian ryegrass (M-W U-test, $p = 0.171$) as was the case for propionic acid. Also *iso*-butyric acid inhibited radicle elongation almost totally at 4.8 and 9.6 mmol/l, as C1 - C3 VFAs did (chapters 3.1.1 - 3.1.3).

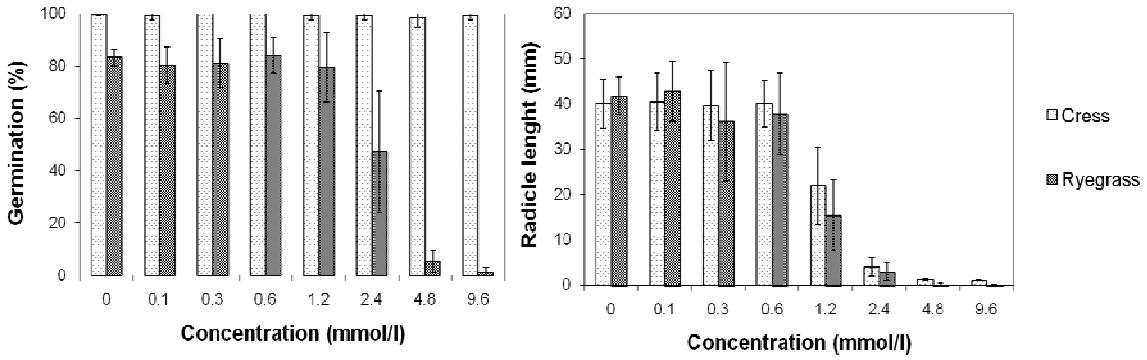



Figure 10. Germination (left) and radicle length (right) in acute exposures to *iso*-butyric acid performed on Italian ryegrass (120 h) and garden cress (72 h). Results represent means with standard deviation error bars.

In subchronic experiments, 30 mmol/kg dw was the lowest concentration at which statistically significant inhibition of seedling emergence was detected in both species (M-W U-test, ryegrass $p = 0.037$; cress $p < 0.001$). Also with *iso*-butyric acid cress was more sensitive than Italian ryegrass. At concentration 40.5 mmol/kg dw no seedling emergence was observed, while 45 mmol/kg dw germination success of Italian ryegrass was on average 33 % and dry biomass 6.9 % of control (Figures 11 and 12).

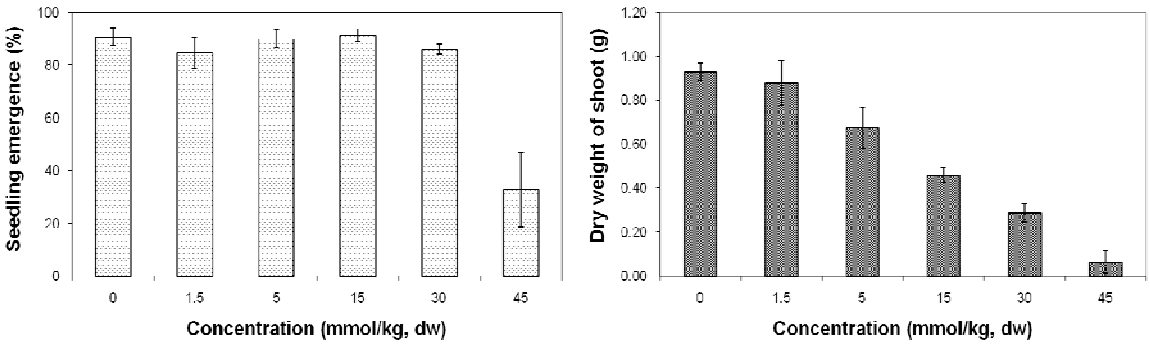



Figure 11. Results of 21-day growth of Italian ryegrass exposed to *iso*-butyric acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass.

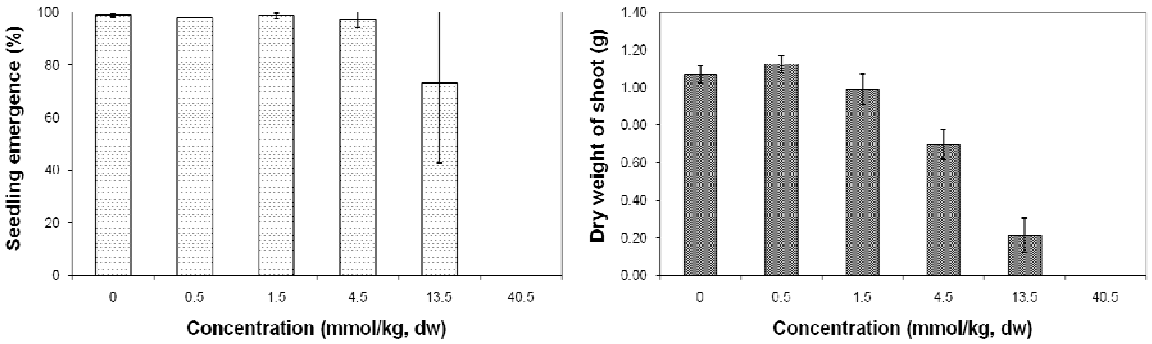



Figure 12. Results of 21-day growth of garden cress exposed to *iso*-butyric acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass.

### 3.1.5 Phytotoxicity of butyric acid

In acute experiments, as with C1 - C3 volatile fatty acids and *iso*-butyric acid, garden cress did not exhibit any inhibition of germination even at highest studied butyric acid concentration 9.68 mmol/l (M-W U-test, $p$ = 0.620). In Italian ryegrass the lowest concentration, at which inhibition was detected, was 2.42 mmol/l (M-W U-test, $p < 0.001$) (Figure 13). It is interesting that germination of Italian ryegrass at 0.1 mmol/l exceeded germination of control by 5 % (M-W U-test, $p = 0.028$).

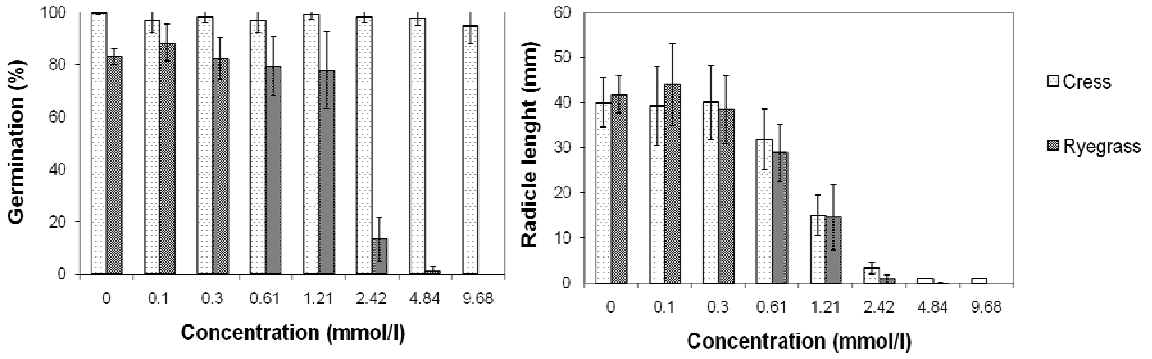



Figure 13. Germination (left) and radicle length (right) in acute exposures to butyric acid performed on Italian ryegrass (120 h) and garden cress (72 h). Results represent means with standard deviation error bars.

Radicle length was more sensitive parameter over germination also in butyric acid experiments. Detection of inhibition was already at concentration 0.61 mmol/l in both tested species (M-W U-test, cress p = 0.007; ryegrass $p < 0.001$) (Figure 13). This kind of low inhibition concentration was observed with C1 - C4 VFAs only with propionic acid and Italian ryegrass. The radicle elongation halted in cress in two strongest concentrations

(4.84 and 9.68 mmol/l) and Italian ryegrass did not exhibit almost any radicle elongation at 2.4 mmol/l.

In subchronic growth tests seedling emergence was inhibited at concentrations 13.5 mmol/kg dw in cress and at 40 mmol/kg dw in Italian ryegrass (M-W U-test, cress $p$ = 0.014; ryegrass $p < 0.001$). Cress showed to be more sensitive in terms of germination and at highest studied concentration (40.5 mmol/kg, dw) on average 32 % of seeds emerged compared to Italian ryegrass, which had at almost the same concentration (40.0 mmol/kg, dw) seedling emergence rate 68 % and at 60 mmol/kg dw still 35 % (Figures 14 and 15).

The average dry biomass showed more sensitive response in subchronic growth experiments over seedling emergence and on average inhibition of growth was observed with every studied butyric acid concentration. Already at 2.5 mmol/kg dw in Italian ryegrass and 4.5 mmol/kg dw in garden cress the average biomass production was lower to control (95 % confidence limits considered) (Figures 14 and 15).

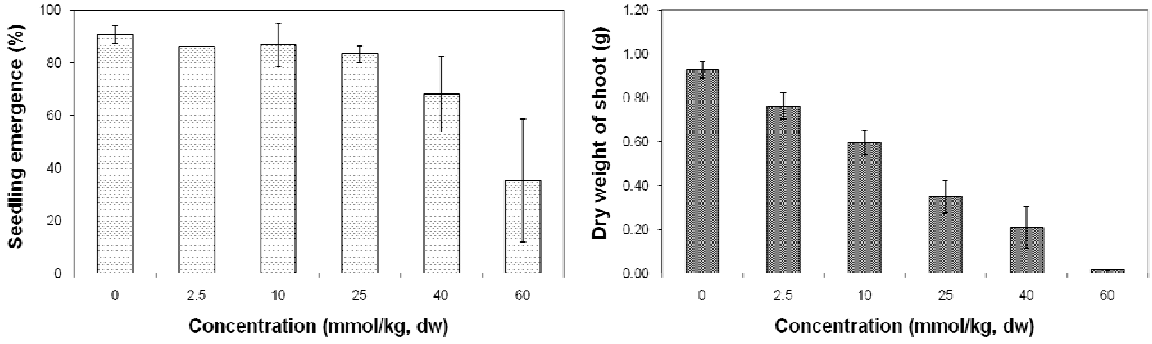



Figure 14. Results of 21-day growth of Italian ryegrass exposed to butyric acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass with exception of 60 mmol/kg dw, where error bars represent standard deviation. The shoot dry weight data of strongest acid concentration was not normally distributed, mean and SD are presented, because normality is still assumed to exist.

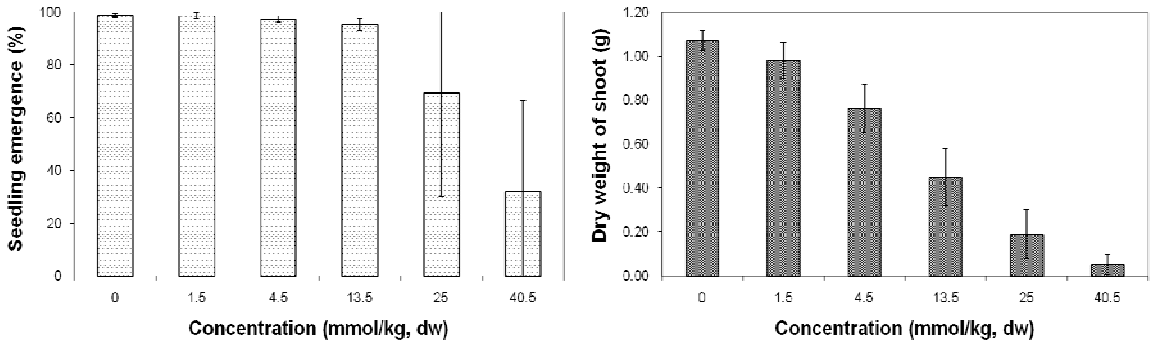



Figure 15. Results of 21-day growth of garden cress exposed to butyric acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass.

### 3.1.6 Phytotoxicity of *iso*-valeric acid

Acute toxicity of *iso*-valeric acid was stronger than observed with C1 - C4 acids. This was especially visible with Italian ryegrass. Inhibition of ryegrass germination in acute experiments was observed already at 1.2 mmol/l (M-W U-test, $p < 0.001$) (Figure 16), which was not the case with previously discussed VFAs (C1 - C4) (chapters 3.1.1 - 3.1.5). No germination was observed at concentrations 4.8 mmol/l and 9.6 mmol/l. Already at 2.4 mmol/l ryegrass germination was on average only 6.6 %. Garden cress did not show any signs of germination inhibition even at strongest concentration, 9.6 mmol/l (M-W U-test, $p = 0.535$).

As with butyric acid, inhibition of radicle elongation in acute testing of *iso*-valeric acid was observed at 0.6 mmol/l in both studied species (M-W U-test, cress $p = 0.001$; ryegrass $p = 0.001$). At three strongest concentrations (2.4, 4.8 and 9.6 mmol/l) radicle elongation was almost halted (Figure 16).

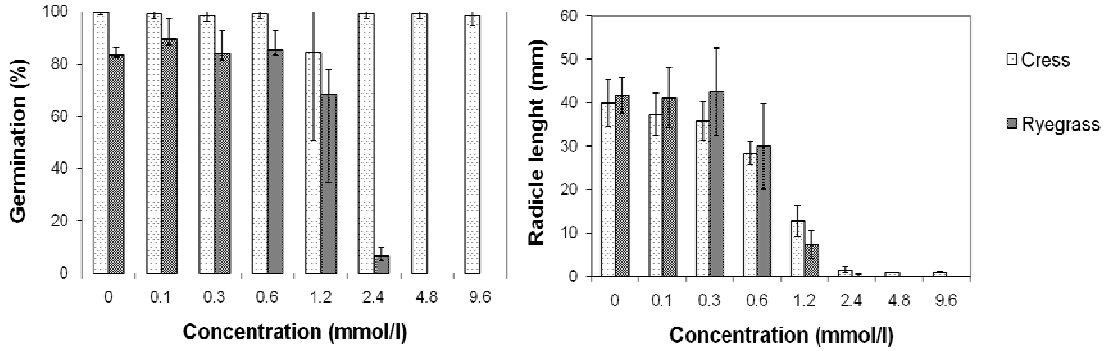



Figure 16. Germination (left) and radicle length (right) in acute exposures to *iso*-valeric acid performed on Italian ryegrass (120 h) and garden cress (72 h). Results represent means with standard deviation error bars.

In subchronic experiments, Italian ryegrass seedling emergence success was at highest studied concentration (40.5 mmol/kg dw) on average 32 % (Figure 17). This was the lowest observed average seedling emergence at this concentration compared to C1 - C4 VFAs. Garden cress was again more sensitive compared to ryegrass in terms of seedling emergence. At concentration of 30 mmol/kg dw cress had average germination rate app. 25 % (Figure 18).

In terms of dry biomass, both tested plant species showed more sensitive response. The dry biomass was negatively effected by *iso*-valeric acid already at 1.5 mmol/kg dw (M-W U-test, cress $p = 0.026$; ryegrass $p = 0.033$) (Figures 17 and 18).

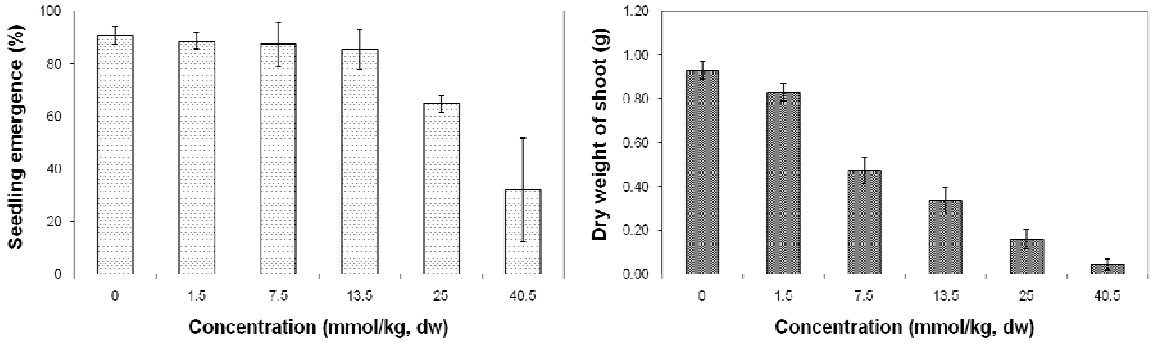



Figure 17. Results of 21-day growth of Italian ryegrass exposed to *iso*-valeric acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass with exception of 1.5 mmol/kg dw, where error bars represent standard deviation. The shoot dry weight data of 1.5 mmol/kg dw acid concentration was not normally distributed, mean and SD are presented, because normality is still assumed to exist.

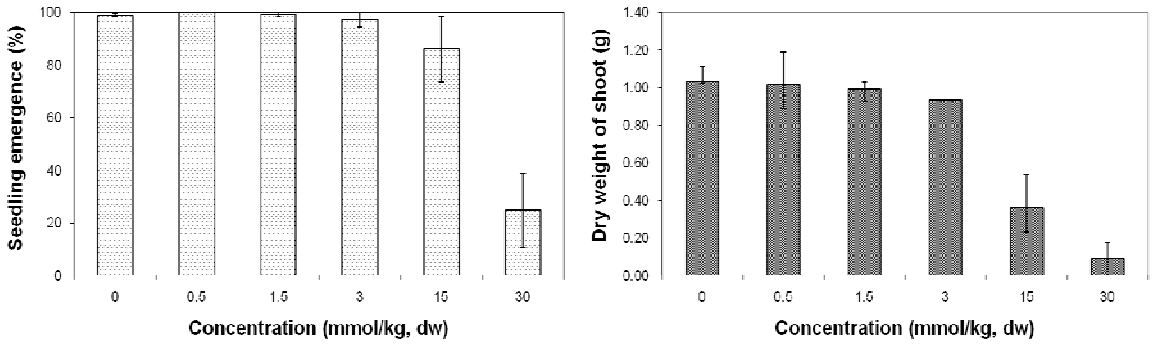



Figure 18. Results of 21-day growth of garden cress exposed to *iso*-valeric acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass with exception of three mmol/kg dw, where error bars represent standard deviation. Transformation of total shoot dry biomass was performed (square root) to obtain a normal distribution of the data. The dry weight data of acid concentration three mmol/kg dw was not normally distributed, mean and SD are given, because normality is still assumed to exist.

3.1.7 Phytotoxicity of valeric acid

As was the case with *iso*-valeric acid also valeric acid totally inhibited germination and growth of Italian ryegrass in two strongest concentrations (4.8 and 9.6 mmol/l) applied in acute tests. At 2.4 mmol/l germination success was on average only 5.9 %. Instead, garden cress did not experience any germination inhibition up to nominal exposure concentration of 9.6 mmol/l (Figure 19).

The response of both species measured as radicle length was more sensitive than germination. Both species were observed to experience inhibition of radicle elongation at concentration 0.6 mmol/l (M-W U-test, cress $p = 0.007$; ryegrass $p = 0.005$), as was the case with butyric, *iso*-valeric acids and in ryegrass also with propionic acid. Although 2.4, 4.8 and 9.6 mmol/l concentrations showed still decrease in radicle length (except for ryegrass at 4.8 and 9.6 mmol/l), it should be stressed that on average over 96 % decrease in radicle length in cress and 98 % in Italian ryegrass had occurred already between concentrations 0.3 - 2.4 mmol/l, with concentration of 0.3 mmol/l not experiencing any statistically significant inhibition of radicle elongation compared to control in both species, M-W U-test, $p > 0.05$) (Figure 19).

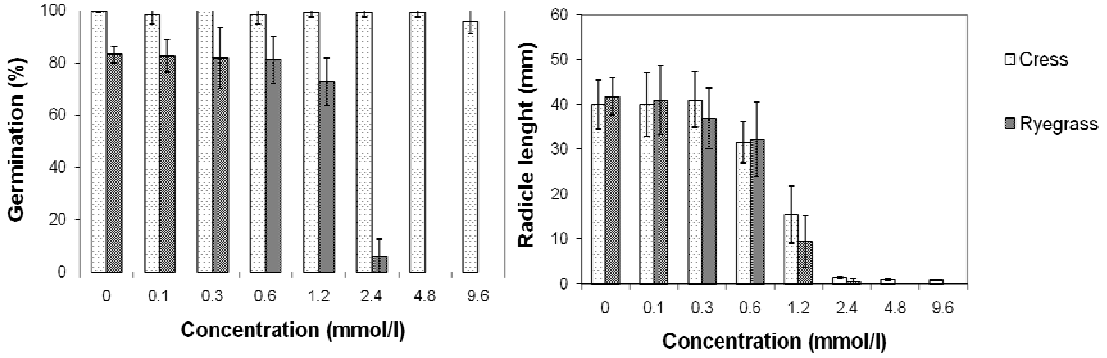



Figure 19. Germination (left) and radicle length (right) in acute exposures to valeric acid performed on Italian ryegrass (120 h) and garden cress (72 h). Results represent means with standard deviation error bars.

In subchronic experiments on the plant growth, (Figures 20 and 21) seedling emergence inhibition was detected at 15 mmol/kg dw in cress and at 20 mmol/kg dw in ryegrass (M-W U-test, cress $p < 0.001$; ryegrass $p < 0.001$). Although in both species the average dry biomass decreased in every studied concentration, statistically significant difference was not detected until 10 mmol/kg dw in Italian ryegrass and 15 mmol/kg dw in garden cress (95 % confidence limits considered). Garden cress was more sensitive in seedling emergence and inhibition of growth compared to Italian ryegrass. At 30 mmol/kg dw

germination rate of cress was on average 6 %, while in ryegrass it was 63 % and at 40.5 mmol/kg dw it was 0 % and 34 %, respectively. Similar trend, although much steeper, was observed for the biomass. Compared to control, at 30 mmol/kg dw cress had gained biomass in three weeks only 1.3 % and ryegrass 21 % (Figures 20 and 21).

