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Evaluation of the aquatic bryophyte *Fontinalis antipyretica* for laboratory and field toxicity studies

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Abstract

Fontinalis antipyretica is an ecologically important aquatic moss in boreal streams. Bioassays using this species provide information of effects at the base of the food chain. The present study assessed toxicological effects to *F. antipyretica*, firstly of the positive controls copper and 3,5-dichlorophenol (3,5-DCP) in separate laboratorial bioassays, and secondly of ecologically relevant pollutants during autumn in a catchment area dominated by agriculture in a field study.

The laboratorial studies assessed effects of five concentrations of copper sulphate ($CuSO_4$, 0-300 µM) and 3,5-DCP (0-9 mg/L) for exposure up to 21 days. This included determination of suitable mode of actions (MoAs) including maximal PS II efficiency (Fv/Fm), pigment concentration and production of reactive oxygen species (ROS), and the adverse outcomes (AO) greenness index (GI) and growth, and toxic effects on these endpoints. Copper caused clear concentration and time-dependent responses for inhibition of GI, Fv/Fm, pigment concentration, and production of ROS. 3,5-DCP caused clear concentration and time-dependent responses for inhibition of GI, Fv/Fm, and pigment concentration, additionally to weak responses for growth. Optimal exposure time was typically 7-14 days for most endpoints; however, 21 days were needed for growth.

The field study assessed effects of environmentally relevant concentrations of pollutants and stressors of the test stream Skuterudbekken, compared to a reference stream with little pollution. Moss tissue was deployed from Skut 1 (located upstream of sedimentation ponds) and Skut 2 (located downstream of sedimentation ponds and European route 18) back into their respective location and additionally to the reference stream. The study additionally included comparison of native and deployed moss in the test stream, and moss sampling for endpoint analysis was done after exposure for up to 14 days. Chemical and physical conditions of streams were monitored during the exposure study. The results indicated no clear deployment status, site or time-dependent responses caused by pollution. However, site-specific negative responses on growth, Fv/Fm, and production of ROS were observed in Skut 2, possibly caused by high water discharge combined with fragmenting of *F. antipyretica* due to the time of year.

Overall, *F. antipyerica* demonstrated to be a suitable study species for bioassays, with limitations including difficulties in obtaining sterile moss cultures for laboratorial assays and short life cycle period for obtaining ideal growth conditions.

Sammendrag

Fontinalis antipyretica er en økologisk viktig akvatisk mose i boreale elver og bekker. Bioassay med bruk av denne arten gir informasjon om effekter på bunnen av næringskjeden. Dette studiet undersøkte toksikologiske effekter på *F. antipyretica*, først av de positive kontrollene kobber og 3,5-diklorofenol (3,5-DCP) i separate laboratoriestudier, og deretter i en feltstudie med økologisk relevante forurensende stoffer på høsten i et nedbørsfelt dominert av landbruk.

Laboratorieforsøkene undersøkte effekter av fem konsentrasjoner av kobbersulfat ($CuSO_4$, 0-300 µM) og 3,5-DCP (0-9 mg/L) etter eksponering i opptil 21 dager. Dette inkluderte å fastslå virkemåtene (eng. Mode of Action, MoA) maksimal PS II effektivitet (Fv/Fm), pigment-konsentrasjon og produksjon av reaktive oksygenforbindelser (ROS), samt de alvorlige effektene (eng. Adverse Outcome, AO) grønnhetsindeks (GI) og vekst. Toksiske effekter på disse endepunktene ble undersøkt. Kobbereksponering førte til klar konsentrasjons- og tidsavhengig respons for hemming av GI, Fv/Fm, pigmentkonsentrasjon og produksjon av ROS. Eksponering for 3,5-DCP førte til klar konsentrasjons- og tidsavhengig respons for hemming av GI, Fv/Fm og pigmentkonsentrasjon, samt svak negativ påvirkning på vekst. Optimal eksponeringstid var typisk 7-14 dager for de fleste endepunkt, men effekt på vekst trengte 21 dager.