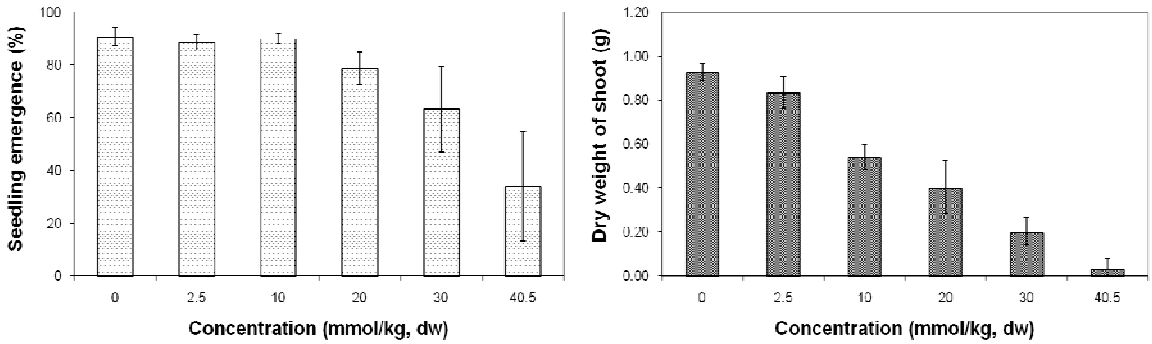



Figure 20. Results of 21-day growth of Italian ryegrass exposed to valeric acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass with exception of, where error bars represent standard deviation. Transformation of total shoot dry biomass weight was performed (square root) to obtain a normal distribution of the data.

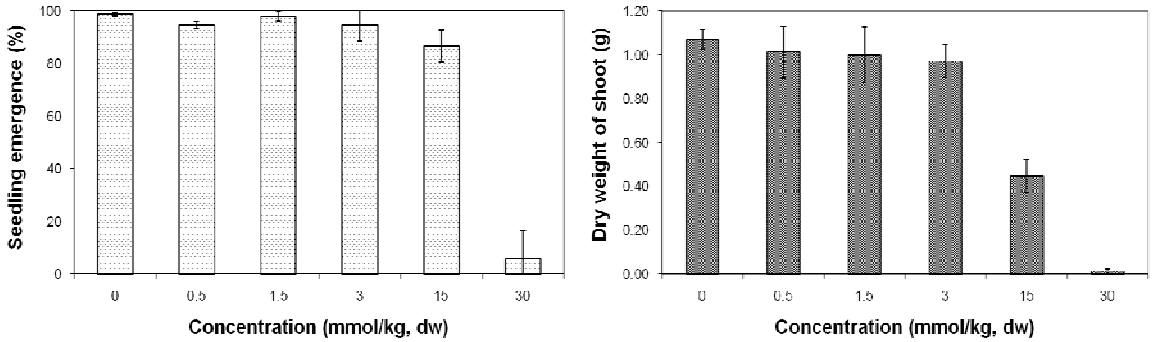



Figure 21. Results of 21-day growth of garden cress exposed to valeric acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass with exception of 30 mmol/kg dw, where error bars represent standard deviation. Transformation of total shoot dry biomass was performed (square root) to obtain a normal distribution of the data. The dry weight data of acid concentration 30 mmol/kg dw was not normally distributed, mean and SD are given, because normality is still assumed to exist.

### 3.1.8 Phytotoxicity of caproic acid

It should be noted that results of garden cress subchronic experiments described in this chapter are results of only two experiments, each bearing two replicates. Previously mentioned dilution series of cress caproic acid (chapter 2.2.2) 0.5, 1.5, 3.0, 15.0, 30.0 mmol/kg dw was not included.

Caproic acid represents the longest carbon skeleton volatile fatty acid (C6). It was interesting to note that inhibition of Italian ryegrass germination was not observed to statistically significant level until concentration of 2.4 mmol/l (M-W U-test, $p < 0.001$), although it was observed with C5 VFAs already at concentration 1.2 mmol/l of acute tests. But at this concentration (2.4 mmol/l) the germination success was on average only 3.0 %. This means that all major decrease in germination occurred between 1.2 - 2.4 mmol/l. As was the case with all other VFAs, garden cress did not show any inhibition of germination at studied concentration range of caproic acid (Figure 22). Radicle elongation was sensitive parameter for both studied plant species (Figure 22) also with exposure to caproic acid. Both species exhibited statistically significant decrease in radicle elongation at concentration 0.6 mmol/l (M-W U-test, cress $p = 0.001$; ryegrass $p = 0.028$), same as the case was with butyric, *iso*-valeric, valeric acid and in ryegrass exposure to propionic acid.

In subchronic growth experiments ryegrass exhibited seedling emergence inhibition at 12.5 mmol/kg dw and garden cress at 16.2 mmol/kg dw (M-W U-test, ryegrass $p = 0.029$; cress $p < 0.001$). Biomass did not show to be more sensitive parameter than germination. Although inhibition of seedling emergence or dry biomass decline was not observed to a statistically significant level in smaller concentrations compared to other VFAs (C1 - C5) it needs to be mentioned that Italian ryegrass at 22.5 mmol/kg dw and cress at 16.2 mmol/kg dw had seedling emergence rate of only 24 % and 29 %, respectively (Figures 23 and 24).

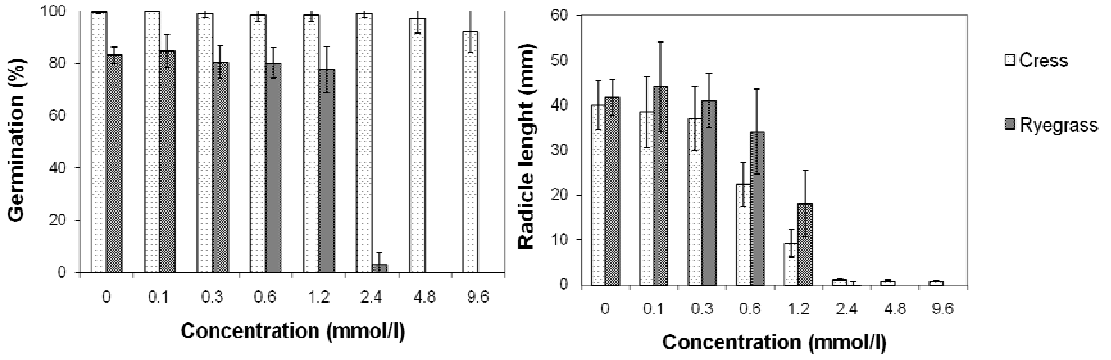



Figure 22. Germination (left) and radicle length (right) in acute exposures to caproic acid performed on Italian ryegrass (120 h) and garden cress (72 h). Results represent means with standard deviation error bars.

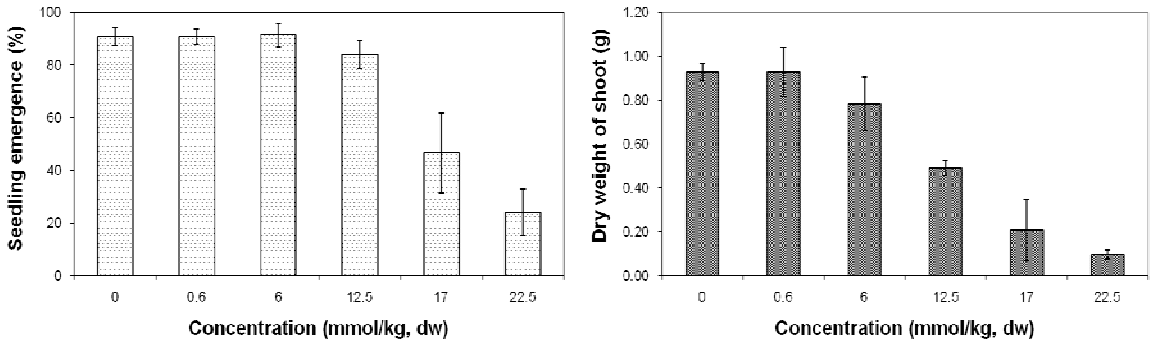



Figure 23. Response results of 21-day growth of Italian ryegrass exposed to caproic acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass.

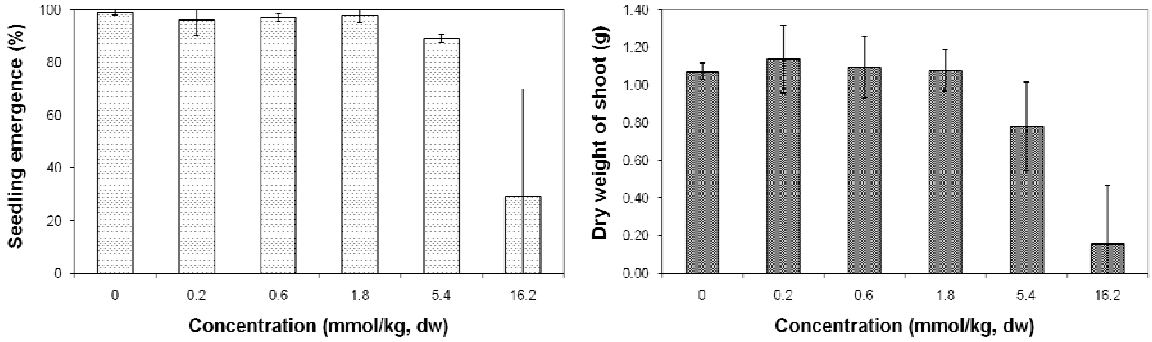



Figure 24. Response results of 21-day growth of garden cress exposed to caproic acid. Error bars represent standard deviation of mean seedling emergence and 95 % confidence limits for average total shoot dry biomass.

## 3.2 Differences in volatile fatty acid toxicities and species sensitivity

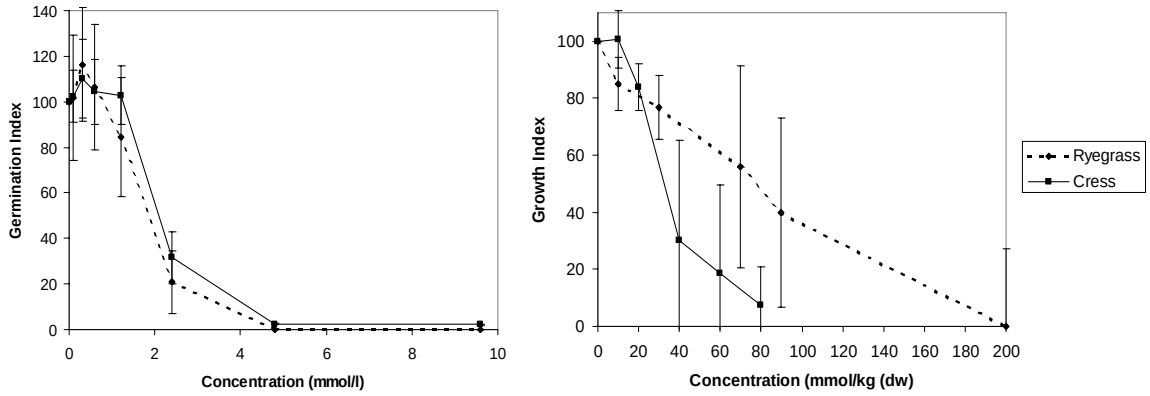
### 3.2.1 Germination and growth indecies

In this chapter presented dose-response relationships of Italian ryegrass (*L. multiflorum*) and garden cress (*L. sativum*) exposed to volatile fatty acids are constructed as indecies. Dose-response curves of germination index (acute tests) and growth index (subchronic tests) can describe toxicity of VFAs in a sensitive way, by uniting (multiplication) two observed response variables and relating the outcome immediately to control. The sensitivity and the usefulness of using indecies has been observed especially after Zucconi et al. (1980a) presented their germination index, which has been extensively cited and associated with compost phytotoxicity studies (chapter 4.8). It is important to note, that indecies omit to give information about natural variability in control data. One should notice that although control values are presented in Figure 25 as values of 100 % with zero deviation this was not the case in actual control results, as could have been observed

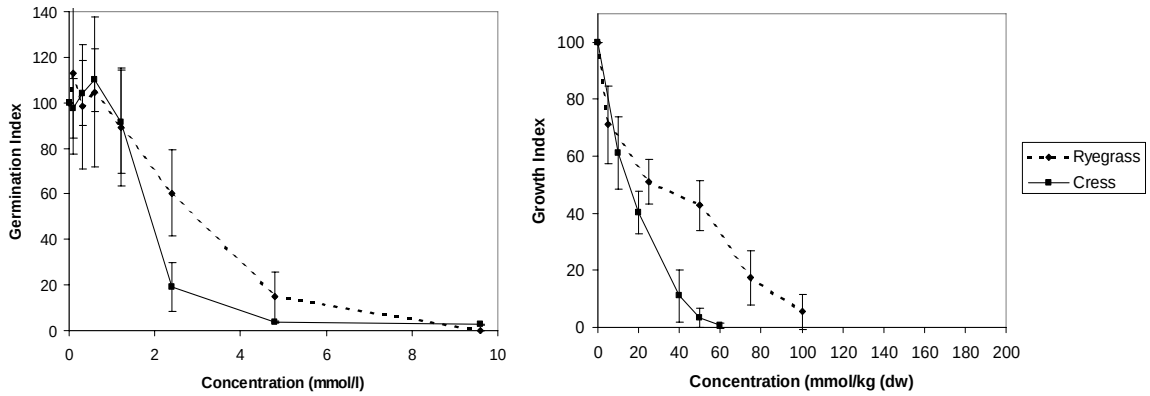
through chapters 3.1.1 - 3.1.8. Most importantly, indecies applied here are able to differentiate between toxicities of different VFAs, which become apparent as a steepness of index curves changes, Figure 25. On average the least toxic VFAs have the shortest carbon skeleton and as the parent carbon chain lengthens the more toxic VFAs become. This is first study, that is known by the author, where a series of VFAs, commonly encountered in composts (C1 - C6), is observed for dose-response relationship for the entire 0 - 100 % response range.

The different sensitivity of plant species to volatile fatty acids becomes apparent especially in subchronic growth tests. Garden cress is more sensitive to VFAs compared to Italian ryegrass. Although control growth of garden cress, measured as dry biomass, was higher than control growth of Italian ryegrass in three-week growth assay, cress exhibited also more rapid inhibition of biomass by VFAs. Also in seedling emergence cress exhibited more sensitive response to VFAs over Italian ryegrass, as was presented in chapters 3.1.1 - 3.1.8. These facts are reasons why growth index of cress exhibits a steeper dose-response curve compared to Italian ryegrass. These observations are further supported by EC50 values of each separate response parameter of subchronic growth tests presented in chapter 3.2.3.

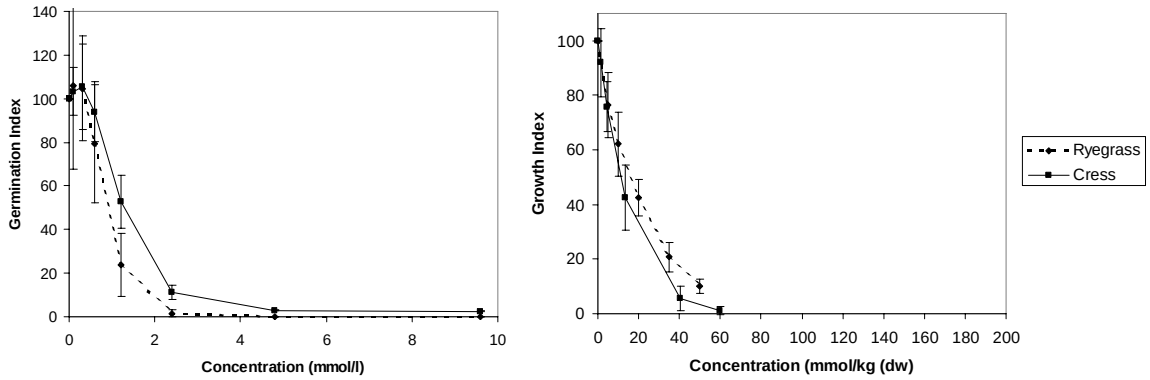
In acute experiments with every studied VFA, concentration limit of approximately five mmol/l can be observed, after which no substantial decrease in response parameter (germination index) on a dose-response curve can be anymore observed. In case of subchronic growth experiments VFA concentration of 50 mmol/kg dw is approximately the concentration point, except for acetic and formic acids, before it all substantial decrease in response parameter (growth index) has already occurred (Figures 25).



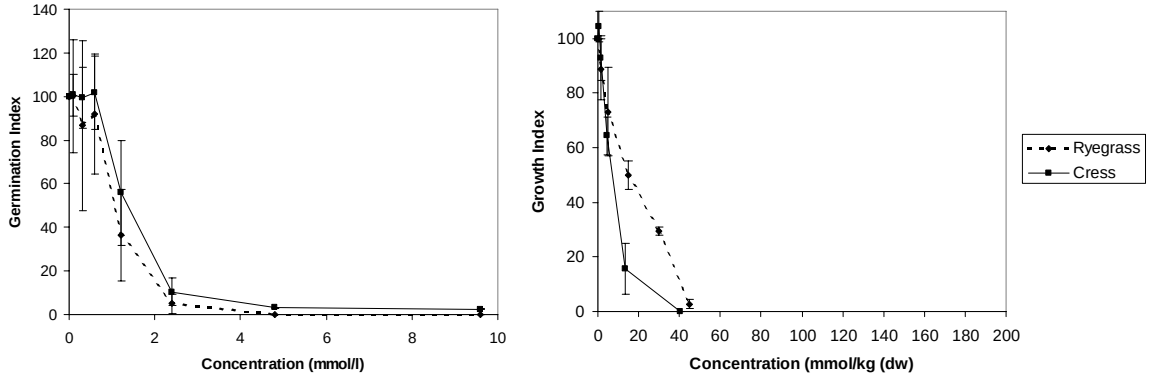

A) Formic acid



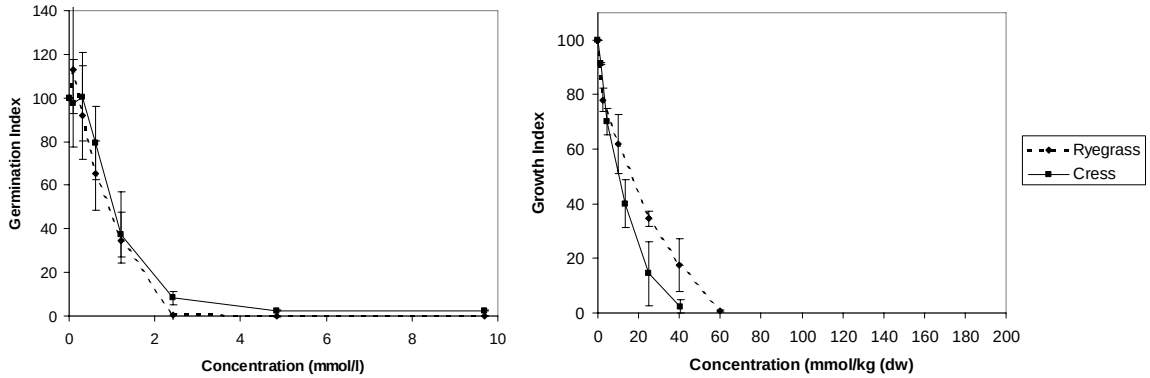

B) Acetic acid



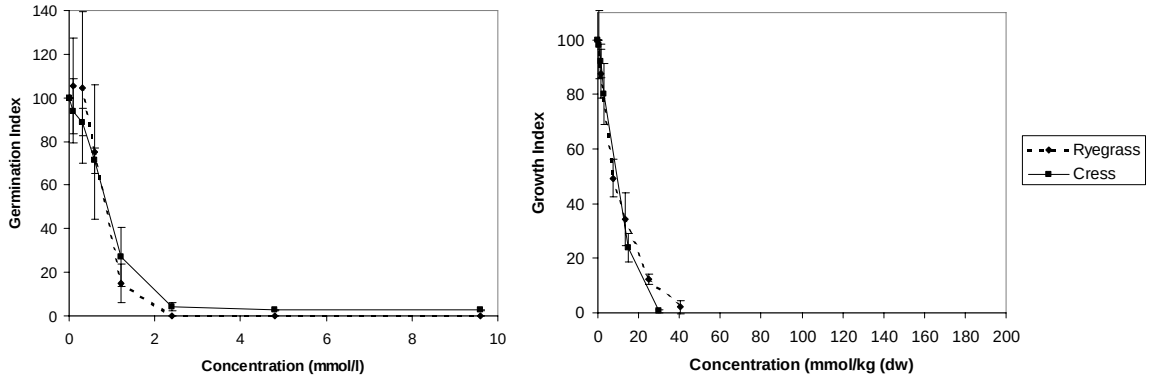

C) Propionic acid



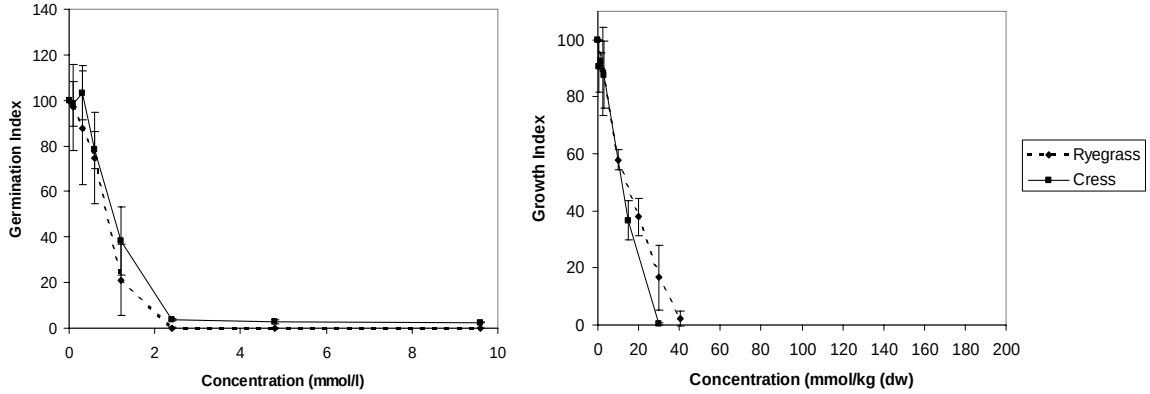

D) *Iso*-butyric acid



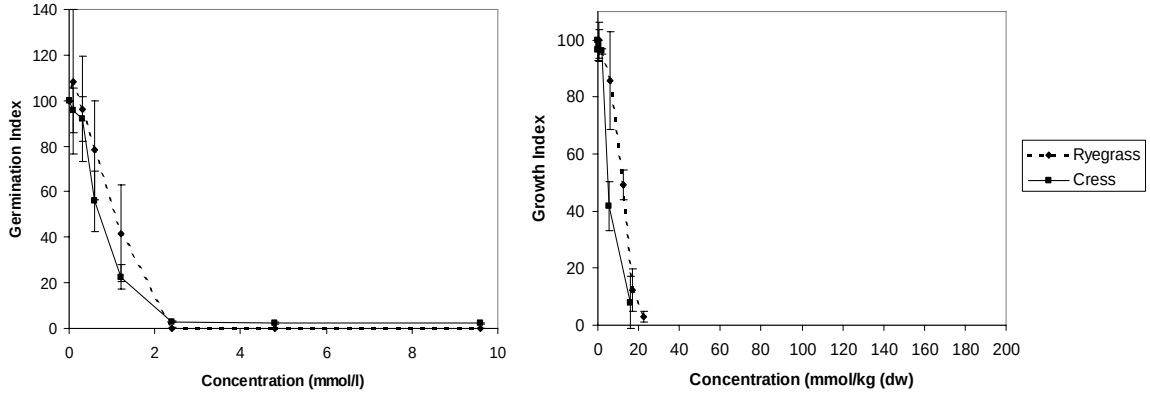

E) Butyric acid




F) *Iso*-valeric acid




G) Valeric acid




H) Caproic acid

Fig. 25. Germination index of acute (left) and growth index of subchronic (right) toxicity of Italian ryegrass and garden cress to volatile fatty acids, C1 - C6, A - H. Curves indicate mean values with standard deviation error bars.

### 3.2.2 Toxicity in acute experiments

In acute experiments, radicle length was more sensitive response parameter than germination (chapters 3.1.1 - 3.1.8). To rank different VFAs according to their toxic potential, VFA concentration of 1.2 mmol/l was chosen for comparisons. The selection of this concentration was based on the lowest VFA concentration, where most acids posed

inhibition to radicle elongation compared to control at the statistically significant level, according to two independent samples t-test ($p$ = 0.05; two-tailed). For Italian ryegrass acetic acid and for garden cress acetic and formic acids were the compounds, where the null hypothesis could not be rejected with this statistical method. Using results of the following concentration, 2.4 mmol/l, would have been more suitable for testing acetic and formic acids, but could not be used for other acids, because inhibition was too strong and a lot of seeds did not germinate.

When comparing radical length of Italian ryegrass in acute exposures the most toxic acid was *iso*-valeric acid, although valeric and propionic acids were in close proximity (Figure 26, Table 7). The least toxic acids were acetic and formic acids. The difference in toxicities between these two acids could not be resolved at studied acid concentration and used statistics (Table 7), but they differed very significantly from the rest of VFAs. Altogether 18 out of 28 statistically significant differences in acid pair comparisons were shown. Although ranking VFAs can not be made to meet the statistical significance level in every individual acid pair comparison, the following coarse list of average inhibition power (toxicity) of volatile fatty acids at 1.2 mmol/l concentrations experienced by Italian ryegrass was: acetic < formic < caproic < butyric < *iso*-butyric < propionic < valeric < *iso*-valeric.

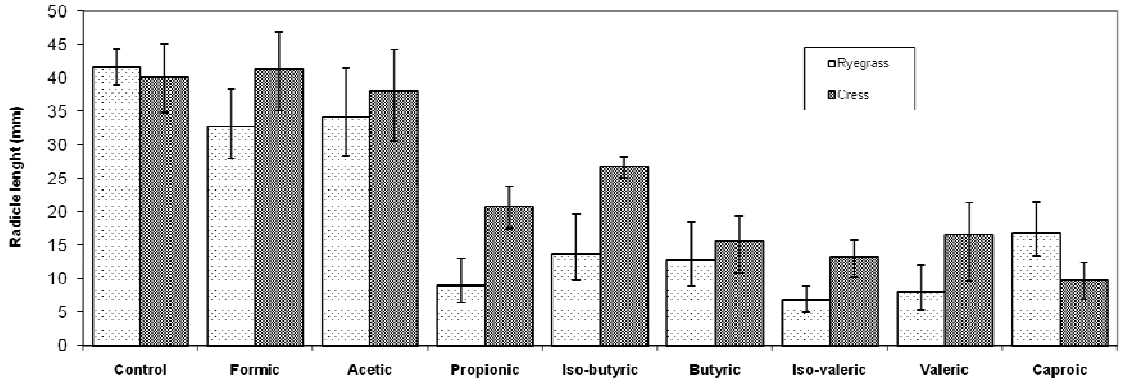



Figure 26. Radicle length of Italian ryegrass and garden cress in acute toxicity tests in VFA concentrations of 1.2 mmol/l. Means and 95 % confidence limits of radicle length (mm) are given. Data on Italian ryegrass was transformed to natural logaritm, to achieve normal distribution. Data on radicle length of garden cress was transformed to the power of two.

Table 7. Comparisons of different VFAs according to their inhibitory effect on Italian ryegrass radicle length in acid concentrations of 1.2 mmol/l. Comparisons are made in acid pairs using t-test. Statistical significance (Sig) (*p*-value of two independent samples t-test) is expressed by * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 from two-tailed normal distribution. Abbreviations of volatile fatty acids are F = formic, A = acetic, P = propionic, Ib = *iso*-butyric, B = butyric, Iv = *iso*-valeric, V = valeric and C = caproic acids.

| Acids | Sig | Acids | Sig | Acids | Sig | Acids | Sig | Acids | Sig | Acids | Sig | Acids | Sig |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F - A | No | A - P | *** | P - Ib | no | Ib - B | no | B - Iv | ** | Iv - V | no | V - C | ** |
| F - P | *** | A - Ib | *** | P - B | no | Ib - Iv | ** | B - V | No | Iv - C | *** | | |
| F - Ib | *** | A - B | *** | P - Iv | no | Ib - V | * | B - C | No | | | | |
| F - B | *** | A - Iv | *** | P - V | no | Ib - C | no | | | | | | |
| F - Iv | *** | A - V | *** | P - C | ** | | | | | | | | |
| F - V | *** | A - C | *** | | | | | | | | | | |
| F - C | *** | | | | | | | | | | | | |

Testing acute response (radicle length) of garden cress in the VFA concentration of 1.2 mmol/l a different toxicity sequence of VFAs was obtained compared to experiments with ryegrass. The most toxic acid was caproic acid (Figure 26, Table 8), although *iso*-valeric acid was in close proximity, but with existing statistically significant difference (Table 8). Out of 28 acid pair comparisons statistically significant difference in radicle length of garden cress was found in 22. *Iso*-butyric was less toxic than propionic acid to a statistically significant level, although the tendency was there also in ryegrass experiments (two independent sample t-test, $p = 0.079$). *Iso*-butyric acid was clearly ($p = 0.001$) less toxic than butyric acid, in contrast to ryegrass. With both species, differences between *iso*-valeric and valeric acid could not be detected to a statistically significant difference. Caproic acid inhibited radicle growth of cress more than valeric acid (two independent sample t-test, $p = 0.003$), also opposite to ryegrass, although not to sufficient statistically significant difference (two independent sample t-test, $p = 0.053$). On the other hand, similar to ryegrass, the effect of acetic and formic acids on cress differed significantly with all other studied VFAs. Although ranking VFAs can not be made to meet the statistical significance level in every individual acid pair comparison, the following coarse list of average inhibition power (toxicity) of volatile fatty acids at 1.2 mmol/l concentrations experienced by garden cress was: formic < acetic < *iso*-butyric < propionic < valeric < butyric < iso-valeric < caproic.

Table 6. Comparisons of different VFAs according to their inhibitory effect on garden cress radicle length in acid concentrations of 1.2 mmol/l. Comparisons are made in acid pairs. Abbreviations of volatile fatty acids are F = formic, A = acetic, P = propionic, Ib = *iso*-butyric, B = butyric, Iv = *iso*-valeric, V = valeric and C = caproic acids. Statistical significance (Sig) (*p*-value of two independent samples t-test) is expressed by * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001 from two-tailed normal distribution.

| Acids | Sig | Acids | Sig | Acids | Sig | Acids | Sig | Acids | Sig | Acids | Sig | Acids | Sig |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F - A | no | A - P | ** | P - Ib | *** | Ib - B | *** | B - Iv | no | Iv - V | no | V - C | no |
| F - P | ** | A - Ib | ** | P - B | ** | Ib - Iv | *** | B - V | no | Iv - C | * | | |
| F - Ib | *** | A - B | *** | P - Iv | *** | Ib - V | *** | B - C | ** | | | | |
| F - B | *** | A - Iv | *** | P - V | no | Ib - C | *** | | | | | | |
| F - Iv | *** | A - V | *** | P - C | *** | | | | | | | | |
| F - V | *** | A - C | *** | | | | | | | | | | |
| F - C | *** | | | | | | | | | | | | |

### 3.2.3 Toxicity in subchronic experiments

#### 3.2.3.1 Differences in seedling emergence

Seedling emergence gives information about seed germination and it's ability to penetrate the thin layer of peat to light without help provided by photosynthesis. On average garden cress was more sensitive to VFAs than Italian ryegrass (Figure 27). Very interestingly *iso*-butyric acid was statistically significantly (95 % confidence limits considered) more toxic than butyric acid with both species. Between acids *iso*-valeric and valeric, statistical difference with either species could not be observed, considering 95 % confidence limits. Both species, however, seem to be most sensitive to caproic acid, i.e. response differed significantly from all other VFAs. Again formic and acetic acid (in this order) posed the least toxic influence on tested species and differed significantly from all other tested VFAs, with exception of seedling emergence response of garden cress on propionic acid, where acetic and propionic acid toxicity could not be resolved to statistical confidence (95 % confidence limits considered). With Italian ryegrass, following toxicity potential ranking of VFAs according to seedling emergence response (EC50), can be made (statistical significance taken into account): formic < acetic < propionic = butyric < *iso*-butyric < valeric = *iso*-valeric < caproic. With garden cress the ranking is in the order formic = acetic = propionic < butyric < valeric = *iso*-valeric = *iso*-butyric < caproic acid. It needs to be stated here that the maxima of seedling emergence values in probit modeling were taken from emergence in control.

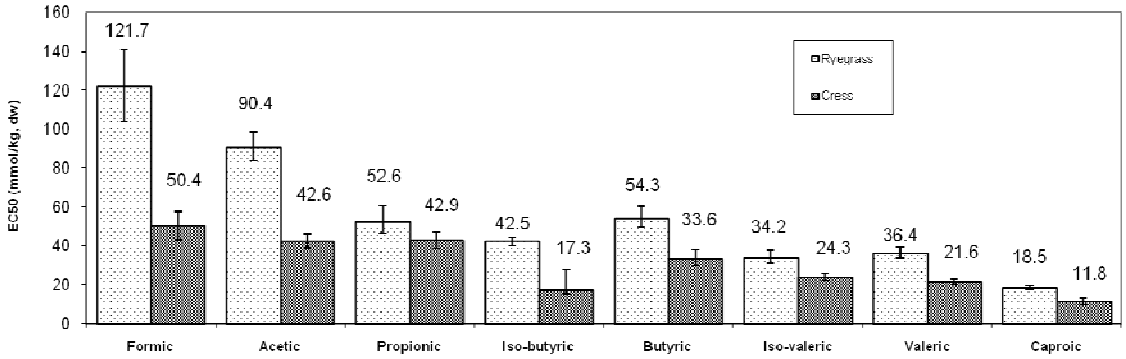



Figure 27. EC50 values for seedling emergence of Italian ryegrass and garden cress in subchronic experiments on growth. Error bars represent 95 % confidence limits. Number above each bar represents the average EC50 value predicted by probit analysis. Natural response was calculated from the data of controls and the maximum value is represented by average control seedling emergence.

### 3.2.3.2 Comparison of plant biomass

As mentioned above, plant biomass was more sensitive parameter over seedling emergence; therefore EC50 values are lower than for emergence (Figure 28). Differences between VFAs are also in a smaller value range, which apparently reduces detection possibilities of differences in toxicities of VFAs by statistics used. Albeit a more sensitive parameter, still similar patterns existed as in seedling emergence data. Formic and acetic acids exhibited least toxic inhibition compared to other VFAs, although with garden cress acetic acid toxicity could not be distinguished from propionic, butyric, *iso*-valeric and valeric acids. There could be seen more toxic potential of *iso*-butyric acid compared to butyric acid, but with ryegrass not to extent of statistical confidence (95 % confidence limits considered). For cress, *iso*-butyric acid represented the most toxic acid together with caproic acid. For ryegrass, caproic and *iso*-valeric acids formed the most toxic pair of VFAs. Still, with ryegrass, propionic, *iso*-butyric, butyric and valeric acids could not be separated according to their inihibition by means of dry biomass response (Figure 28).

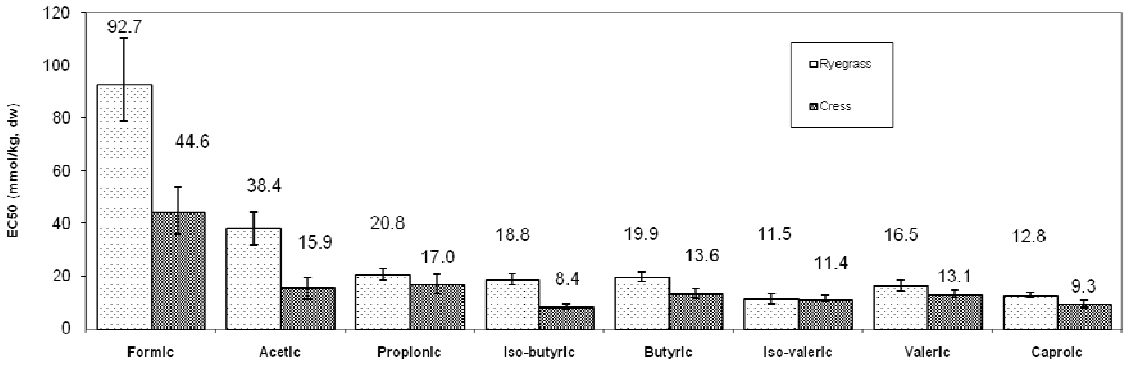



Figure 28. EC50 values of dry biomass in subchronic growth tests of Italian ryegrass and garden cress. Error bars represent 95 % confidence limits. Number above each bar represents the average EC50 value predicted by probit analysis. Natural response was calculated from the data (controls) and the maximum value ($EC_0$) is represented by average control dry biomass.

### 3.2.4 Differences in pH and EC

Acidity and conductivity of growth and germination media represent critical factors affecting plant development by their impact on e.g. nutrient availability and water potential (Taiz & Zeiger 1998, Bewley & Black, 1994). As VFAs are weak carboxylic acids, when they dissociate, they influence both these essential characteristics of growth and germination media.

The difference in pH of various different volatile fatty acids at the same concentration was negligible in the acute experiments (Appendix 4, Table 1). Only formic acid, as a stronger carboxylic acid differed from other acids used in experiments. In solutions with the highest concentration of 9.6 mmol/l pH was 2.91. With other acids the pH of the highest concentration stayed around 3.40 - 3.50. There was even smaller difference between most dilute concentration (0.1 mmol/l), from formic acid of about 4.24 to valeric acid of about 4.65. The same pattern is noticeable in values of electrical conductivity (EC). The EC values stayed in low levels even with highest concentrations of formic acid (Appendix 4, Table 1).

In the subchronic experiments, pH and EC determinations revealed at the end of the experiments, after 21 days, that the pH had risen from acidic values to neutral or slightly alkaline values. In the results of pH differences of formic acid experiment (Appendix 4, Table 3) such pattern of pH change could be detected: the lower was the pH at the beginning of the growth experiments the higher it got at the end. This trend was not clearly detectable with every VFA and was better visible with experiments performed on Italian ryegrass (Appendix 4, Tables 2 - 9). Garden cress control pots (no VFAs added) had, at the end of the experiments, already higher pH compared to ryegrass controls, which made it

more difficult to observe any trend of pH. Also the two strongest VFA concentrations did not always fit the pH-trend, although the other concentrations did. But the most important thing was that the pH of substrate in every single VFA concentration on both studied plant species at the end exceeded always 7, which means that it rose from initial acidic values up to 4 - 5 pH units over 21 day incubation period. Also pH in control pots could be observed to have slightly risen at the end, although controls had a neutral pH already at the beginning of the experiments.

Although VFA concentrations applied in subchronic exposure experiments were not the same for all VFAs and thereby electrical conductivity showed large differences between acids, after 21 days the conductivity values became more even. Interestingly with long carbon chain VFAs and the higher the concentration of an acid the probability of getting smaller EC values increased. Most of the experiments (Appendix 4, Table 2 - 9) had at the end EC values clearly under 100 µS/cm with all experimented acid concentrations, quite low. Cress showed some exceptions. Only in control pots the electrical conductivity raised during the experiment. Formic acid was the only acid were at concentration 200 mmol/kg dw the electrical conductivity raised at the beginning above 1000 µS/cm. At the end the conductivity was still higher compared to other VFAs, close to 300 µS/cm, although this was not visible in pH, which rose to neutral ranges.

### 3.3 Results of experiments A - E

#### 3.3.1 Experiment A

Initial addition of 80 mmol/kg dw of formic acid to sand mixture growth media resulted in decrease of pH from neutral (seven) to about three. The electrical conductivity (EC) rose from about 13 µS/cm to around 660 µS/cm. It needs to be noted that no seeds were planted into any pots in this experiment. After 21 days incubation at room temperature, very interesting results were obtained. At the end of the experiment the pH of sand mixture layer rose by 5 pH units to slightly alkaline values in pots spiked with the acid, which had two layers of peat. The elevation of pH was higher with pots, which were watered with fertilizer solution. In pots with no peat layers the pH rose less than one pH unit. This small rise in the pH indirectly suggested pots to contain some formic acid after three weeks of free evaporation and watering procedure. This suggestion is in addition supported by the high EC values measured in pots, which were watered only with pure deionized water (Figure 29).

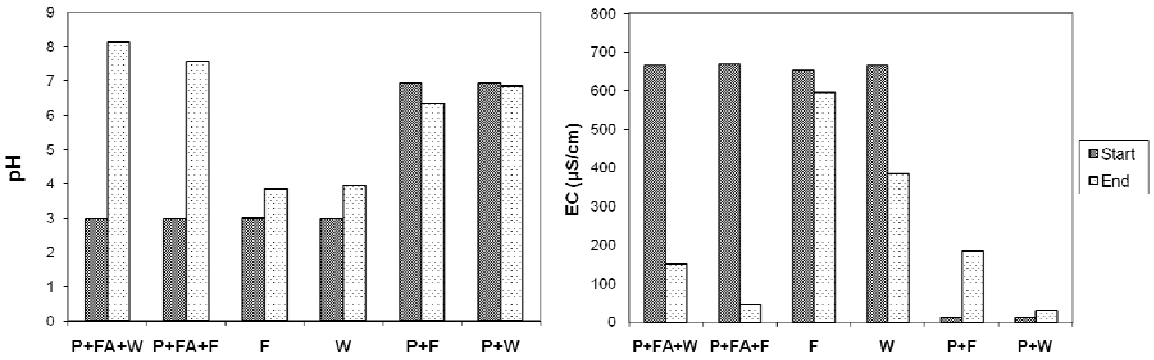



Figure 29. Acidity (pH, left) and electrical conductivity (EC, right) results in experiment A at the start and after 21 day incubation at room temperature. Measurements were taken only from the sand mixture layer of the growth media. Every treatment included sand mixture layer. Treatment abbreviations: P = peat layers on the top and bottom of the pot, FA = formic acid (80 mmol/kg, dw) spiked sand mixture layer, F = irrigation performed with fertilizer, W = irrigation performed with deionized water.

This was the first experiment indicating elevation of pH through the three-week exposure time in the subchronic experiments, not to be considered as an effect induced by plant growth, but clearly peat layers used in the pots had a significant effect on the outcome together with volatile fatty acids. Based on the results of the experiment, two questions arose: what was the neutralizing capability of fertilized limed peat against volatile fatty acids (complex forming, chelating?) and did fertilizing solution have effect on acidity exhibited by VFAs?

### 3.3.2 Experiment B

To understand the neutralizing capability of the sand mixture in a pot with peat top and bottom layers and its sole contribution to the clear rise in pH (while evaporation prohibited), the experiment B was performed. The growth medium content of a single pot was incubated in sealed glass flasks. Results of the experiments indicate that the evaporation of volatile fatty acids has to be taken also in to consideration in understanding of the elevated pH values at the end of subchronic growth experiments (Table 9). This may be one reason, why elevated pH values were most clearly seen with most volatile VFA, formic acid, (chapter 3.2.4).

The quick neutralizing capability of peat exerted on a whole suspension was determined after one hour mixing, which rose pH of the suspension about 0.6 units. Remaining still strongly acidic (3.6). The usage of fertilized solution (pH balanced to 5.5) had virtually no effect on acidity at the start of experiment, although it affected EC values. One should note the high EC values also in treatments with formic acid + sand and peat (Table 9), although

not comparable with previous EC determinations and determined directly from the filtered suspension, were quite high.

After three week incubation period in tightly closed glass flasks the pH rose only about 0.8 pH units, but electrical conductivity rose substantially. Since evaporation of VFAs was restricted and the only evaporation possible was to space above sand-peat mixture (ca. 0.5 l), which has to be considered, it can be concluded that utilized peat has on average small capability of neutralizing formic acid. The acidity of sole peat was neutral with small electrical conductivity potential (determined according to the CEN standards 1999a, 1999b).