Feltforsøket undersøkte effekten av miljømessig relevante konsentrasjoner av forurensende stoffer og stressorer i testbekken Skuterudbekken, sammenlignet med en referansebekk med lite forurensing. Mosen ble transplantert fra Skut 1 (plassert oppstrøms for sedimentasjonsdammer) og Skut 2 (plassert nedstrøms for sedimentasjonsdammer og Europavei 18), tilbake til sine respektive bekker og i tillegg referansebekken. Forsøket inkluderte også sammenligning av transplantert og frittvoksende mose i testbekken, og endepunktsanalyser ble gjort etter eksponering i opptil 14 dager. Målinger av kjemiske og fysiske variabler i bekkene ble gjort gjennom eksponeringsstudiet. Resultatene indikerte ingen klar transplantasjonsstatus-, lokasjons- eller tidsavhenging respons på grunn av forurensing. Derimot ble lokasjonsavhenging respons observert for vekst, Fv/Fm og produksjon av ROS i Skut 2, muligens på grunn av høy vannføring kombinert med fragmentering av *F. antipyretica* på grunn av årstiden.

Alt i alt har *F. antipyretica* vist seg å være en passende art for bioassay, selv om det kan være begrensinger inkluderende vanskeligheter med å oppnå en steril mosekultur for laboratorieforsøk og kort periode med ideelle vekstforhold i livssyklusen.

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1. Introduction

Large amounts of pollution in nature tend to eventually end up in aquatic systems. When present in high enough concentrations, particular speciation, or combinations, pollution cause toxic effects to organisms. These effects can in turn lead to reduced health of organisms and possible effects on ecosystems. For this reason, toxic effects of pollutants to aquatic organisms and ecosystems has become an increasing concern (Weiner & Matthews, 2003).

Aquatic plants (macrophytes) are key species with important ecologically roles and are constantly exposed to natural and anthropogenic toxicants and stressors. For this reason, it is necessary to assess pollutant toxicity to macrophytes, including mode of actions (MoA) and adverse outcome (AO), to increase knowledge of effects at the cellular and individual level. Additionally, assessing toxicity in various species of macrophytes living in different habitats is necessary to increase knowledge of effects in multiple ecosystems. This assessment can be done by using macrophytes as bioindicators through field and laboratorial studies.

The aim of this thesis is to assess effects of pollutants to the globally distributed aquatic moss *Fontinalis antipyretica,* through laboratorial and field studies.

1.1. Pollution in aquatic systems

1.1.1. Sources and abundance

Pollution can find its way into aquatic systems from multiple sources, including air and land runoff. It is unintentionally leached or spilled from land, or deliberately used to control organisms in water. This includes pollution originating from industry or wastewater treatment plants through channels or pipes, or land runoff from sources such as agriculture or construction sites (Weiner & Matthews, 2003). On a global scale, main sources of pollution are urbanisation, infrastructure, industry, and agriculture (Mateo-Sagasta et al., 2017).

Historically, aquatic pollution has been considered a threat to human health due to spread of diseases. However, in developed countries the threat to aquatic life has become an equally big concern (Weiner & Matthews, 2003). Aquatic pollution could include oxygen-demanding wastes, organic substances, metals, sediments, suspended material, nutrients or heated wastewater (Weiner & Matthews, 2003).

1.1.2. Distribution and fate

The pollutants fate in aquatic systems are diverse; they can be dissolved in water, suspended as droplets, adsorbed to particles or interact with biota (Walker et al., 2016). For locations where

the stream flow rate slows down, including sedimentation ponds or when entering a lake, particles such as soils and decaying organic matter can be precipitated to the stream bottom. Liquid droplets such as oil tend to float on the stream surface or are transported to the stream bottom with sedimented particles (Wetzel, 2001).

Pollution of rivers and streams are diluted or degraded immediately downstream of the discharge point, which can be observed through gradually decreasing biological impact (Walker et al., 2016). Additionally, the transport length in rivers is dependent of the pollutants physical state, stability in water and water flow rate. Pollutants with high chemical stability tend to stay present in the environment, however less chemical stable substances could be degraded to other toxic products (Walker et al., 2016).

The fate of different types of pollutants in water is diverse, such as for metals and organic pollutants. The solubility and bioavailability of metals commonly increased at lower pH (Walker et al., 2016). Higher pH tends to result in precipitation (sedimentation) of metals. The fate of organic pollutants in water, on the other hand, are mostly dependent on physical property of the substance, including lipophilicity, chemical stability and vapor pressure (Walker et al., 2016). Furthermore, availability to organisms are dependent on factors such as presence of other heavy metals, anions, particles and organic chelators in the water, temperature or light intensity (Tessier & Turner, 1995).