Table 9. Results of pH and electrical conductivity (EC, µS/cm) measurements (average ± standard deviation) in the 21-day experiment B. Where indicated formic acid was used in concentration of 80 mmol/kg dw of sand mixture.

| Test material | Start pH | End pH | Start EC | End EC |
|---|---|---|---|---|
| Sand + Peat + Water + Formic Acid | 3.59 ± 0.01 | 4.43 ± 0.01 | 2870 ± 0 | 3645 ± 21 |
| Sand + Peat + Fertilizer + Formic Acid | 3.60 ± 0.01 | 4.42 ± 0.01 | 3630 ± 0 | 4470 ± 42 |
| Peat | 7.07 ± 0.03 | | 145.7 ± 0.14 | |
| Sand + Formic Acid | 3.01 ± 0.00 | | 677 ± 2.83 | |

### 3.3.3 Experiment C

To highlight the effect of fertilizer and complexing and chelating agents of peat used in the experiments on pH and only on pH, peat as an only representative of the growth medium was tested. As Figure 30 reveals, peat or EDTA did not really support the neutralizing effect observed in the subchronic VFA growth experiments. Interestingly peat in fertilized water solution raised pH on a $10^{th}$ day of the experiment above the deionized water treatment, already starting pH of nearly 0.5 units lower compared to deionized water replicates! One should keep in mind, that the glass flasks were opened throughout the incubation time of the experiment. Although evaporation of formic acid was made possible this had minimal effect on pH at the end of the experiment, taking also into consideration results from previous experiment B (elevation altogether about 1.2 pH units, compared to about 0,8 pH units e.g. in experiment B). The sole EDTA solution had no effect on pH.

One should be careful in interpretation of these results, because pH values represented in Figure 30 might involve also distortion from microbial community (fungi or bacteria). At least, at the end of this experiment, treatments T4, T5, T6 showed some small, possible, sings of fungi contamination.

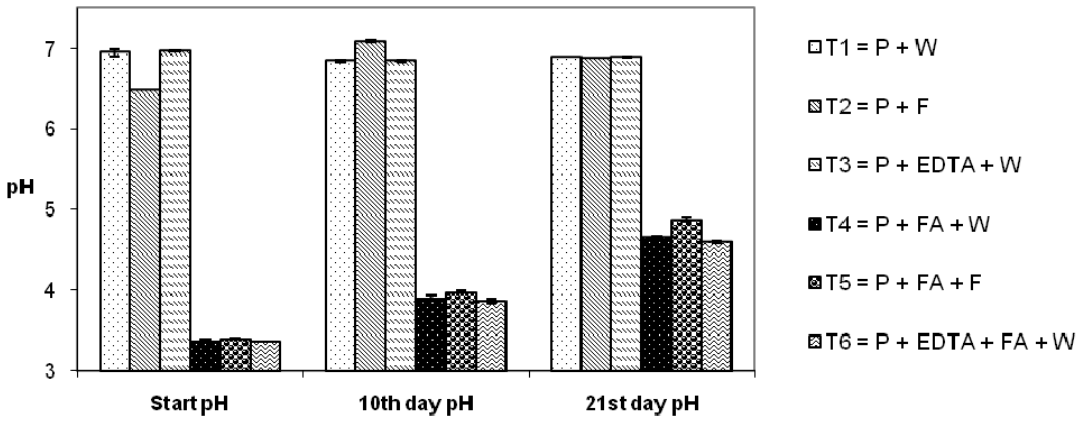



Figure 30. Results of a 21-day experiment C with peat. Acidity values (pH) with ± standard deviation determined at the star of the experiment, on a 10th day and at the end. Treatments 1,3,4 and 6 were prepared into deionized water, W, (300 ml) and treatment 2 and 5 were prepared in fertilized water solution, F, (300 ml). P = peat, FA = formic acid. Formic acid, where indicated, was provided in the quantity of pot sand mixture layer concentration (80 mmol/kg, dw).

### 3.2.4 Experiment D

In experiment D the contribution of evaporation of VFAs to observed increased end pH values measured in subchronic growth experiments was monitored from formic acid spiked sand mixtures. These were left to evaporate in open buckets and watered according to methods in actual subchronic experiments, but only with deionized water, prohibiting pH adjusted fertilizer solution from influencing. It should be noticed that although the results of experiment D and experiment A are not comparable, in both experiments, at the end of 21-day exposure time the pH of the sand mixture, spiked with formic acid 80 mmol/kg dw, was still acidic (Table 10). As said, this was observed also in experiment D, where before every pH determination the sand mixture content was mixed and therefore evaporation of formic acid with every pH determination event was enhanced. Furthermore, the experiment was performed in buckets with thinner sand layers compared to pots. It was, however, less acidic than in experiment A performed in pots, which were not mixed throughout the 21 day period. Somewhat interesting was the pH result of control buckets on 10th day after experiment start, which had risen about 0.3 pH units. One might ask why? Fertilized water was not used, as was the case with peat experiments on a 10th day in experiment C (Figure 30, treatment “Peat + Fertilizer”).

Table 10. Results of 21-day experiment D, pH changes in pure sand mixtures. Sand mixture pH values. Treatment T1 = control buckets with 200 ml deionized water, T2 = buckets with 80 mmol/kg dw formic acid in 200 ml deionized water.

| Treatments | Star pH | 3rd day pH | 10th day pH | End pH |
|---|---|---|---|---|
| T1 | 6.94 ± 0.00 | 6.98 ± 0.03 | 7.29 ± 0.09 | 7.18 ± 0.04 |
| T2 | 2.91 ± 0.00 | 3.30 ± 0.00 | 3.99 ± 0.01 | 4.78 ± 0.03 |

### 3.2.5 Experiment E

Experiment E was performed to mimic the actual 21 day growth experiments, accurately, to establish the start and end pH and EC values of pure growth medium in a pot, without the effects of VFAs or plants. In the experiment E, it was found that after 21 days the pH of seedless pots (in sand mixture layer) was with standard deviation 7.14 ± 0.15 and electrical conductivity (EC) 115.06 ± 25.44 µS/cm. The effect of plants on pH and EC of growth media in subchronic growth tests can be compared against these values.

## 4 DISCUSSION

There is a need to distinguish between adverse effects posed by VFAs and by other factors possibly influencing the results of present study. This is addressed in the first part of discussion (chapters 4.1 - 4.7). The question of representativeness of applied methods and procedures is discussed in chapter 4.8 after which the effects of VFAs are reviewed (4.9). Some new ideas on the role of VFAs as plant regulators and their resemblance to auxins are brought up in chapter 4.10. Problematic aspects associated with cress germination definition and influence of VFAs on gibberellins induced germination events are addressed in 4.11. In chapter 4.12 the ability of present study to support phytotoxic effects observed in environment and composts is discussed. Conclusions are reported in chapter 4.13, highlighting some future research lines encouraged for a better understanding of VFA toxic processes under light of current study.

### 4.1 Electrical conductivity

As it was addressed in introduction (chapters 1.1 and 1.4), dissociation of volatile fatty acid increases also conductivity. Electrical conductivity was considered a crucial factor in both subchronic and acute toxicity experiments possibly affecting response parameters. This was especially true, because in both tests (acute and subchronic) the plant development process began with germination of seeds, which is known to be, on average, salt sensitive stage in plant development (Bewley & Black, 1994) (chapter 1.4). But also

later development and growth could have been affected by higher salinity. Especially garden cress (*L. sativum*) is known to be a salt intolerant species (TMECC, 2001). According to Kotuby-Amacher et al. (2000) at values up to 2000 µS/cm plant response is mostly negligible and at 2000 - 4000 µS/cm the growth of salt sensitive plants may be restricted. Values over 1000 µS/cm were encountered in present study only in subchronic growth experiment with formic acid concentrations of 200 mmol/kg dw (1234 µS/cm, Appendix 4, Table 2). In acute tests the highest EC exhibited formic acid (9.6 mmol/l), about 450 µS/cm (Appendix 4, Table 1). In all cases of subchronic growth experiments the conductivity values decreased substantially, usually to values below 100 µS/cm, till the end of experiments. End conductivity measurements on acute experiments were not performed, but it was considered unlikely to have resulted in higher conductivity values compared to starting values. Thus, overall, the electrical conductivity can not be seen as a reason for observed phytotoxicity.

### 4.2 Concentration of $H^+$

The second aspect of this study was the difficulty to distinguish between the effects of high $H^+$ concentration and VFA phytotoxicity. On could detect a clear negative correlation between increasing VFA concentration and lowered pH values of growth media/solution (Appendix 4, Tables 1 - 9). Questions considering the role of lowered pH arise. What kind of role plays the lowered pH in observed phytotoxic effects? And of course, the imminent fundamental question has to be brought up also: are not the adverse effects of VFAs merely conjugated to lowered pH of growth media/solution?

The question of pH is not easily answered. In acute tests, the pH values of growth media at the beginning of experiments showed for the highest acid concentrations values from 2.9 to 3.5 and in subchronic tests from 3.0 to 4.2. The question is: does such low pH stress the plant and add to the exhibited adverse effects? Through the review by Stevenson (1967) and discussion by Chandrasekaran & Yoshida (1973), there is evidence, that many different organic acids are part of root exudates and can naturally lower the pH in the immediate vicinity of rhizosphere in addition to microbes of rhizosphere, where acid producing and acid tolerant species are known to reside (Stevenson, 1967; International Rice Research Institute, 1970). Taiz & Zeiger (1998) state that slightly acidic soil conditions, pH (5.5 - 6.5), would favor plants root grow. But the low start pH values, as stated above, could have impacted results by affecting e.g. enzymes in germination and

seedling emergence (chapter 1.4) and further it is recognized that $H^+$ ions are directly (*per se*) toxic to most plants at concentration above 1 mM (below pH 3) (Fitter & Hay, 1987).

This study supports the idea that declination of pH was not the primary cause of phytotoxic effects, as was proposed also by e.g. Lee (1977), Lynch (1977) and Shiralipour and McConnell (1997). There were statistically significant differences between the same response parameters of different VFAs (chapter 3.2) at the same pH values (Appendix 4, Tables 1 - 9), and decline in the dry biomass can be detected in subchronic growth experiments in some VFAs treatments, which already have high starting pH of above 5 - 6. Adverse effects of VFAs on e.g. rice plants have been reported also in neutral (pH 6.5 - 7.5) environments (Chandrasekaran & Yoshida, 1973; Rao & Mikkelsen, 1977).

Although, as stated above, potentially harmful low starting pH values were observed in subchronic growth tests, the pH rose over three week period to neutral or slightly alkaline values. And the key question is: how fast this change in pH had happened? When had ended the possible pH stress caused by applied VFAs? It is clear that in acute experiments at least germination could have been affected by lower pH values and in subchronic experiments germination and seedling emergence may have initially been exposed adversely to high $H^+$ concentrations.

Using data of the preliminary subchronic experiment it is possible to get an idea how quickly the pH changed to neutral ranges. The experiment lasted for only seven days (data not shown). In garden cress (*L. sativum*), preliminary experiment showed that at least up to 13.5 mmol/kg concentration of every applied acid, neutral levels were reached in sand mixture layer in one week or earlier! And for acetic acid, at concentration of 40.5 mmol/kg the pH rose in just seven days from 3.98 up to 8.00. With caproic acid 16.2 mmol/kg concentration (highest applied concentration) the final pH was elevated to 7.66. Although with other VFAs the highest applied concentration in these preliminary experiments, 40.5 mmol/kg, did not show such a large increase in pH, as was observed in the case of acetic and caproic acids, pH values of 5 - 6 were observed. Thus, even in this one week experiment, all studied VFAs (8) showed increased end pH values, in pattern of: the more acid the pots received at the beginning of the experiment (that is, the lower the pH) the higher were the end pH values, except for the highest concentrations, as stated above.

This is interesting. Why pH of sand mixture layer rose at the end to higher pH values in pots receiving more acid than in pots that received less acid and had higher starting pH? It

is clear, that after one week, the highest concentrations, 40.5 mmol/kg, could have left some VFAs still in the sand mixture layer and therefore the end determination of pH of this highest concentration showed in preliminary experiments still some lower pH values than for the rest of the concentration series. As mention, this was except for acetic acid and caproic acid, were end pH was always in neutral ranges and in the line with the remarkable observed patter of: the higher the VFA concentration to begin with, the higher the end pH values. Since the highest applied VFA concentrations with the longest carbon skeleton acids did not differ remarkably from concentrations used between pre-experiments and actual subchronic experiments, it can be speculated that at the end of second week the sand mixture layer of pots could have reached neutral pH in all applied VFA concentrations. This conclusion is also supported by qualitative odor notifications, since after second week the inner VFA absorption apparatus of growth chamber was dismantled due to disappeared malodors. Malodors of VFAs can be substantially decreased by neutralizing them. Formed salts are not evaporable. One could approximate that two pH units above VFAs $pK_a$-value means practically full dissociation of acids (Harris, 2003). This would be true for all VFAs at pH approximately around seven and, only for formic acid, already at pH six.

### 4.3 Chlorosis of shoots and other damages

There have been reported cases of VFAs exhibiting visual damages on plant shoots, especially on rice. Dying of lower leaves in rice plants has been reported by Chandrasekaran & Yoshida (1973), while Rao & Mikkelsen (1977) reported withering of leaf tips at one mmol/l and bronzing of leafs at five mmol/l of acetic, propionic and butyric acid concentrations and dying of whole rice seedlings at 10 mmol/l within 24 hours of exposure, when studied VFAs individually. Faded leaf color has been reported also by Takijima (1964). The effects were stronger as acid concentration increased (Takijima, 1964; Rao & Mikkelsen, 1977). In ryegrass (*L. multiflorum*) subchronic growth experiments of present study, no chlorotic shoots were observed, although diminished lengths of shoots and roots were detected. The color of shoots of highest VFA concentrations was as green as color of shoots of control pots by visual inspection. This is of interest, since on the opposite, cress showed chlorotic shoot damage, deformations of small seedlings and number of death plant individuals (Appendix 5, Fig. 1 - 2) at the end of subchronic growth experiments. Chlorotic shoots were not recorded until second week. Most of the yellowing and withering happened during the last week of the experiments (days 15 - 21). Although in some pots experiencing chlorotic damage and withering at the

end of subchronic experiments, at the beginning of tests the seedling emergence was high (almost equivalent to control pots) and no pale green coloring was observed. This gives rise to a question of, why the seedling emergence or condition of shoots was not affected by VFAs, when the amount VFAs in the sand mixture layer was highest and pH lowest?

Then again, one should ask, why in cress the increase in pH toward the end of experiments did not counteract against withering, dying and yellowing, which progressively proceeded towards the end of tests? Was the time too short for the recovery of plants to be observable? Sanderson and Armstrong (1980) observed that with spruce (*Picea sitchensis*) and pine (*Pinus contorta*) recovery of root growth is possible after acetic and butyric acid exposure to rooted cuttings. But the resumed growth rate could not be achieved until after several days or weeks and it was concentration and species dependent. The highest studied concentrations (about two mmol/l in acetic acid or 0.6 mmol/l in butyric exposures) the 21 day recovery period was not enough to resume the growth rate of the roots compared to control in study by Sanderson and Amrstrong (1980). On a more acute scale, Ulbright et al. (1982b) found that 24 - 48 hours exposure of C5 - C7 fatty acids on primary roots of lettuce (*L. sativa*) at concentrations 3 - 4 mmol/l, recovered root elongation only partly. Prolonged exposure and stronger concentration affected the rate and magnitude of recovery.

Interestingly, the yellowing of garden cress shoots was observed only in pale way in whole experiment replicate number two. Other interesting feature was that the inhibition of seedling emergence in subchronic experiments with both plant species was observed to be a somewhat nonreversible (quite persistent) in the three-week exposure period. The seedling emergence was counted on day seven and at the end of experiments (21 days). In some cases the seedling emergence doubled from day seven to the end of experiments, although the increasing pH towards the end of experiments (in most cases probably in neutral levels by the end of the second week, as speculated earlier) did not resume the seedling emergence even distantly to levels of control pots.

### 4.4 Effects of microbes and molds

As this study was not performed in sterilized conditions, this led to the third question about effects of microbes, especially fungus, on tested plants. As was stated earlier in introduction, the VFAs represent high energy rich compounds for respirating organisms (Madigan et al., 2003). Due to low pH, growth media could be seen to favor growth of fungi, as fungi predominate in acidic soils over bacteria (Taiz & Zeiger, 1998). Metabolic

activity of microbes could lead to decomposition of VFAs and accelerated loss of VFAs from the treatment pots. And still one should not forget that these micro-organisms are capable of forming phytotoxic compounds themselves, like organic acids (Stevenson, 1967). The three weeks of incubation, with fertilizer and organic material (peat) amendment and possible extensive rhizosphere with root exudates (Stevenson, 1967) could have contributed to favorable conditions of microbial growth. In this study, the effects of micro-organisms on VFAs and their phytoxicity were not studied and can not be assessed. Although some VFAs can act as antifungal agents (Tenuta, 2002; Browning, 2006; Evira, 2006) this was not evident in experiments performed. In acute experiments, Italian ryegrass exhibited some very small signs of mold growth, in rare occasions. By comparing these results to same treatment without fungal infection none treatment was discarded. Molds were also a small nuisance in subchronic experiments. This was especially noticeable in pots with the highest VFA concentrations, with the exception of caproic acid. As with acute experiments, also in subchronic experiments there was an estimation made that observed molds had only limited, if any, effect on experiment results. All treatments were grown in same closed space, with same fan made air distribution and therefore same chances of getting infected with fungal spores. If some VFAs treatments would serve a better conditions for fungal growth/colonization (e.g. some VFAs were more prone to fungal infection) this was out of control possibilities. It needs to be although stressed that the impossibility to control fungal infection and growth could have broadened the deviation of results. And standard deviation indeed was sometimes high in results (chapters 3.1.1 - 3.1.8).

The differences in sensitivity and in other characteristics of tested plant species are also important features to be noted. To come back to the question of fungus infecting pots or Petri dishes a striking feature was found to accompany acute toxicity tests performed on garden cress. No visible mold colonization was ever found in Petri dishes with garden cress! One possible explanation was that the acute tests performed on garden cress were just 72 hours and this fact could therefore limit the timeframe needed for visible mold growth to occur compared to 120 hour acute testing on Italian ryegrass. The other explanation would be that the garden cress produces or leaks some compounds that could be inhibitory against some fungi species minimizing chances of fungal growth. Although this observation was not the focus of the study some qualitative (visual) indication of possible inhibitory effects against some molds were received, when seeds of both tested

plant species were incubated for 3 days on Petri dishes with gelatinous culture medium of meat extract and starch in the vicinity of same Petri dishes infected by water extracts of ordinary molded bread (incubated in closed compartment with heavy air circulation). The pH was lowered to about 4.0 - 4.5 to inhibit bacterial growth. Visible differences between these two species were observed already by the day two in visible in mold growth (Appendix 6, Fig. 1 - 2). Even if this was an only single three day incubation test with two replicates of each treatment, it could be speculated that species sensitivity to fungus infections could have resulted in some differences observed between species, if fungal infection had some adverse effects on measured exposure parameters.

**4.5 Seed size**

There is also a difference in seed size, which could have attributed to difference observed between tested species. Garden cress seeds were smaller compared to ryegrass seeds. The small nutrient and energy reserves are more limited with smaller seeded species (Bewley & Black, 1994). Whether this has negatively influenced the garden cress against Italian ryegrass is questionable. As results show (chapter 3.1) cress had better germination success in acute exposures, virtually with no effect of even highest applied VFA concentrations contrary to ryegrass, where dose-response curve could have been modeled. Then again, in subchronic experiments garden cress showed more sensitive response to VFAs (chapter 3.2.3). The concentrations needed for 50 % response (seedling emergence and pot dry biomass) were on average mostly statistically significantly lower compared to Italian ryegrass. The coverage of cress seeds (with peat), which was not suggested by seed provider, could have meant one more stress agent in the subchronic experiments. In fact, although very rarely, in some occasions at the end of experiments, during the cleaning process, a cress seedling was observed under the peat coverage, which had not penetrated the peat layer. Whether this was just a question of delayed germination or really a problem of emergence through peat layer, since of cress's seed's limited and small energy reserves, is debatable. Peat layer, used for seed coverage, was assumed to provide even moisturized conditions for seed germination. The usage of e.g. plastic coverage to maintain moisture for the seed germination was on a large scale technically impossible due to heavy air circulation in the growth chamber without effecting the air circulation and other even physical conditions (temperature ranges, humidity, light etc.). The need for small seeded species to germinated near the soil surface has been described also by Bewley & Black (1994).

### 4.6 Peat and variability of results

There is a one important aspect in subchronic growth experiments, which may have played an essential role in enlarging deviations of results, namely peat. The applied peat was fertilized and limed and represented a healthy, commercial growth substrate. According to experiments A - E, peat had a role to play in elevating pH towards the end of tests, it had provided also nutrients, buffering capacity and organic material for plants and possible microbes residing in rhizosphere. The visual approximation of addition of same quantity of peat by hand in to each pot by two (≈ one centimeter) layers is not a precise one. Especially the coverage of seeds by peat could have resulted in seeds burrowing deeper into the sand (with VFAs) or incorporating seeds into the layer of peat, away from the VFA contaminated sand mixture layer. This could have resulted in differences observed in seedling emergence success. And since the limed peat provided also buffering or neutralizing capacity against low pH determined by VFAs, the percolation of irrigation water or, when watered on a saucer, capillary rise, through different amounts of peat could have also played a role in variability of results. One should also note the high variability of different response parameters inside one pot or Petri dish (represents one treatment replicate) as indicated by shoot length and radicle length in Appendix 7, Fig. 1 - 2. If the variability in response parameters is high, one should increase the number of replicates and use more individuals in one replicate to obtain also some rare events and neutralize the effect of variation in the data. From this point of view we come to the question of statistics. The number of replicates and repeated whole experiments were perhaps not enough to dissipate the higher variability in results and the sensitivity to find statistically significant differences between closely related acids. Especially using three different incubators in acute testing with only one treatment replicate in each repeated, whole, experiment could have increased higher variability in results, when thinking retrospective.

There is a need to highlight also the importance of choosing statistical or mathematical models for obtained dose-response results. Even small changes in scale or data transformations could lead to different results to be achieved even with the same model. Every model has its own assumptions, and is usable only, when criteria are met. For example probit modeling, used in this study, could not include induced effects of response parameters (the so called hormesis) of low VFA concentrations visible in acute testing. Therefore comparisons in acute testing had to be made on concentration basis, without statistical/mathematical modeling dose-response curve for obtaining EC50 values. What

would be good to stress here is that with different statistical methods different results may have been achieved. And as a personal opinion of the author can be stated that the statistical idea of real world, the meanings of words “true” and “real” results (statistically significant results), vary. These merely just depend on ones judgments and assumptions of relevant statistical models and results.

**4.7 Results of experiments A - E**

Experiments A - E did not give an ultimate answer to question about pH rise in subchronic growth experiments. However, they indicated indirectly that even after three weeks of incubation formic acid (the most volatile VFA) in only pure sand mixture (present at 80 mmol/kg dw) did not result in pH rise to neutral levels (D experiment supported by findings of A experiment). This was observed, although sand mixture layer was much thinner than present in real test pots and mixed during incubation. The conclusion can be made that, without peat layers and evaporation, the pH rise would not have possibly occurred. A role had also the fertilizer solution used for irrigation, because its pH was buffered with KOH to 5.5 and therefore provided some neutralizing effect against volatile fatty acids. As stated earlier, the rate of VFA-degradation by microbes in the growth media was not assessed in this study, although it may had affected results. It could be hypothesized that the observed interesting pH pattern (the lower starting pH the higher end pH) could be attributed to the fact that both, VFAs and fertilizer solution, were acidic in nature. These acidic conditions released alkaline buffering compounds from the peat layers to sand mixture layers. The used peat, Kekkilä Kasvuturve B2, was limed with dolomite limestone, which would leave after hydration unevaporable inorganic, alkaline $Ca(OH)_2$, $Mg(OH)_2$. As a weak carboxylic acids, VFAs are in a constant dynamic equilibrium with their dissociated and undissociated (the volatile form of VFAs) forms determined by surrounding pH (Formula 1, chapter 1.1). As the protonated form evaporates, it also stimulates a reverse reaction to replace the protonated form to maintain the dynamic equilibrium. This means that there will be smaller concentrations of dissociated forms of VFAs and thus less $H^+$ ions to contribute acidity. The question of why the pH rose more at the end with higher VFA application at the start of experiments could be perhaps explained by fact that more acidic environment also dissolves more alkaline compounds. The reason, why this effect was most visible with formic acid could be a contribution of the highest volatility, because easy volatile formic acid evaporates from sand mixture to the peat layer

or air more rapidly than other VFAs and as a stronger acids also dissolves alkaline compounds more easily.

Why the trend was not obvious in all VFAs after 21-day experiment? There could be several reasons. First of all the acid concentrations differed, formic acid being applied in highest concentrations. Then there are the possible effects of microbes degrading at different rate different VFAs or producing organic acids themselves (Stevenson, 1967) and the question is complicated still by different volatility rates of VFAs. Perhaps the most important aspect is the ability of three week incubation period to even out some end pH differences, whether by physical or biological way. This last fact was supported by the garden cress one week pre-experiment results, where the trend of pH rise was clearly noticeable with all VFAs up to the second highest concentrations (acetic and caproic acids up to the highest concentrations), but in three weeks actual subchronic growth experiments the differences were levelled off. Interestingly, the pH in control pots of garden cress (no VFAs) was of about 0.5 pH units higher than would be suggested by the results of experiment E, the average pH of pots without any seeds. This same effect was not observed with Italian ryegrass. Same experiment E suggested that the electrical conductivity (EC) would rise to somewhat higher values than observed at the end of subchronic growth experiments. On average, plants lowered the EC of sand mixture layer, by absorbing some nutrient electrolytes from supplied fertilizer solution, lowering electrical conductivity by 30 - 60 µS/cm, compared to seedless replicates.

### 4.8 Reliability, representativness and selection processes

This chapter of discussion will clarify some choices made in the selection process of materials and methods described in chapter 2 to give credence to performed experiments. As the background aim of this work was to study and better understand the toxic potentials of different VFAs identified to be present in immature composts, the phytotoxicity tests conducted on composts, found in literature, served here as models.

*The representativness of selected volatile fatty acids*

The volatile fatty acids were selected according to information found in literature on analysis of different composts (Schuman & McCalla, 1976; DeVleeschauwer et al., 1981; Chanyasak et al., 1982; García et al., 1991; Marambe et al., 1993; Baziramakenga & Simard, 1998; Himanen et al., 2006). Although not all studies have measure or even tried to measure all VFAs employed under present study, the desire was to include all carbon

skeleton lengths (C1 - C6) VFAs into the comparative toxicity testing. Paying attention to the purpose of present study to provide basic toxicological information about the phytotoxic potentials of different volatile fatty acids, the aim was to conduct all work only with pure substances (VFA exposure).

*Selection process of plant species*

Several criteria were employed in the selection process of test plant species. Standardized guides for terrestrial plant toxicity tests were not to be ignored, species selected should have had agrological significance and be arable in the Finnish climatic conditions and the selected species should have been recorded in previous studies on compost phytotoxicity. By these criteria the results of this study could be of some practicality too. It was a desire that the test species used should have been the same in acute and in subchronic tests, to record a possible difference in toxicity potential of VFAs as substratum and length of the exposure changes. Speed and success of germination was also emphasized. To be suitable for acute and subchronic testing seeds should have germinated reasonably quickly, with in few days.

It was decided to select two test species for this study. According to ISO 11269 - 2 (ISO, 1995) these should include one monocotyledon and one dicotyledon species (selectable species listed on the standard). The number of species needed varies among different standards and their aims, e.g. guidelines of OECD 208 (OECD, 1984) requires minimum of three species, and ATSM standard E 1598 - 94 (ASTM, 1994) requires at least three dicotyledon and two monocotyledon species, and still ASTM E 1963 - 02 (ASTM, 2002) does not state any particular number of required test species. It has to be stated here that literature contains numerous studies on compost phytotoxicity, where terrestrial plant tests have been applied. But at least one method should be here mentioned and that is the TMECC ’s Biological Assays 05.05 (TMECC, 2001), which is designed to measure phytotoxicity of compost with cucumber (in chapter A and B of this method). This was also considered for this study.

From the dicotyledonous species it was easy to select garden cress (*L. sativum*) for this study. Cress is mentioned in all above mentioned standards and has been widely used for compost phytotoxicity and maturity assessment. Especially after Zucconi et al. (1981a) published their famous germination index of cress bioassay by which they evaluated phytotoxicity and maturity of compost. Reference to other studies associated with cress

and compost phytotoxicity can be given to e.g. Zucconi et al. (1981b), DeVleeschauwer et al. (1981), Lilja et al. (1986), Saviozzi et al. (1987), Keeling et al. (1994), Ligneau & Watt (1995), Gajdos (1997), Helfrich et al. (1998), Warman (1999), Marchiol et al. (1999), Brinton & Tränkner (1999), Brinton & Evans (2002), Himanen et al. (2006). As a novel further advantage can be also seen the close resemblance of germination of cress (*L. sativum.*) and famous *Arabidopsis thaliana* and the interest for adopting cress as a model system in germination studies of Brassicaceae (instead of *Arabidopsis*), namely in understanding germination process (Müller et al., 2006). Although cress is known to be salt intolerant species (TMECC, 2001) this was not considered to be a problem since dose-response studies on VFAs would not be carried out with pH buffered VFAs. Neutralizing of VFAs can substantially increase the conductivity, at least in experiments with liquid pure VFA solutions (Shiralipour & McConnell, 1997).

In the selection process of monocotyledon species preliminary incubation experiments were used (data not shown), since there was no personal experience in germination of these species and there was desire for monocotyledon species to be able to grow in the same conditions as the selected dicotyledon species, cress ("Incubation conditions", see below). For this purpose there were 4 species selected for preliminary germination testing. Barley (*H. vulgare*), Italian ryegrass (*L. multiflorum*), oat (*Avena sativa*) and an onion (*Allium cepa*) were chosen. These species were selected from standards ISO 11269 - 2 (ISO, 1995) and ASTM E 1598 - 94 (ASTM, 1994) and OECD test guideline 208 (OECD, 1984) as a possible representatives of monocotyledons. It needs to be stated that ryegrass mentioned in these standards was perennial (*Lolium perenne*) and the chosen Italian ryegrass (*L. multiflorum*), although also perennial in nature, was not listed. But the Italian ryegrass (*L. multiflorum*) has an agronomic value in Finland (Niskanen & Niemeläinen, 2006), although it does not overwinter successfully in Finnish climate conditions. It has also been studied in relation to phytotoxicity of composts and found to be in some aspects sensitive species (Marchiol at al., 1999).

As a result of pre-tests on monocotyledon species, it was concluded that Italian ryegrass (*L. multiflorum*) would be used in further experiments. This was due to the ability to germinate in sufficient percentage in 5 days and species ' lower variability of seedling length (root and shoot measured) and germination compared to other monocotyledons evaluated. It has to be mentioned, that the seed of Italian ryegrass is much smaller compared to e.g. oat or barley. This was also considered to be beneficial, since big seed size and high energy

reserves have been speculated to withstand toxicity exerted by VFAs, e.g. acetic acid (Lynch, 1977), and therefor sensitivity of tests could be reduced. Comparisons of the results could be also made easier or more straightforwardly with small seeded dicotyledonous garden cress.

It should be also mentioned that every seed in all experiments in present study went through laborious, hand made (tweezers), selection process, where each seed was inspected visually. Any observed visual differences in the shape or color of the seed resulted in rejection of the seed. This was done to minimize variation in test results as suggested by standards (e.g. ASTM E 1963 - 02 (ASTM, 2002)).

*Incubation conditions*

Incubation conditions for acute tests were selected 25 °C and darkness. It this was done to support rapid germination and try to avoid influence of light and photosynthesis on toxicity exhibited by VFAs. These conditions were chosen according to literature on cress germination experiments (e.g. Helfrich et al. (1998)). Most of the agricultural and vegetable seeds according to International seed testing association (1996) have studied germination temperatures of 20 - 30 °C and ASTM E 1598 - 94 (ASTM, 1994) standard recommends the same temperature range for seedling studies. It should be realized that as temperature increases metabolic rate also rises, which can affect the toxicity response. E.g. for abscisic acid it was found that growth inhibition of cress seed reached maximum around 26 - 28 °C in studied temperature range of 18 - 30 °C (Zucconi et al., 1981a). Also with studied C6 - C9 fatty acids there has been reported more inhibitive (germination inhibition of lettuce seeds) response at higher temperatures, at studied temperature range 12 - 28 °C (Stewart & Berrie, 1979).

For comparability, same temperature rage was considered necessary for incubation condition of subchronic growth experiments, although this was not achieved to the same precision (chapters 2.1.1 and 2.1.2.1). Lightning conditions vary in literature, but conditions were selected to follow recommendation of standard ASTM E1963 - 02 (ASTM, 2002) with 100 - 200 µmol $m^{-2}$ $s^{-1}$ of photosynthetically active radiation with diurnal cycle 16 hours light 8 dark (also supported by ISO 11269 - 2 (ISO, 1995).

*Germination and growth substrates in acute and subchronic experiments*

Two inert materials, quartz sand and filter paper (Whatman No. 1), were considered and tested for the acute VFA exposure experiments made on Petri dishes. These materials are

commonly encountered in plant toxicity tests (sand/quartz e.g. Lynch (1977), DeVleeshauwer et al. (1981), ASTM E 1963 - 02 (ASTM, 2002), International Seed Testing Assosiation (1996); filter paper e.g. by Zucconi et al. (1981a), Linglau and Watt (1995), Helfrich et al. (1998), International Seed Testing Assosiation (1996)). Quartz sand was discarded after the preliminary experiments with monocotyledonous species, as it was found to be difficult to use for accurate measurements of seedlings due to adherence of sand particles on seedlings.

It needs to be stressed that with the selection of different research frames and procedures the main focus was on the phytotoxicity induced by pure substances (VFAs) and not e.g. phytotoxicity of soil. This could be seen, for example in selection of the substrate (artificial soil), since the effect of substrate, through which the toxicity of VFAs acts, was not the focus of present study! It should be realized that chemical and physical environments of different soils may alter the toxicities of VFAs (coarsely observed with different growth media of present study) and may perhaps alter even the relative toxicities of VFAs; which can be deduced e.g. from the works of Chandrasekaran & Yoshida (1973) and Takijima (1964). Initially, in subchronic growth experiments, peat based growth substrate was considered to serve best the purpose, goals and aims (chapter 1.5) of present study. Peat based growth media are very common in horticultural cultivations. For example, according to questionnaire study made in Finland by Grönroos & Nikander (2002), more than 40% of greenhouse cultivators use peat based substratum as an only growth media and in even more cases it is one of principal growth substrates among Finnish greenhouse cultivators.

In scientific literature peat and peat based substrata are also very commonly encountered with compost and its phytotoxicity studies. Peat or peat based growth media have been used as dilution material for composts and/or as control substrata in compost phytotoxicity studies (Juste et al., 1987; Keeling et al., 1994; Gajdos, 1997; Brinton & Tränkner, 1999; Brinton & Evans, 2002; Gariglio et al., 2002; Himanen et al., 2006). As the background of the present study was based on compost phytotoxicity, it was chosen that a peat based growth medium would serve as a representative organic material and some assumption could be perhaps extrapolated to composts or horticultural substrata (peat based) amended with composts. Thus giving some insight into the poor growth of some compost amended horticultural soils commonly encountered (Brinton & Tränkner, 1999). Unfortunately, inconsistency in results of preliminary subchronic experiment on peat (chapter 2.1.2.1) led to decision to change it to more inert growth media, sand mixture. Although limitations in

use and representativness of real world conditions, sand has been approved by standards on conducting terrestrial plant toxicity tests, e.g. ASTM (2002).

It needs to be noted that two separate peat layers were applied in experiment pots (see chapter 2.1.2.2). Although the growth media was not anymore homogeneous, use of only sand mixture in a pot showed less growth (fresh weight) of cress (data not shown). Especially the seedling emergence had been much slower, when covered only with sand-quartz mixture than with peat. Desiccation of seeds could have been one reason for the observed adverse effects.

*Response parameters*

In acute exposures germination and length of the primary root have been stated in the literature to have different sensitivity to toxic substances (Schuman & McCalla, 1976; Zucconi et al. 1981a). In the present study they were statistically tested separately, but also Zuccini's et al. (1981a) germination index (Formula 3, chapter 1.1), which considers both parameters, was brought into play. This is due to the fact that the germination index is extensively cited and used in compost phytotoxicity studies (Zucconi et al., 1981a; Zucconi et al., 1981b; Helfrich et al., 1998; Marchiol et al., 1999; Ozores-Hampton et al., 1999; Lau et al., 2001; Gariglio et al., 2002). It has also been shown to be very sensitive parameter. According to Zucconi et al. (1981a) it is able to detect inhibition of 25 ppb of abscisic acid on garden cress.

The response parameter of germination is subjected to somewhat arbitrariness of own decision making. In literature, there can be found different endpoints for seeds to be considered germinated. For example Ozores-Hampton et al. (1999) in their compost maturity and weed germination studies followed considerations of Peterson & Harrison (1991) that seeds are germinated, when the length of a radicle equals to the length of a longest dimension of a seed. Marambe et al. (1993) considered seed with "visible" radicle to be germinated and Marchioll et al. (1999) refers to the U.S. Food and Drug Administration (1987) for their suggestion of three millimeter radicle length to be assigned as germinated. Not all germination studies give information or definitions on how they understand germination to have taken place. This can also lead to variation in the results mentioned in the literature. Despite the different views on radicle length, one should keep in mind that radicle elongation is not considered to be anymore a germination event and germination event is regarded to have ended by the time of radicle elongation starts

(Bewley & Black, 1994). As the selected seeds in this study were quite small and the measurement capability was one millimeter, it was assessed to use one millimeter radicle length to represent germination limit-value, although some problematic aspects, especially with garden cress, can be also found with this definition (discussed in chapter 4.11).

In subchronic experiments, dry weight was seen to represent the desired production parameter of plant. Although fresh weight of shoots was also weighted, dry weight was considered to be superior over fresh weight, omitting errors from different and possible uneven watering of pots etc.

### 4.9 Effects of volatile fatty acids on plants

Concentration range of VFAs applied in acute experiments of present study is supported well with studies presented in literature (Takijima et al., 1960; Jackson & Taylor, 1970; Lee, 1977; Rao & Mikkelsen, 1977; Ulbright et al., 1982a; 1982b). Some response parameters or plant species may be more sensitive to VFAs than others. In literature especially plant roots have shown sensitive inhibitive response to VFAs (Prill et al., 1949; Ulbright et al., 1982a).

Root length was not measured in the present study, however correlation between root and shoot lengths was visually observed (Appendix 8, Fig. 1 - 3). Lee (1977) and Rao and Mikkelsen (1977) pointed that the toxicity of VFAs correlates with the mole concentration of undissociated form of VFAs and addressed the importance of protonated form in eliciting phytotoxicity. They calculated the concentration of undissociated form of VFAs exhibiting phytotoxic effects. In fact, Lee (1977) suggested that as an only comparable method to assess the different toxicity potentials of VFA is to study them with same concentration of undissociated form. According to formula 1 (chapter 1.1) this would mean slightly different pH for the same concentrations of each VFA.

The protonated VFA concentrations for pH-values observed in the beginning of the tests, of present study, are calculated according to Henderson-Hasselbalch equation (Formula 1) and presented in Appendix 9, Tables 1 - 3. In the calculations $pK_a$-values reported in Table 1 (chapter 1.1) for infinite dilutions, utilized in theoretical calculations, were used. Calculated values might be different in reality, where different ions of different salts with polar or nonpolar artificial soil substances can influence the real $pK_a$-values of VFAs. At present study, there were also some problems associated with usage of protonated VFA concentrations as a part of toxicological explanations and therefore one can argue that the

comparisons can not really be made. One would have to compare the concentrations of undissociated VFAs of their EC50 concentrations. This can not be achieved, since for particular EC50 value there is no corresponding pH value. And further, it would be good to stress that even if concentrations of each undissociated VFA for EC50 value would exist there is a comparison problem. One would make coarse simplification of $pK_a$-values, since it changes, as mentioned, with ionic strength. E.g. between values 0 - 3 mol/l of ionic strengths of pure propionic acid solutions, $pK_a$ can receive values from 4.63 to 5.16 (Martell & Smith, 1979). This means that using the same ionic strength $pK_a$-values for calculating the concentration of protonated form of different VFAs in EC50 comparisons would be doubtful. EC50 concentration (in moles) can be almost one fifth or sixth smaller for longest VFAs than for formic acid (see chapter 3.2.3).

The question of whether undissociated or dissociated forms are the toxic ones was complicated further in subchronic experiments by the pH increase as the experiments proceed. In many occasions only one week was enough to achieve a neutral pH in pots of subchronic exposures experiments (as pointed earlier), and still no substantial resumed increase in seedling emergence could be detected by termination time, after two weeks of neutral pH in many of the pots of subchronic experiments. Conspicuous attitude against the sole toxic importance of undissociated VFA forms is also supported by the work of e.g. Chandrasekaran & Yoshida (1973) and also from the microbial field, where VFA salts have been shown to pose antimicrobial activity (Cherrington et al., 1991). Chandrasekaran & Yoshida (1973) found adverse effects on rice growth in soils of neutral pH (7.1 - 7.5) already at VFA concentration 5 mmol/kg. Still further, Ulbright et al. (1982a) did not found any difference on the effects of C5 - C7 VFAs on root growth in lettuce at pH 4.8 or 6.0. They concluded that the total VFA concentration in the medium might be more important compared to the proportion concentration of protonated VFA molecules. This can be supported also by results of present study, although initial pH in the pots of subchronic growth experiments had been substantially lower.

Interesting is the fact that not all studies support the view of increased toxicity accompanied with increase in parent carbon chain of VFA molecule. In fact, Jackson and Taylor (1970) imply that the toxicity is greatest as the molecular weight is lowest in studied C1 - C3 VFAs. And further, Ulbright et al. 1982a stated that root growth of lettuce was invariant in studied C3 - C8 short chain fatty acid sequence in spite of the fact that inhibition of radicle emergence and shoot differed according to carbon chain length.

### 4.10 Volatile fatty acids and auxins

To continue the discussion, about toxicity of undissociated and dissociated forms of VFAs, as can be deduced from Henderson-Hasselbalch equation (Formula 1, chapter 1.1), the lower the pH the higher is the proportion of undissociated forms of VFAs. As a neutral form, the undissociated form, of VFAs are more lipid soluble (Lee, 1977) therefore able to interact with cell lipid membrane. According to Cherrington et al. (1991) literature review, in microbial world, neutral VFAs have been assumed to diffuse freely across the cell membrane, although other modes of crossing semipermeable cell membrane are also suspected. In this chapter of discussion, plant hormones, auxins, will be introduced with some interesting features resembling VFAs, although speculative, but supported by the idea of some chemical resemblance with VFAs.