1.1.3. Agricultural pollution

Due to increasing demand of food for the growing human populations, the intensity and land used for agriculture have increased. Consequently, the need of pesticides to control unwanted plants and pests, and fertilizers to optimise and enlarge the crop, has increased. Agricultural pollutants includes nutrients from fertilization (typically phosphorous and nitrogen), organic matter, sediments, faecal coliform bacteria and agricultural chemicals such as pesticides and drug residues (Mateo-Sagasta et al., 2017; Weiner & Matthews, 2003). Pesticides are especially concerning when leaked into nature, as they are intended to destroy, prevent, repel or reduce unwanted organisms (The United States Environmental Protection Agency (U.S. EPA), 2019). In areas with high use of pesticides and fertilizers, agricultural runoff can contaminate both surface and ground water (Weiner & Matthews, 2003). Within the European Union, agricultural pollution affected 38 % of all water bodies in 2015 (WWAP, 2015), and have in countries with high income overtaken as the main source of pollution of aquatic systems, over industry and settlements (Mateo-Sagasta et al., 2017).

In this thesis, the focus of the field study is agricultural pollution such as pesticides from autumn spraying and nutrients from fertilization. In the laboratorial study, $CuSO_4$ (previously used as a pesticide (Amdur et al., 1993)) and 3,5-DCP (model compound, may originate as a residue of pesticides (Igbinosa et al., 2013)) were used as positive controls due to their well-known toxicity to plants.

1.2. Pollution and aquatic macrophytes

Pollution is a major threat to aquatic ecosystems and can affect organisms throughout the food chain. Macrophytes are macroscopic plants in aquatic environments, including non-vascular and vascular plants, some large algae, lichens and mosses (Wilzbach & Cummins, 2019). They can be free-floating, attached to the substrate or have roots growing into the substrate, and can be partly or fully submerged (Wilzbach & Cummins, 2019; Aarnes, 2016). Macrophytes are important organisms at the base of the food chain, with ecological roles including oxygen production, carbon dioxide uptake, source of food or habitats for other species, and stabilisation of sediments (Mohan & Hosetti, 1999).

Bryophytes (mosses) tend to be the dominating macrophyte in boreal streams (Turunen et al., 2020) and in acidic and soft-watered (low concentration of ions) aquatic systems (Wetzel, 2001; Aarnes, 2016). They have been used as bioindicators for a long time where primarily bioaccumulation and its mechanisms have been studied (Ah-Peng & De Traubenberg, 2004). In Norway, aquatic bryophytes are relatively common in rivers and streams, and species such as *F. antipyretica* can be found throughout the country (Artsdatabanken, n.d.; Lye, 1968).

1.2.1. Uptake and effects of pollution

Uptake of chemicals in macrophytes is dominated by direct uptake from sediments and water via roots and leaves (Walker et al., 2016). Adsorption on the plant cuticle (protective outer layer) depends on the chemical properties, cuticle surface properties, strength of adsorption bond, plant species and properties of the surrounding aquatic environment (Walker et al., 2016). Bryophytes lack root systems and cuticle layer, resulting in easier absorption of metal ions (Koz & Cevik, 2014; Little & Martin, 1974; Sun et al., 2009). After uptake, chemicals can be stored in the plant tissue, metabolized, and possibly excreted, or interact with internal molecules and structures resulting in an effect on the plant (Figure 1). The principle of storage in plant tissue is used in phytoremediation, where plants are used to take up heavy metals from the soil (Pilon-Smits, 2005). For instance, bryophytes are known to effectively accumulate metals such as copper (Stankovic et al., 2018).

Analysis of chronic and sublethal effects can be assessed through endpoint analysis, including assessment of the pollutant's MoA and AO. Metal toxicity typically cause reduced photosynthetic efficiency, reduced pigment concentration, and increased oxidative stress as MoAs in macrophytes (Chen et al., 2015; Rau et al., 2007; Shakya et al., 2008). This can in turn lead to AOs such as reduced growth and chlorosis (yellow shoots due to loss of chlorophyll) (Dumont et al., 2019b; Guo et al., 2020; Krayem et al., 2021). Organic pollutants such as chlorophenols (including 3,5-dichlorophenol) can cause long-term adverse effects to macrophytes (Zagorc-Koncan et al., 2002), through reduced photosynthetic efficiency, pigment concentration and increased ROS production as MoAs, and reduced growth and reproduction as AOs (Xie et al., 2018). Model for fate of chemicals in organisms (Walker et al., 2016).