There are varieties of natural and synthetic auxins and antiauxins, acting as phytohormones at very small concentrations. They act on plant growth by e.g. on cell wall deformations and elongation and protein synthesis (Devlin & Witham, 1983). Antiauxins are auxin analogs inhibiting the effects of auxins. They may themselves possess little auxin activity (Taiz & Zeiger, 1998). The only structural resemblance is some kind of ring structure (usually planar, aromatic; not always necessary) and weak acid side chain, which is negatively charged in neutral pH (Taiz & Zeiger, 1998). The side chain resemblance to VFAs is evident from the structure and associated names of these compounds, e.g. indole-3-acetic acid, phenylacetic acid, indole-3-butyric acid (auxins), α-(p-chlorophonoxy)isobutyric acid (antiauxin) etc. Auxins do enter the cells also by passive diffusion across cell membranes, in protonated form (Taiz & Zeiger, 1998). This is enhanced by the lower apoplastic pH (near cell walls 5, xylem sap around 6.3) compared to cytoplasm, where it is 7.0 - 7.5 (Taiz and Zeiger, 1998). As is obvious from Henderson-Hasselbalch equation (Formula 1) the more acidic environment the more there is of protonated (neutral) forms of weak acid. Taiz and Zeiger (1998) and Lee (1977) cited the work of Rubery and Sheldrake (1973) that there is tendency to increase the amount of weak acid in cell cytoplasm, if there is a pH difference, with extracellular lower pH compared to inner cytoplasm. This would favor also VFA accumulation into cell cytoplasm. And as protonated VFAs enter higher pH environment of cytoplasm cause this dissociation and lose of $H^+$ (acidification), according to Henderson-Hasselbalch equation (Formula 1). It is worth mentioning that the lower apoplastic pH causes dissociated VFA molecules entering cells down the concentration gradient to become protonated and thus

transforming to more lipid (membrane) soluble form and sequestering on the way to cell apoplastic $H^+$ ions. This could contribute to the imbalance in proton gradient, and thus electrochemical gradient, across the membrane and cellular acidosis experienced by the plant cell.

The aim of the discussion and involvement of auxins into it is to try to highlight some apparent similarities of VFAs and auxins. The question is not only, that these groups of compounds are weak organic acids, as was pointed above. But they pose other similarities, such as the lipid solubility of the protonated form and the possible diffusion across the lipid membrane. They both tend to accumulate in the more alkaline compartments of the cell. And this is not all. Although studies with VFAs and their action on plants have been scarce, there are other interesting resembling features. For example, most of the principal auxin of higher plants, indole-3-acetic acid (IAA), is covalently bound (conjugated) to an inactive form in plants. The carboxylic functional group of auxins forms conjugates, e.g. esters and amide conjugates, which are not active forms (Taiz & Zeiger, 1998). Interestingly Lee (1977) also synthetized ester and amide derivatives of VFAs and included also these into the phytotoxicity studies. Lee (1970) found that, although lipid soluble, even more than parent VFAs, these derivatives were inactive to produce ionic and UV-light absorbing material loss from barley roots. Some similarity there too. Second important feature is that auxin activity is very sensitive to concentrations. At small concentrations (about 0.001 to 0.01 mmol/l) IAA induces growth of young stems and over 0.1 mmol/l there can be inhibitory effects that lead to less growth than in controls, without auxin treatment (Taiz & Zeiger, 1998). In fact, some synthetic auxins are used as herbicides (Taiz & Zeiger, 1998; Mallory-Smith & Retzinger, 2003).

There are other similarities, e.g. auxins are known to be very potent in root inhibition (Taiz & Zeiger, 1998), although not the same context as with VFAs, plant roots have been observed also sensitive to VFAs (Prill et al., 1949; Rao & Mikkelsen, 1977; Lee, 1977). As is known with auxins (IAA), plastids also accumulate these lipid soluble weak acids to the same concentrations as in cytoplasm. Chloroplasts are essential for energy harvesting for photosynthesizing green plants. The whole plastid is full of membrane-separated compartments. Especially thylakoid membranes inside the chloroplasts, which are the important sites for ATP synthesis depending on proton gradient, potential difference, between opposite thylakoid membrane sides, are crucial in cell energy production. Proton gradient has to be maintained, low proton $H^+$ concentration in stroma of chloroplasts and

higher $H^+$ in the lumen of thylakoids to produce ATP. Although not entirely the same idea, Helfrich et al. (1998) found that VFAs (only acetic and propionic studied) did indeed show in millimolar concentrations inhibition of thylakoid membrane photosynthetic electron transport. More so, with combined action of both tested VFAs.

When one observes the results of acute toxicity studies of present work, one can find an induction, i.e. hormesis, (revealed best by germination index) in many cases. The germination index is of course composed of two variables, germination and radicle elongation. But the probit analysis utilized to find EC50 even just for the radicle length of acute experiments was not applied here, because in too many cases, in small VFA concentrations, the elongation of radicle was higher than that of control. Although hormesis is a well known phenomenon, were small concentration of otherwise toxic compound can induce stimulation of response parameter, the question can be asked, whether the case is similar to concentration-sensitive auxins? The concentrations of VFAs, were the inductions have been apparent, were from 0.1 - 1.2 mmol/l. This is approximately two orders of magnitude higher than that for natural IAA auxin. But, as the lipophilicity of short carbon skeleton VFAs can be smaller compared to auxins, does not this mean that for the effects a greater quantity is needed since more lipophilic auxins are readily quicker penetrating the cells? VFAs do not have an auxin characteristic ring structure (Taiz & Zeiger, 1998), usually an aromatic ring, which is highly lipid soluble. Could this prevent comparisons between VFAs as auxins? Perhaps not. For example, carboxylic acids possessing planar aromatic ring structure, like benzoic acid, phenyl acetic acid, have been shown to poses same effect, even higher (Takijima et al., 1960; Lee, 1977), than aliphatic VFAs. Could it be merely just question of compounds lipophilic nature and concentration? It would not be hard to apply the acid growth theory, normally used to explain growth induction by natural auxins (Taiz & Zeiger, 1998), to VFAs, at least from the short term effect point of view. The ability of VFAs to decrease pH in the vicinity of the cell wall, as is suggested for the implicit action of auxins (Devlin & Witham, 1983), could possible for VFAs.

In the paper by Mallory-Smith and Retzinger (2003) the authors classified herbicides by the site of action. Although no VFAs are mentioned, the group of carboxylic acid is mentioned as one chemical family, where site of herbicidic action is to act as “synthetic auxins”. Listed compounds of “carboxylic acid” - chemical family have in their structure pyridine ring, no aliphatic fatty acids are present. One can not label these compounds to be

chemically similar with aliphatic carboxylic acids, such as VFAs. But one could ask oneself again, could not also aliphatic carboxylic acid pose a similar action?

### 4.11 Cress germination and effect of VFAs on actions induced by gibberellins

Based on results of acute toxicity experiments made on garden cress (*L. sativum*) it can be noticed that the effects of VFAs on germination have not had a substantial impact. But if one inspects the average radicle lengths, detection of almost none growth (average of radicle length close to one millimeter, detection limit) with two to three highest concentrations of any studied VFA can be observed. As a more sensitive parameter, radicle elongation was entirely halted with these VFA concentrations. There is a possibility to unit the works of Buller et al. (1976) and Müller et al. (2006) to hypothetically explain this observation. Müller et al. (2006) studied garden cress germination. To complete germination, garden cress has to go through two stages, testa rupture and micropylar endosperm rupture, which covers at about two cell layers thick the radicle tip, preventing it from elongating. This cell layer has to be ruptured before germination is completed. The article by Müller at al. (2006) is provided with some fantastic pictures and drawings. When comparing these informative pictures of different stages of germination, one can come to a conclusion that observed one millimeter radicle length, the measure of germination, does not necessary always support the idea of non-inhibitive effect on germination in cress acute experiments. It was shown by Mülller at al. (2006) that the endosperm cap surrounding the radicle tip has to weaken, before it can rupture. They showed that abscisic acid (ABA), which plays an essential role in dormancy of seeds and development (Taiz & Zeiger, 1998), is responsible for prevention of endosperm weakening and finally the rupture, which is induced by gibberellins. The ratio of ABA and gibberellins is the determinant key factor of success in germinations. As Müller at al. (2006) states: "*Endosperm weakening therefore appears to require de novo gibberellin biosynthesis in the micropylar endosperm cap*."

The work of Buller et al. (1976) gives also one possible explanation (tested concentration were one mmol/l) for observed inhibition of radicle elongation of cress and ryegrass or low germination success of Italian ryegrass exposed to VFAs in present study. They found that the inhibition of gibberellins induced amylolysis in barley (*H. vulgare*) increased as the length of carbon chain became longer. All studied fatty acids caused reduction in the gibberellin induced sugar release. VFAs overcame the effect induced by gibberellins in a pH buffered solutions. Since VFAs could overcome the effects of gibberellins in

amylolysis, the question rises, whether this could be true also for gibberellin induced cress endosperm weakening and sequential rupture of micropylar endosperm cap over radicle tip. This suggestion is supported by the observations of present study on garden cress, where radicle elongation was measured and found to halt the elongation at one millimeter (endosperm still covering the cap, according to pictures provided by Müller et al. 2006), at higher VFA concentrations. In the present study, the VFAs did not have effect on cress testa rupture as did not have the abscisic acid in Müller et al. (2006) work.

### 4.12 Inhibitive VFA concentrations and their relevance

In this chapter results of present study are connected to observations found in actual real environments. The main question is: have results of present study any relevance in understanding phytotoxicity exhibited by VFAs in soils and composts?

Chandrasekaran and Yoshida (1973) report on rice growth, in Philippine soils, adverse effects already at 5 mmol/kg concentrations. These experiments were subchronic in nature (lasting 23 to 28 days) and dry weight of whole rice plants was used as response parameter. Present study, conducted on artificial growth medium, supports these findings and in some cases indicates that even smaller concentrations can inhibit growth of shoots. The next question, to which Chandrasekaran and Yoshida (1973) study answers, is the question of VFA formation in soils. And indeed they showed that after amendment of soils with green manure high concentration of VFA could be detected. Sometimes soils generated over fivefold concentration of a particular VFA compared to concentrations exhibiting 50 % decrease in whole plant dry weight. The production of VFAs of such high quantities was shown to vary in different soils after amendment with green manure (laboratory scale experiments). There was also an interesting notion that is somewhat supported also by present study of subchronic growth experiments (although not always with in 95 % confidence limits) and this is the indication of *iso*-butyric acid being more toxic than its four carbon isomer *n*-butyric acid. This is consistent with the more lipophilic nature of *iso*-butyric acid compared to *n*-butyric acid (Table 4).

The presence of VFAs in raw manures has been detected by many studies. Schuman and McCalla (1976) reported fresh beef cattle manure to contain, before composting, large amounts of VFAs. The reported (dry weight basis) VFAs were (if using the approximate conversion factors in Appendix 2): acetic ≈ 5.80 mmol/kg dw, propionic 11.8 mmol/kg dw, butyric ≈ 6.55 mmol/kg dw, *iso*-butyric (traces), valeric ≈ 0.19 mmol/kg dw and *iso*-valeric ≈ 0.33 mmol/kg. Schuman and McCalla (1976) used pure VFAs to test wheat and sorghum

germination and root number affected by VFAs. Highest concentration tested was 500 mg/l, which corresponds to about 8.3 mmol/l acetic, 6.7 mmol/l of propionic and 5.7 mmol/l of butyric acid. Germination was affected by concentrations of already 1/10 of these concentrations, but showed almost none decrease thereafter. These results are consistent with the findings of present study, if one takes into consideration different test species. Interestingly, even highest concentrations did not have effect on root numbers. Schuman and McCalla (1976) made also an important observation, since also pure VFA mixtures were examined, the mixture of acetic, propionic and butyric acid (in "equal" concentration, measured by mass) could cause much greater (up to almost 30 %) germination inhibition than equivalent concentration of one VFA applied alone, when studied at highest concentration (500 mg/l)! This means that one should only extrapolate results of present study, when bearing in mind the fact that in soils and composts the inhibitory effects are always mixture effects. This finding is supported also by Berrie et al. (1976) and Helfrich et al. (1998).

Studies on composting have shown different quantities of VFAs being formed or to be present at the beginning of composting. Chanyasak et al. (1982) studied two commercial scale composting plants and concentration up to almost 400 mmol/kg dw of acetic acid could be detected (acetic acid constituting about 60 - 90% of total VFAs present). DeVleeschauwer et al. (1981) found also high amounts of VFAs, especially acetic acid up to 350 mmol in kg, in unmaturated composts. Phytotoxicity studies on garden cress (DeVleeschauwer et al., 1981) confirm the results of the present study, as it was difficult to obtain really notable germination inhibition of cress (if one millimeter radicle elongation can be accepted to represent germination success) with applied VFA concentrations. DeVleeschauwer et al. (1981) observed that to get total germination inhibition over three times as high acetic acid concentration should be used. Kirchmann & Widén (1994) have obtained much lower VFA concentrations in their study of separately collected organic household waste composts. Highest observed acetic acid concentrations were about 50 mmol/kg dw, propionic 7 mmol/kg dw, butyric 11 mmol/kg dw and valeric acid 33 mmol/kg dw. Even if studied every VFA separately, the present study supports the idea that composts may contain, in unmaturated state, sufficient amounts of even one, single, VFA to pose adverse effects on growth of plants.

### 4.13 Conclusions and future research

Present study gives an opportunity to draw some conclusions adressing some aspects of VFA toxicities.

1. There are statistically significant differences in phytotoxicities of different VFAs. On average, the importance of C3 - C6 VFAs compared to C2 - C1 VFAs in compost phytotoxicity assessments should be stressed and acknowledged.

2. Although formic and acetic acids are the least toxic VFAs, followed by propionic - caproic acids, the hypothesis of increasing phytotoxicity with increasing length of carbon chain can not be always consistently supported, especially in acute exposures in studies on germination and radical elongation.

2. Sensitivity between response parameters and plant species to VFAs varies. Garden cress (*L. sativum*) exhibited more sensitive response, especially in biomass growth, compared to monocotyledon Italian ryegrass (*L. multiflorum*).

3. Single application of VFAs can decline the pH of a growth medium to plant harmful levels, but the ability of a growth medium to recover from pH shock can be rather quick. This does not necessary mean recovery observed in plants in a short term.

4. Small concentrations of VFAs may in acute exposures induce germination and growth of radicle.

Some future research lines for comprehensive understanding of phytotoxicities of VFAs can be suggested. The assessment of phytotoxicities of mixed VFAs, as they usually appear in the growth media, can influence the knowledge of their real inhibitive nature and role in real environments. As the exogenous exposure of VFAs is mediated by soils or growth substrates, the understanding of different growth media to support or neutralize toxicities exhibited by VFAs should be addressed. Also whole plant lifecycle studies are heavily outnumbered by acute and subchronic studies and the long term importance of phytotoxicity of VFAs and their influence on plants should be the focus. The definitive answer of toxicity mechanism is still waiting to be described. Especially interest is the allocation and compartmentalization of VFAs from the soil medium into different parts of plant and in plant cell. The understanding of the distortion of cell membrane function, suggested in many papers, and the ultimate cause of cell death upon VFA exposure are waiting to be described more accurately.

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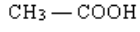
**Appendix 1**

## Information on Chemicals and Seeds

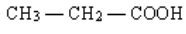
Present study was conducted on 8 volatile organic acids. These where:

Formic (methanoic) acid, by Riedel-de Haën, Germany
Standard of purity = 98 – 100 %
Mr = 46.03 g/mol
Density = 1.22 g/ml

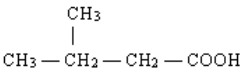
Acetic (ethanoic) acid, by Merck, Germany
Standard of purity = 100 %
Mr = 60.05 g/mol
Density = 1.05 g/ml



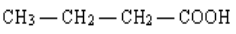
Propionic (propanoic) acid, by Fluka Chemie, Germany
Standard of purity > 99.5 %
Mr = 74.08 g/mol
Density = 0.992 g/ml



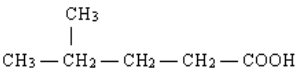
*Iso*-butyric (*iso*-butanoic) acid, by Merck-Schuchardt, Germany
Standard of purity > 98 %
Mr = 88.11 g/mol (not marked, assumed the same as butanoic acid)
Density = 0.95 g/ml



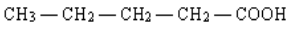
Butyric (*n*-butanoic) acid, by Fluka Chemie, Germany
Standard of purity > 99.5 %
Mr = 88.11 g/mol
Density = 0958 g/ml



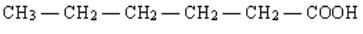
*Iso*-valeric (*iso*-pentanoic) acid, by Sigma-Aldrich, Germany
Standard of purity = 99 %
Mr = 102.13 g/mol
Density = 0.937 g/ml



Valeric (*n*-pentanoic) acid, by Merck-Schuchardt, Germany
Standard of purity > 99 %
Mr = 102.13 g/mol (not marked, assumed the same as *iso* - valeric acid)
Density = 0.94 g/ml



Caproic (*n*-hexanoic) acid, by ICN Biomedicals Inc., USA
Standard of purity = 100 % (not marked, assumed)
Mr = 116.16 g/mol
Density = 0.927 g/ml



The tested seed species were:

Italian ryegrass (*Lolium multiflorum v. fabio*)
Seeds packed in 2004
Country of origin: Denmark
Germination certificate by Tilasiemen OY
Germination on 25.8.2005 = 87 % normally germinated in 14 days

Garden cress (*Lepidium sativum*)
”Siemen”, 5 grams
Packed for Habitec OY
Germination = 90 %
Seeds packed in November 2005
Best before: November 2008

In the preliminary experiments garden cress seeds were the same except for the best before date (October 2004), packing date (October 2002) and germination percentage (95 %).

# Appendix 2

Table 1. Incubators used in toxicity experiments. Abbreviations: cress = garden cress (*Lepidium. sativum*); rye = Italian ryegrass (*Lolium. multiflorum*); peat = peat as an only growth substratum; Termaks = Termaks Cooling Incubator KB2, Termaks AS, Norway; Bio 1600 = Bio 1600, Weiss Umwelttechnik GmbH, Germany; Friocell = Friocell Mo 55, MMM Medcenter Einrichtungen GmbH, Germany.

| Experiment | Incubator |
|---|---|
| Preliminary cress acute exposure experiments 1 - 3 | Termaks |
| Preliminary ryegrass acute exposure experiment 1 - 2 | Termaks |
| Actual cress acute exposure experiment 1 - 2 | Termaks |
| Actual cress acute exposure experiment 3 - 5 | Bio 1600 |
| Actual cress acute exposure experiment 6 - 7 | Friocell |
| Actual ryegrass acute exposure experiment 1 - 2 | Termaks |
| Actual ryegrass acute exposure experiment 3 - 5 | Bio 1600 |
| Actual ryegrass acute exposure experiment 6 - 12 | Friocell |
| Preliminary ryegrass / cress subchronic growth experiment with peat | Bio 1600 |
| Preliminary ryegrass subchronic growth experiment 1 | Bio 1600 |
| Preliminary cress subchronic growth experiment 1 | Bio 1600 |
| Actual cress acute exposure experiment 1 - 3 | Bio 1600 |
| Actual ryegrass acute exposure experiment 1 - 3 | Bio 1600 |

## Appendix 3


Figure 1. Growth chamber Bio 1600 Weiss in operation with visible outlet absorption apparatus. The tests were not influenced by room lightning or room temperature.

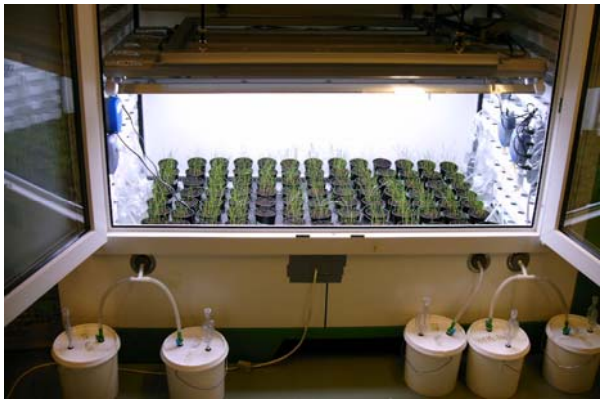


Figure 2. Apparatus for VFA absorption and management of the malodourous nuisance. All air pumps were installed to inner part of the growth chamber. Absorption liquids were on in - and outside of the chamber.

Figure 3. Closer look at the inner pumping and absorption apparatus in growth chamber.


Figure 4. The outlet, VFA containing, air from the growth chamber was purified by absorption to 0.1 M NaOH solution, when bubbled through liquid containing tightly closed, water lock bearing 5 liter buckets.

Figure 5. The condensation water was dropping directly into alkaline solution minimizing malodour formation into the room.

Table 1. Values of pH and electrical conductivity (EC, µS/cm) of test solutions in acute toxicity experiments (mean ± standard deviation). F = formic, A = acetic, P = propionic, IB = *iso*-butyric, B = butyric[1], IV = *iso*-valeric, V = valeric, C = caproic acid. [2]

| | 0.1 mM | 0.3 mM | 0.6 mM | 1.2 mM | 2.4 mM | 4.8 mM | 9.6 mM |
|---|---|---|---|---|---|---|---|
| Garden Cress | | | | | | | |
| F pH | 4.24 ± 0.09 | 3.86 ± 0.04 | 3.64 ± 0.06 | 3.44 ± 0.02 | 3.26 ± 0.02 | 3.09 ± 0.02 | 2.91 ± 0.02 |
| F EC | 23.2 ± 3.7 | 48.0 ± 7.7 | 75.6 ± 13.9 | 111.0 ± 17.4 | 161.7 ± 31.6 | 288.2 ± 33.2 | 446.2 ± 49.0 |
| A pH | 4.51 ± 0.05 | 4.24 ± 0.04 | 4.07 ± 0.03 | 3.90 ± 0.03 | 3.73 ± 0.03 | 3.57 ± 0.03 | 3.42 ± 0.04 |
| A EC | 12.7 ± 1.1 | 23.4 ± 1.4 | 33.5 ±1.8 | 49.5 ± 1.7 | 71.0 ± 1.7 | 102.5 ± 4.2 | 147.7 ± 5.0 |
| P pH | 4.58 ± 0.07 | 4.31 ± 0.03 | 4.13 ± 0.03 | 3.96 ± 0.03 | 3.79 ± 0.03 | 3.64 ± 0.02 | 3.47 ± 0.02 |
| P EC | 11.7 ± 1.2 | 20.4 ± 1.2 | 29.8 ± 1.3 | 42.9 ± 2.1 | 62.1 ± 1.9 | 88.4 ± 3.2 | 127.0 ± 4.1 |
| IB pH | 4.61 ± 0.07 | 4.30 ± 0.03 | 4.12 ± 0.03 | 3.94 ± 0.03 | 3.78 ± 0.03 | 3.63 ± 0.03 | 3.45 ± 0.03 |
| IB EC | 11.8 ± 1.5 | 20.6 ± 1.2 | 31.2 ± 1.8 | 43.7 ± 2.2 | 62.8 ± 2.3 | 93.6 ± 6.0 | 132.4 ± 3.1 |
| B pH | 4.55 ± 0.03 | 4.29 ± 0.03 | 4.11 ± 0.03 | 3.94 ± 0.02 | 3.78 ± 0.03 | 3.61 ± 0.03 | 3.45 ± 0.02 |
| B EC | 12.2 ± 1.1 | 21.2 ± 0.9 | 31.2 ± 1.3 | 44.8 ± 1.7 | 65.1 ± 2.6 | 94.0 ± 4.6 | 136.5 ± 4.1 |
| IV pH | 4.54 ± 0.06 | 4.27 ± 0.04 | 4.09 ± 0.04 | 3.92 ± 0.02 | 3.75 ± 0.02 | 3.58 ± 0.02 | 3.42 ± 0.02 |
| IV EC | 12.3 ± 1.3 | 21.9 ± 1.1 | 31.9 ± 1.2 | 47.1 ± 2.4 | 68.2 ± 3.2 | 98.6 ± 4.3 | 141.6 ± 4.5 |
| V pH | 4.68 ± 0.18 | 4.29 ± 0.03 | 4.11 ± 0.01 | 3.94 ± 0.03 | 3.77 ± 0.02 | 3.61 ± 0.03 | 3.44 ± 0.02 |
| V EC | 11.4 ± 1.4 | 20.2 ± 1.3 | 30.3 ± 1.7 | 43.7 ± 1.5 | 63.8 ± 2.0 | 92.4 ± 3.3 | 134.1 ± 4.9 |
| C pH | 4.61 ± 0.07 | 4.33 ± 0.03 | 4.15 ± 0.03 | 3.96 ± 0.03 | 3.80 ± 0.03 | 3.63 ± 0.02 | 3.47 ± 0.03 |
| C EC | 10.8 ± 1.1 | 19.3 ± 1.0 | 27.8 ± 0.9 | 40.9 ± 1.4 | 60.4 ± 2.6 | 86.0 ± 3.0 | 125.5 ± 3.7 |
| Italian Ryegrass | | | | | | | |
| F pH | 4.24 ± 0.08 | 3.86 ± 0.04 | 3.64 ± 0.03 | 3.45 ± 0.02 | 3.26 ± 0.02 | 3.09 ± 0.02 | 2.91 ± 0.02 |
| F EC | 22.3 ± 3.9 | 45.8 ± 7.9 | 72.2 ± 13.2 | 102.4 ± 21.7 | 180.5 ± 27.7 | 295.5 ± 30.9 | 454.1 ± 44.0 |
| A pH | 4.53 ± 0.06 | 4.24 ± 0.04 | 4.08 ± 0.03 | 3.90 ± 0.03 | 3.74 ± 0.03 | 3.57 ± 0.03 | 3.42 ± 0.04 |
| A EC | 12.4 ± 1.1 | 23.4 ± 1.2 | 33.9 ± 1.4 | 49.3 ± 1.6 | 71.0 ± 1.8 | 102.7 ± 3.6 | 147.8 ± 4.3 |
| P pH | 4.58 ± 0.05 | 4.31 ± 0.03 | 4.13 ± 0.03 | 3.96 ± 0.03 | 3.80 ± 0.03 | 3.64 ± 0.02 | 3.47 ± 0.02 |
| P EC | 11.5 ± 1.2 | 20.3 ± 1.1 | 29.9 ± 1.2 | 43.2 ± 1.9 | 62.1 ± 1.8 | 88.9 ± 3.0 | 127.4 ± 3.7 |
| IB pH | 4.61 ± 0.06 | 4.30 ± 0.04 | 4.12 ± 0.03 | 3.95 ± 0.03 | 3.79 ± 0.03 | 3.63 ± 0.03 | 3.46 ± 0.02 |
| IB EC | 11.5 ± 1.4 | 20.5 ± 1.2 | 31.0 ± 1.7 | 43.7 ± 1.9 | 62.8 ± 2.1 | 92.9 ± 5.2 | 131.7 ± 3.0 |
| B pH | 4.56 ± 0.03 | 4.28 ± 0.03 | 4.11 ± 0.03 | 3.94 ± 0.02 | 3.78 ± 0.03 | 3.61 ± 0.03 | 3.45 ± 0.02 |
| B EC | 11.8 ± 1.1 | 21.1 ± 0.9 | 31.1 ± 1.1 | 44.9 ± 1.5 | 65.4 ± 2.4 | 94.1 ± 3.9 | 136.4 ± 3.7 |
| IV pH | 4.54 ± 0.04 | 4.26 ± 0.03 | 4.09 ± 0.04 | 3.92 ± 0.02 | 3.75 ± 0.02 | 3.58 ± 0.02 | 3.42 ± 0.02 |
| IV EC | 12.2 ± 1.1 | 22.0 ± 0.9 | 32.2 ± 1.0 | 47.1 ± 2.1 | 68.2 ± 2.8 | 98.9 ± 3.7 | 142.1 ± 3.9 |
| V pH | 4.65 ± 0.16 | 4.30 ± 0.03 | 4.11 ± 0.02 | 3.94 ± 0.02 | 3.78 ± 0.02 | 3.62 ± 0.03 | 3.45 ± 0.03 |
| V EC | 11.3 ± 1.2 | 20.3 ± 1.1 | 30.4 ± 1.6 | 44.1 ± 1.6 | 63.9 ± 1.7 | 91.8 ± 3.1 | 133.6 ± 4.9 |
| C pH | 4.59 ± 0.04 | 4.31 ± 0.02 | 4.14 ± 0.03 | 3.96 ± 0.02 | 3.79 ± 0.02 | 3.63 ± 0.02 | 3.47 ± 0.02 |
| C EC | 10.7 ± 0.8 | 19.5 ± 0.8 | 28.2 ± 1.1 | 41.4 ± 1.5 | 61.5 ± 3.2 | 87.2 ± 3.3 | 126.5 ± 3.6 |

[1] Note the different concentrations of butyric acid: 0.10, 0.30, 0.61, 1.21, 2.42, 4.84, 9.68 mM

[2] Control values were pH ≈ 5.5 and EC < 2.5 µS/cm

Table 2. Mean ± standard deviation of pH and electrical conductivity (EC, µS/cm) values in the formic acid subchronic growth experiments. Rye = Italian ryegrass, Cress = garden cress, Start = start of the experiment, End = end of the experiment.

| Conc. mmol/kg | Control Rye Control Cress | 10.0 Rye 10.0 Cress | 30.0 Rye 20.0 Cress | 70.0 Rye 40.0 Cress | 90.0 Rye 60.0 Cress | 200.0 Rye 80.0 Cress |
|---|---|---|---|---|---|---|
| | | | pH | | | |
| Rye Start | 7.02 ± 0.14 | 4.01 ± 0.05 | 3.51 ± 0.05 | 3.25 ± 0.05 | 3.17 ± 0.05 | 2.96 ± 0.02 |
| Rye End | 7.11 ± 0.04 | 7.46 ± 0.02 | 7.82 ± 0.12 | 8.17 ± 0.19 | 8.23 ± 0.30 | 7.78 ± 0.47 |
| Cress Start | 6.96 ± 0.09 | 4.01± 0.01 | 3.65 ± 0.01 | 3.39 ± 0.01 | 3.28 ± 0.02 | 3.19 ± 0.02 |
| Cress End | 7.76 ± 0.12 | 7.63 ± 0.26 | 7.90 ± 0.25 | 8.11 ± 0.25 | 8.30 ± 0.15 | 8.34 ± 0.08 |
| | | | EC | | | |
| Rye Start | 12.2 ± 0.4 | 207.2 ± 21.0 | 435.7 ± 26.6 | 702.0 ± 42.4 | 811.3 ± 19.0 | 1233.5 ± 37.5 |
| Rye End | 80.7 ± 8.0 | 85.8 ± 7.0 | 87.3 ± 11.3 | 99.1 ± 1.17 | 99.3 ± 16.8 | 282.6 ± 120.8 |
| Cress Start | 14.0 ± 2.4 | 202.3 ± 10.5 | 327.7 ± 13.6 | 503.0 ± 19.3 | 625. 3 ± 11.6 | 751.7 ± 14.6 |
| Cress End | 82.3 ± 9.4 | 70.2 ± 7.8 | 72.0 ± 9.2 | 103.0 ± 35.0 | 107.9 ± 10.0 | 119.0 ± 19.1 |

Table 3. Mean ± standard deviation of pH and electrical conductivity (EC, µS/cm) values in the acetic acid subchronic growth experiments. Rye = Italian ryegrass, Cress = garden cress, Start = start of the experiment, End = end of the experiment.

| Conc. mmol/kg | Control Rye Control Cress | 5.0 Rye 10.0 Cress | 25.0 Rye 20.0 Cress | 50.0 Rye 40.0 Cress | 75.0 Rye 50.0 Cress | 100.0 Rye 60.0 Cress |
|---|---|---|---|---|---|---|
| | | | pH | | | |
| Rye Start | 7.02 ± 0.14 | 4.87 ± 0.01 | 4.18 ± 0.02 | 3.93 ± 0.02 | 3.79 ± 0.03 | 3.70 ± 0.03 |
| Rye End | 7.11 ± 0.04 | 7.49 ± 0.015 | 7.94 ± 0.01 | 7.71 ± 0.07 | 7.62 ± 0.06 | 7.43 ± 0.12 |
| Cress Start | 6.96 ± 0.09 | 4.53 ± 0.05 | 4.24 ± 0.03 | 3.97 ± 0.01 | 3.87 ± 0.01 | 3.83 ± 0.02 |
| Cress End | 7.76 ± 0.12 | 7.86 ± 0.24 | 7.86 ± 0.22 | 7.76 ± 50.08 | 7.73 ± 0.18 | 7.75 ± 0.05 |
| | | | EC | | | |
| Rye Start | 12.2 ± 0.4 | 85.6 ± 0.6 | 173.7 ± 12.5 | 228.8 ± 16.3 | 265.5 ± 11.4 | 294.3 ± 11.7 |
| Rye End | 80.7 ± 8.0 | 85.7 ± 9.9 | 91.9 ± 8.8 | 97.0 ± 10.7 | 94.4 ± 5.9 | 99.1 ± 12.2 |
| Cress Start | 14.0 ± 2.4 | 125.1 ± 13.0 | 159.9 ± 11.4 | 195.4 ± 5.3 | 217.9 ± 10.0 | 223.7 ± 16.4 |
| Cress End | 82.3 ± 9.4 | 87.6 ± 7.1 | 93.6 ± 19.0 | 103.1 ± 3.8 | 99.2 ± 16.9 | 115.1 ± 7.1 |

Table 4. Mean ± standard deviation of pH and electrical conductivity (EC, µS/cm) values in the propionic acid subchronic growth experiments. Rye = Italian ryegrass, Cress = garden cress, Start = start of the experiment, End = end of the experiment.

| Conc. mmol/kg | Control Rye Control Cress | 5.0 Rye 1.5 Cress | 10.0 Rye 4.5 Cress | 20.0 Rye 13.5 Cress | 35.0 Rye 40.5 Cress | 50.0 Rye 60.0 Cress |
|---|---|---|---|---|---|---|
| | | | pH | | | |
| Rye Start | 7.02 ± 0.14 | 4.91 ± 0.02 | 4.61 ± 0.01 | 4.33 ± 0.02 | 4.12 ± 0.02 | 4.01 ± 0.02 |
| Rye End | 7.11 ± 0.04 | 7.63 ± 0.12 | 7.85 ± 0.15 | 7.78 ± 0.13 | 7.82 ± 0.10 | 7.70 ± 0.10 |
| Cress Start | 6.96 ± 0.09 | 5.61 ± 0.07 | 4.90 ± 0.02 | 4.44 ± 0.01 | 4.02 ± 0.01 | 3.90 ± 0.02 |
| Cress End | 7.76 ± 0.12 | 7.64 ± 0.05 | 7.83 ± 0.11 | 7.94 ± 0.22 | 7.93 ± 0.49 | 7.77 ± 0.47 |
| | | | EC | | | |
| Rye Start | 12.2 ± 0.4 | 78.6 ± 4.0 | 104.5 ± 4.9 | 140.2 ± 6.1 | 173.5 ± 9.8 | 196.5 ± 5.1 |
| Rye End | 80.7 ± 8.0 | 82.6 ± 3.3 | 79.2 ± 11.1 | 88.3 ± 3.4 | 86.5 ± 7.4 | 94.5 ± 4.2 |
| Cress Start | 14.0 ± 2.4 | 41.5 ± 1.4 | 70.4 ± 0.9 | 109.8 ± 2.6 | 163.7 ± 7.1 | 195.5 ± 2.8 |
| Cress End | 82.3 ± 9.4 | 75.1 ± 3.4 | 74.4 ± 6.0 | 97.0 ± 10.8 | 117.6 ± 20.6 | 134.3 ± 39.6 |

Table 5. Mean ± standard deviation of pH and electrical conductivity (EC, µS/cm) values in the *iso*-butyric subchronic growth experiments. Rye = Italian ryegrass, Cress = garden cress, Start = start of the experiment, End = end of the experiment.

| Conc. mmol/kg | Control Rye Control Cress | 1.5 Rye 0.5 Cress | 5.0 Rye 1.5 Cress | 15.0 Rye 4.5 Cress | 30.0 Rye 30.0 Cress | 45.0 Rye 45.0 Cress |
|---|---|---|---|---|---|---|
| | | | pH | | | |
| Rye Start | 7.02 ± 0.14 | 5.63 ± 0.04 | 4.88 ± 0.01 | 4.41 ± 0.01 | 4.15 ± 0.01 | 4.00 ± 0.01 |
| Rye End | 7.11 ± 0.11 | 7.23 ± 0.15 | 7.67 ± 0.14 | 7.79 ± 0.20 | 7.49 ± 0.40 | 7.50 ± 0.23 |
| Cress Start | 6.96 ± 0.09 | 6.47 ± 0.04 | 5.66 ± 0.13 | 4.91 ± 0.03 | 4.42 ± 0.02 | 4.01 ± 0.01 |
| Cress End | 7.76 ± 0.12 | 7.64 ± 0.17 | 7.74 ± 0.13 | 7.89 ± 0.09 | 7.94 ± 0.14 | 7.72 ± 0.18 |
| | | | EC | | | |
| Rye Start | 12.2 ± 0.4 | 39.1 ± 1.2 | 77.1 ± 4.5 | 123.5 ± 8.8 | 158.0 ± 7.4 | 180.7 ± 5.3 |
| Rye End | 79.5 ± 8.7 | 75.3 ± 3.4 | 83.3 ± 2.7 | 84.6 ± 1.1 | 84.3 ± 8.5 | 78.8 ± 16.6 |
| Cress Start | 14.0 ± 2.4 | 24.0 ± 5.6 | 41.5 ± 3.6 | 72.3 ± 4.6 | 111.2 ± 4.4 | 165.4 ± 3.9 |
| Cress End | 82.3 ± 9.4 | 75.3 ± 5.4 | 72.7 ± 5.4 | 75.0 ± 6.6 | 84.6 ± 8.2 | 114.0 ± 34.1 |

Table 6. Mean ± standard deviation of pH and electrical conductivity (EC, μS/cm) values in the butyric acid subchronic growth experiments. Rye = Italian ryegrass, Cress = garden cress, Start = start of the experiment, End = end of the experiment.

| Conc. mmol/kg | Control Rye Control Cress | 2.5 Rye 1.5 Cress | 10.0 Rye 4.5 Cress | 25.0 Rye 13.5 Cress | 40.0 Rye 25.0 Cress | 60.0 Rye 40.5 Cress |
|---|---|---|---|---|---|---|
| | | | pH | | | |
| Rye Start | 7.02 ± 0.14 | 5.24 ± 0.03 | 4.53 ± 0.02 | 4.16 ± 0.03 | 4.00 ± 0.02 | 3.86 ± 0.01 |
| Rye End | 7.11 ± 0.04 | 7.36 ± 0.10 | 7.72 ± 0.17 | 7.82 ± 0.29 | 7.67 ± 0.40 | 7.42 ± 0.25 |
| Cress Start | 6.96 ± 0.09 | 5.75 ± 0.14 | 4.89 ± 0.01 | 4.41 ± 0.02 | 4.18 ± 0.03 | 3.99 ± 0.02 |
| Cress End | 7.76 ± 0.12 | 7.61 ± 0.13 | 7.85 ± 0.16 | 7.91 ± 0.04 | 7.70 ± 0.14 | 7.49 ± 0.21 |
| | | | EC | | | |
| Rye Start | 12.2 ± 0.4 | 51.2 ± 7.0 | 102.4 ± 7.5 | 146.1 ± 12.6 | 176.1 ± 10.3 | 205.0 ± 6.4 |
| Rye End | 79.5 ± 8.7 | 77.8 ± 18.7 | 74.1 ± 1.1 | 82.5 ± 12.6 | 79.9 ± 10.0 | 81.6 ± 19.0 |
| Cress Start | 14.0 ± 2.4 | 45.8 ± 4.5 | 70.3 ± 1.0 | 114.4 ± 3.3 | 148.4 ± 4.0 | 168.0 ± 5.3 |
| Cress End | 82.3 ± 9.4 | 75.5 ± 6.1 | 65.3 ± 6.1 | 76.0 ± 6.1 | 92.3 ± 11.3 | 96.2 ± 18.8 |

Table 7. Mean ± standard deviation of pH and electrical conductivity (EC, μS/cm) values in the *iso*-valeric acid subchronic growth experiments. Rye = Italian ryegrass, Cress = garden cress, Start = start of the experiment, End = end of the experiment.

| Conc. mmol/kg | Control Rye Control Cress | 1.5 Rye 0.5 Cress | 7.5 Rye 1.5 Cress | 13.5 Rye 3.0 Cress | 25.0 Rye 15.0 Cress | 40.5 Rye 30.0 Cress |
|---|---|---|---|---|---|---|
| | | | pH | | | |
| Rye Start | 7.02 ± 0.14 | 5.56 ± 0.03 | 4.63 ± 0.03 | 4.39 ± 0.01 | 4.14 ± 0.01 | 3.96 ± 0.01 |
| Rye End | 7.11 ± 0.04 | 7.26 ± 0.11 | 7.72 ± 0.12 | 7.82 ± 0.14 | 7.83 ± 0.17 | 7.75 ± 0.14 |
| Cress Start | 6.96 ± 0.09 | 6.59 ± 0.22 | 5.69 ± 0.08 | 5.07 ± 0.01 | 4.32 ± 0.02 | 4.05 ± 0.02 |
| Cress End | 7.76 ± 0.12 | 7.55 ± 0.14 | 7.56 ± 0.10 | 7.71 ± 0.03 | 7.83 ± 0.06 | 7.79 ± 0.19 |
| | | | EC | | | |
| Rye Start | 12.2 ± 0.4 | 37.6 ± 4.5 | 90.4 ± 6.0 | 110.3 ± 7.0 | 143.5 ± 11.4 | 167.6 ± 13.1 |
| Rye End | 79.5 ± 8.7 | 70.7 ± 9.7 | 80.7 ± 10.9 | 77.3 ± 14.1 | 72.2 ± 15.8 | 74.5 ± 17.0 |
| Cress Start | 14.0 ± 2.4 | 22.2 ± 2.2 | 41.9 ± 3.6 | 56.6 ± 2.1 | 117.6 ± 5.4 | 148.4 ± 1.1 |
| Cress End | 82.3 ± 9.4 | 66.2 ± 9.8 | 64.8 ± 1.5 | 63.4 ± 10.3 | 76.3 ± 11.9 | 79.6 ± 11.4 |

Table 8. Mean ± standard deviation of pH and electrical conductivity (EC, µS/cm) values in the valeric acid subchronic growth experiments. Rye = Italian ryegrass, Cress = garden cress, Start = start of the experiment, End = end of the experiment.

| Conc.<br>mmol/kg | Control Rye<br>Control Cress | 2.5 Rye<br>0.5 Cress | 10.0 Rye<br>1.5 Cress | 20.0 Rye<br>3.0 Cress | 30.0 Rye<br>15.0 Cress | 40.5 Rye<br>30.0 Cress |
|---|---|---|---|---|---|---|
| | | | pH | | | |
| Rye Start | 7.02 ± 0.14 | 5.23 ± 0.04 | 4.55 ± 0.02 | 4.26 ± 0.02 | 4.09 ± 0.02 | 3.98 ± 0.03 |
| Rye End | 7.11 ± 0.04 | 7.24 ± 0.06 | 7.66 ± 0.11 | 7.84 ± 0.05 | 7.97 ± 0.09 | 7.94 ± 0.06 |
| Cress Start | 6.96 ± 0.09 | 6.52 ± 0.20 | 5.70 ± 0.09 | 5.15 ± 0.05 | 4.37 ± 0.01 | 4.08 ± 0.02 |
| Cress End | 7.76 ± 0.12 | 7.61 ± 0.17 | 7.73 ± 0.19 | 7.70 ± 7.70 | 8.07 ± 0.19 | 7.98 ± 0.42 |
| | | | EC | | | |
| Rye Start | 12.2 ± 0.4 | 52.1 ± 4.1 | 98.3 ± 5.9 | 125.9 ± 5.4 | 141.6 ± 4.9 | 156.1 ± 2.8 |
| Rye End | 79.5 ± 8.7 | 63.3 ± 3.4 | 69.8 ± 6.2 | 69.1 ± 7.7 | 71.4 ± 5.7 | 65.1 ± 19.0 |
| Cress Start | 14.0 ± 2.4 | 24.1 ± 5.2 | 40.4 ± 0.6 | 59.0 ± 5.9 | 113.0 ± 6.2 | 141.2 ± 3.8 |
| Cress End | 82.3 ± 9.4 | 71.6 ± 5.8 | 67.3 ± 3.4 | 66.4 ± 5.1 | 69.4 ± 5.0 | 88.5 ± 17.6 |

Table 9. Mean ± standard deviation of pH and electrical conductivity (EC, µS/cm) values in the caproic acid subchronic growth experiments. Rye = Italian ryegrass, Cress = garden cress, Start = start of the experiment, End = end of the experiment.

| Conc.<br>mmol/kg | Control Rye<br>Control Cress | 0.6 Rye<br>0.2 Cress | 6.0 Rye<br>0.6 Cress | 12.5 Rye<br>1.8 Cress | 17.0 Rye<br>5.4 Cress | 22.5 Rye<br>16.2 Cress |
|---|---|---|---|---|---|---|
| | | | pH | | | |
| Rye Start | 7.02 ± 0.14 | 6.32 ± 0.16 | 4.83 ± 0.05 | 4.48 ± 0.04 | 4.34 ± 0.03 | 4.22 ± 0.03 |
| Rye End | 7.11 ± 0.04 | 7.22 ± 0.22 | 7.54 ± 0.22 | 7.82 ± 0.24 | 7.93 ± 0.11 | 7.88 ± 0.17 |
| Cress Start | 6.96 ± 0.09 | 6.72 ± 0.12 | 6.25 ± 0.33 | 5.41 ± 0.15 | 4.68 ± 0.26 | 4.23 ± 0.13 |
| Cress pH E | 7.76 ± 0.12 | 7.57 ± 0.07 | 7.60 ± 0.17 | 7.56 ± 0.13 | 7.75 ± 0.13 | 7.79 ± 0.07 |
| | | | EC | | | |
| Rye Start | 12.2 ± 0.4 | 23.5 ± 1.5 | 74.2 ± 4.3 | 92.8 ± 4.8 | 100.9 ± 10.1 | 108.5 ± 6.9 |
| Rye End | 79.5 ± 8.7 | 58.9 ± 6.7 | 60.3 ± 6.9 | 67.5 ± 4.4 | 65.9 ± 7.2 | 56.7 ± 6.4 |
| Cress Start | 14.0 ± 2.4 | 22.0 ± 11.7 | 33.0 ± 17.7 | 50.2 ± 13.7 | 77.7 ± 17.4 | 102.2 ± 11.6 |
| Cress End | 82.3 ± 9.4 | 62.6 ± 7.3 | 57.2 ± 8.1 | 54.3 ± 5.7 | 56.5 ± 2.8 | 66.2 ± 21.3 |

## Appendix 5


Figure 1. Photograph of final day (day 21) of subchronic exposure experiment performed on garden cress (*L. sativum*). Photograph is from dilution series of *iso*-valeric acid. On the far left are the control pots (no VFAs added). Concentrations are indicated in the picture in mmol/kg (dry weight). The yellowing of cress leaves was evident after application of higher concentrations of *iso*-valeric acid

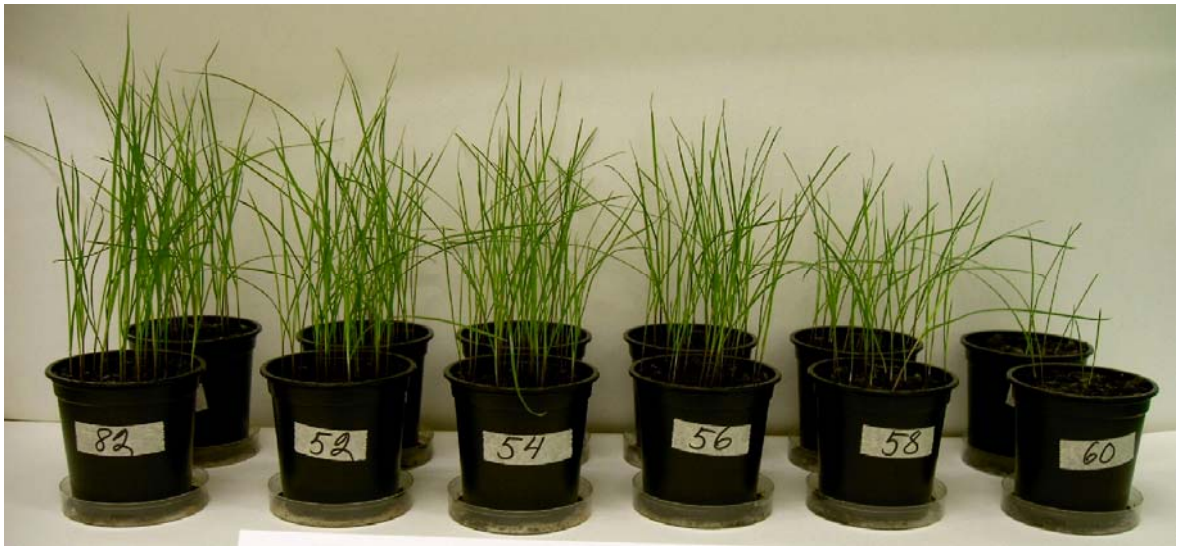



Figure 2. Photograph of day 13 of 21 day subchronic exposure experiment on Italian ryegrass (*L. multiflorum*). Photograph is from *iso*-valeric acids dilution series. On the far left are control pots (no acid added). Concentrations are from the right 40.5, 25.0, 13.5, 7.5, 1.5 mmol/kg (dry weight). No yellowing or dying of growing plants was ever apparent with ryegrass with any tested VFA.

## Appendix 6

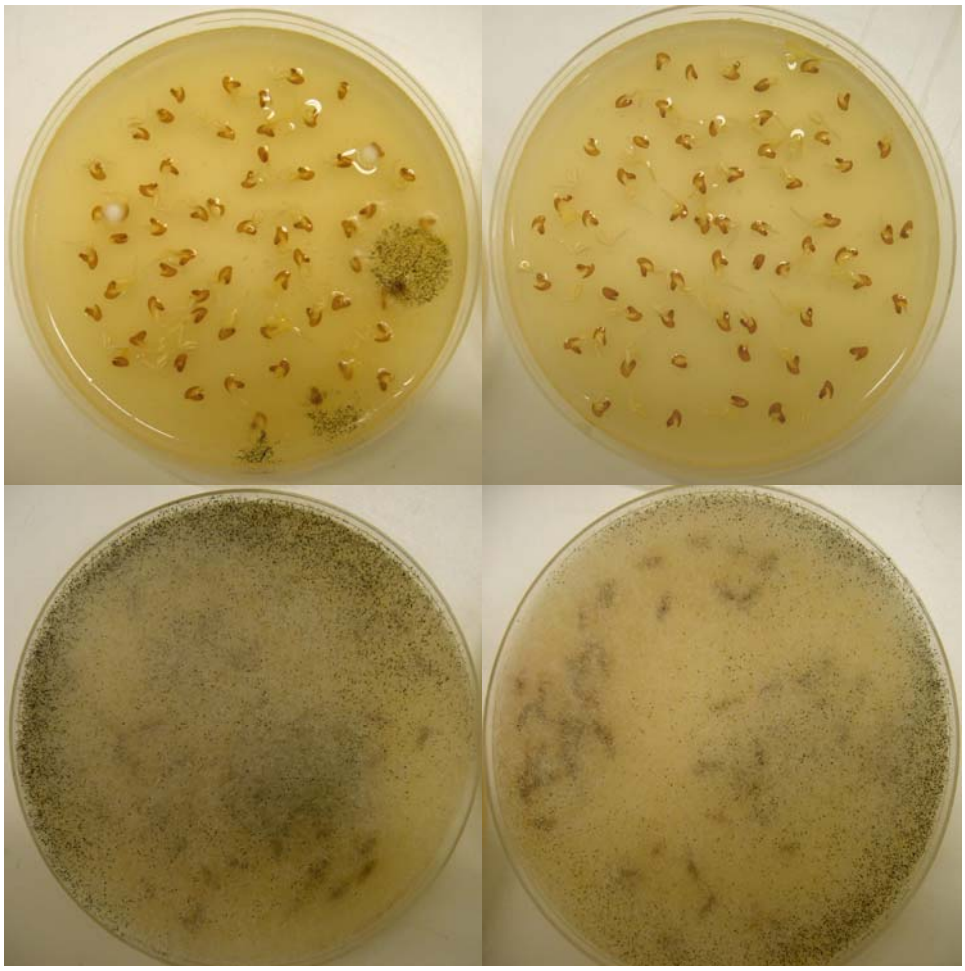
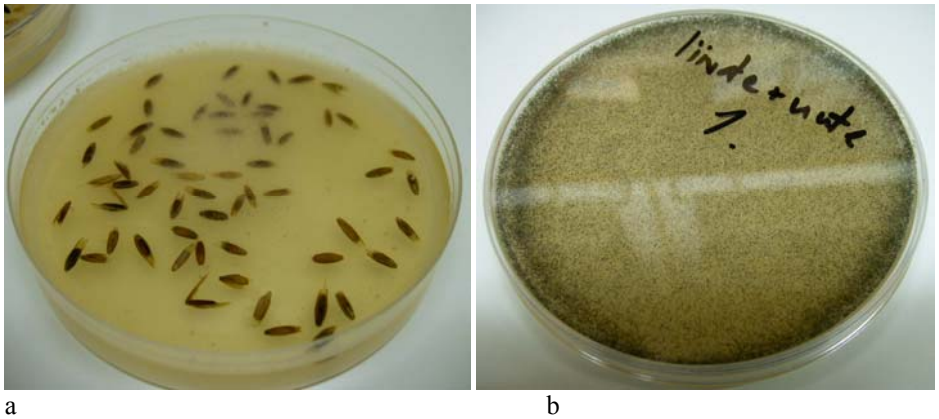


Figure 1. Three days of incubation period showed substantial difference in mold growth between garden cress (*L. sativum*) (upper photographs) and Italian ryegrass (*L. multiflorum*) (photographs below). Two replicates of each are shown. In one of the garden cress Petri dishes (upper left) 3rd day showed 4 small visible areas of mold growth, which were not visible on a day two. Visible examination showed structural features (color (white) and growth pattern) differences from molds infecting Italian ryegrass (front).



a b

Figure 2. (a,b). Two day incubation (darkness, 25°C ) of Italian ryegrass showed already visible mold growth., which was not the case with garden cress The effectiveness of used methods for growth of mold inoculum was checked by control plates with no seeds (b). The effectiveness of inoculum and growth substratum to support rapid growth of inoculated mold was substantial, with the mold raising the lids of Petri dishes by third day of incubation (b).

**Appendix 7**

Figure 1. Example of the different lengths of shoots found in one control pot (no VFAs applied) in Italian ryegrass (*L. multiflorum*) subchronic growth experiment three after 21 day incubation time. The longest shoots were up o 50 cm long.

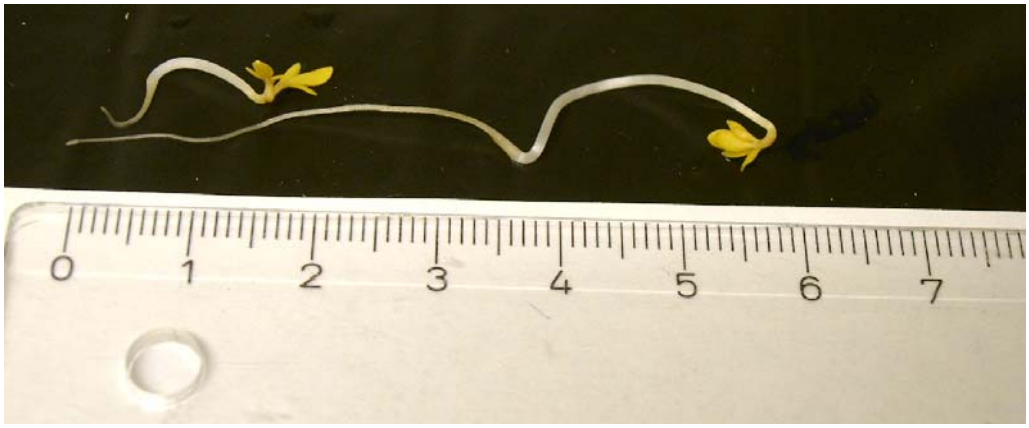



Figure 2. Example of the variability in radicle lengths of garden cress (*L. sativum*) found on one control (no VFAs applied) Petri dish (replicate). Although not stretched to limits, as is done in real measurements, the difference in root and shoot length in one and the same treatment replicate plate is obvious and huge. The importance of enough number of replicates and individuals is the basis for good dose-response estimation.

**Appendix 8**


Figure 1. An example of the visible root system of Italian ryegrass (*L. multiflorum*) after 21 day subchronic exposure to butyric acid. Concentrations in the pots were (from left): 0 (control), 2.5, 10.0, 25.0, 40.0, 60.0 mmol/kg (dw).


Figure 2. Washed root system of Italian ryegrass after 21 day exposure experiment with different VFAs. The different pots of VFAs were selected for photograph to the closest same acid concentration. From left (concentrations are in parenthesis in mmol/kg (dw)): control (0), formic acid (30), acetic acid (25), propionic acid (20), *iso*-butyric acid (30), butyric acid (25), *iso*-valeric acid (25), valeric acid (30) and caproic acid (22,5).

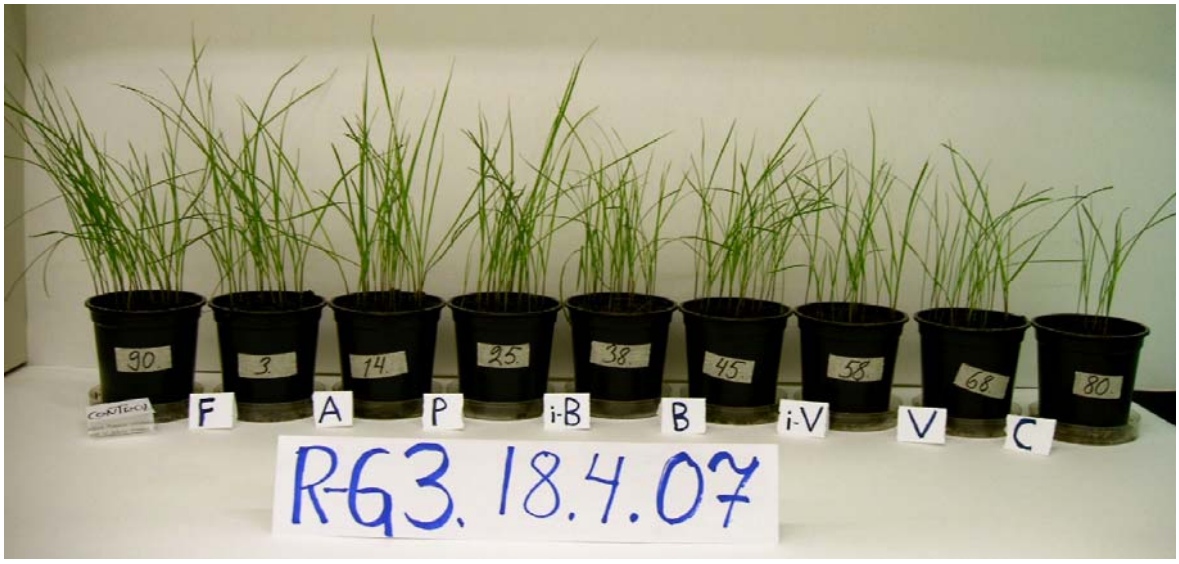



Figure 3. Same concentration pots of Italian ryegrass as in Figure 2. Photograph was taken on a day 13 of 21 day subchronic exposure experiment. The different pots of VFAs were selected for photograph to the closest same acid concentration. From left (concentrations are in parenthesis in mmol/kg (dw)): control (0), formic acid (30), acetic acid (25), propionic acid (20), *iso*-butyric acid (30), butyric acid (25), *iso*-valeric acid (25), valeric acid (30) and caproic acid (22.5).

Table 1. Volatile fatty acid concentrations of undissociated (UD) VFA forms in each total VFA concentration (undissociated + dissociated) applied in acute exposure experiments of garden cress (*L. sativum*). The pH values used for calculations are found in Appendix 4. Concentrations (Conc.) are expressed in mmol/l. The total initial concentrations of butyric acid are 0.10, 0.30, 0.61, 1.21, 2.42, 4.84, 9.68 mmol/l.

| VFA | Total VFA Conc. 0.1 | Total VFA Conc. 0.3 | Total VFA Conc. 0.6 | Total VFA Conc. 1.2 | Total VFA Conc. 2.4 | Total VFA Conc. 4.8 | Total VFA Conc. 9.6 |
|---|---|---|---|---|---|---|---|
| Formic UD | 0.02 | 0.13 | 0.34 | 0.80 | 1.81 | 3.93 | 8.38 |
| Acetic UD | 0.06 | 0.23 | 0.50 | 1.06 | 2.19 | 4.51 | 9.18 |
| Propionic UD | 0.07 | 0.24 | 0.51 | 1.07 | 2.22 | 4.54 | 9.23 |
| *Iso*-butyric UD | 0.06 | 0.23 | 0.51 | 1.07 | 2.21 | 4.53 | 9.23 |
| Butyric UD | 0.07 | 0.23 | 0.51 | 1.07 | 2.22 | 4.56 | 9.29 |
| *Iso*-valeric UD | 0.06 | 0.23 | 0.50 | 1.05 | 2.20 | 4.51 | 9.20 |
| Valeric UD | 0.06 | 0.23 | 0.51 | 1.07 | 2.21 | 4.54 | 9.23 |
| Caproic UD | 0.06 | 0.23 | 0.50 | 1.07 | 2.21 | 4.53 | 9.22 |

Table 2. Volatile fatty acid concentrations of undissociated (UD) VFA forms in each total VFA concentration (undissociated + dissociated) applied in acute exposure experiments of Italian ryegrass (*L. multiflorum*). The pH values used for calculations are found in Appendix 4. Concentrations (Conc.) are expressed in mmol/l. The total initial concentrations of butyric acid are 0.10, 0.30, 0.61, 1.21, 2.42, 4.84, 9.68 mmol/l.

| VFA | Total VFA Conc. 0.1 | Total VFA Conc. 0.3 | Total VFA Conc. 0.6 | Total VFA Conc. 1.2 | Total VFA Conc. 2.4 | Total VFA Conc. 4.8 | Total VFA Conc. 9.6 |
|---|---|---|---|---|---|---|---|
| Formic UD | 0.02 | 0.13 | 0.34 | 0.80 | 1.81 | 3.92 | 8.38 |
| Acetic UD | 0.06 | 0.23 | 0.50 | 1.05 | 2.19 | 4.51 | 9.18 |
| Propionic UD | 0.07 | 0.24 | 0.51 | 1.07 | 2.21 | 4.54 | 9.23 |
| *Iso*-butyric UD | 0.06 | 0.23 | 0.50 | 1.07 | 2.21 | 4.53 | 9.23 |
| Butyric UD | 0.06 | 0.23 | 0.51 | 1.07 | 2.22 | 4.56 | 9.28 |
| *Iso*-valeric UD | 0.06 | 0.23 | 0.50 | 1.05 | 2.20 | 4.51 | 9.20 |
| Valeric UD | 0.06 | 0.23 | 0.51 | 1.07 | 2.21 | 4.53 | 9.23 |
| Caproic UD | 0.06 | 0.23 | 0.50 | 1.07 | 2.21 | 4.53 | 9.22 |

Table 3. The volatile fatty acid (VFA) concentrations of undissociated VFA forms in each concentration as applied in subchronic exposure experiments of Italian ryegrass (R) and garden cress (C). The pH values used for calculations are found in Appendix 4. Numbers 1 - 5 represent total initial VFA concentrations (dissociated + undissociated), where 1 represents the lowest concentration and number 5 the highest concentration in increasing order. Each initial total VFA concentration is found in Appendix 4. Concentrations (Conc.) of undissociated forms of VFAs are expressed in mmol/kg (dw).

| VFA | 1 C | 2 C | 3 C | 4 C | 5 C | 1 R | 2 R | 3 R | 4 R | 5 R |
|---|---|---|---|---|---|---|---|---|---|---|
| | Conc. | Conc. | Conc. | Conc. | Conc. | Conc. | Conc. | Conc. | Conc. | Conc. |
| Formic | 3.54 | 11.09 | 27.75 | 44.60 | 62.67 | 3.50 | 18.91 | 53.13 | 70.97 | 172.09 |
| Acetic | 6.26 | 15.31 | 34.42 | 44.22 | 53.70 | 2.19 | 19.73 | 43.52 | 67.65 | 91.99 |
| Propionic | 0.23 | 2.17 | 9.89 | 35.49 | 54.28 | 2.40 | 6.49 | 15.56 | 29.72 | 44.02 |
| *Iso*- butyric | 0.01 | 0.20 | 2.10 | 21.91 | 39.34 | 0.21 | 2.42 | 11.00 | 25.03 | 39.46 |
| Butyric | 0.16 | 2.08 | 9.71 | 20.36 | 35.24 | 0.69 | 6.62 | 20.48 | 34.70 | 54.06 |
| *Iso*-valeric | 0.01 | 0.17 | 1.01 | 11.12 | 25.27 | 0.21 | 4.38 | 9.62 | 20.32 | 35.22 |
| Valeric | 0.01 | 0.18 | 0.99 | 11.22 | 25.58 | 0.73 | 6.61 | 15.86 | 25.53 | 35.65 |
| Caproic | 0.00 | 0.02 | 0.39 | 3.24 | 13.09 | 0.02 | 3.10 | 8.80 | 13.01 | 18.28 |