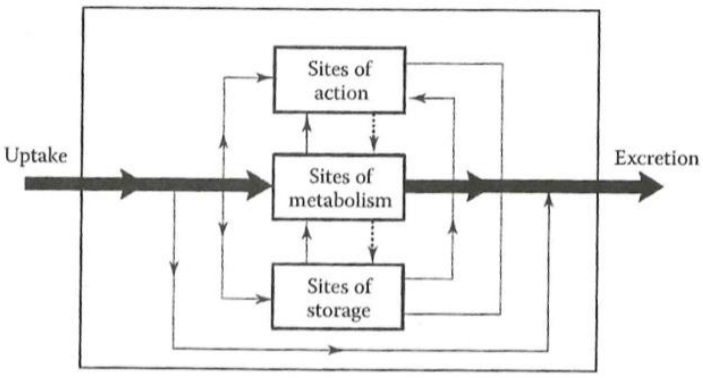

Figure 1: Model for fate of chemicals in organisms (Walker et al., 2016).

As aquatic organisms are exposed to multiple chemicals and stressors at the same time, both single chemical effects and combined effects can occur. Chemical mixtures can interact on several biological levels through similar or different MoAs with a multitude of combined effects (Beyer et al., 2014; Kortenkamp et al., 2009). They were previously believed to only pose additive effects (mixture toxicity equals sum of individual chemical toxicity), however this tend to be the case only for chemicals with similar MoAs or toxicity mechanism in organisms (Kortenkamp et al., 2009; Walker et al., 2016). Mixture toxicity can additionally be synergistic or antagonistic (mixture toxicity exceeds or fall below the sum of individual chemical toxicity, respectively). This tends to happen if there are additional stressors present or toxicokinetic factors (e.g. temperature) change the chemicals toxicity mechanism, often resulting in synergistic effects (Løkke et al., 2013).

1.2.2. Ecotoxicological effects

Pollution can have various impacts on the aquatic ecosystem. The pollutants can cause acute toxicity with immediate death of organisms, or chronic toxicity with reduced health of organisms after a certain time period (Weiner & Matthews, 2003). Additionally, adverse effects can be indirectly caused, such as degradation of organic wastes where decomposers use high amounts of oxygen, causing oxygen depletion (Weiner & Matthews, 2003). Many aquatic

organisms are dependent of oxygen and are affected even by short periods of oxygen depletion (Weiner & Matthews, 2003). Longer periods (anaerobic) can additionally cause production of harmful gases such as ammonia (NH_3) and hydrogen sulphide (H_2S) (Weiner & Matthews, 2003). Whatever the mechanism, pollution and habitat related effect can cause a shift in number and type of species in the ecosystem.

Macrophytes are considered as highly threatened organisms in limnological ecosystems (Lacoul & Freedman, 2006). When macrophytes suffer toxic effects, the whole ecosystem is affected. Toyama et al. (2020) demonstrated that species richness and phylogenetic diversity of macrophytes decreased with increased contamination. Death or reduced health of aquatic macrophytes has consequences throughout the food chain through reduced oxygen production, carbon dioxide uptake, source of food and loss of habitats for other species.

1.2.3. Bioindicators and toxicological principles

Bioindicators are living organisms used to investigate effects of pollutants and provide information of the environmental quality, through field or laboratorial studies (Halleraker & Ratikainen, 2020). Field studies are done to assess effects of ecologically relevant pollutant concentrations and stressors, under natural exposure conditions in the test organism's habitat. Laboratorial studies are done to assess effects known concentrations of specific single or combined pollutants or stressors, under controlled exposure conditions (standardized test regimes). Copper and 3,5-DCP are examples of chemicals with known toxicity to plants and are for this reason used as positive controls in laboratorial toxicity tests.

Macrophytes such as aquatic bryophytes have been used as bioindicators to monitor heavy metal pollution for decades (Cesa et al., 2009; Little & Martin, 1974; Shaw et al., 1989). Aquatic bryophytes are good bioindicators due to their wide geographical and ecological distribution, long life cycles, easy sampling, identifying and transplantation and low variation of chemical composition within a population (Censi, 2000; Deben et al., 2017; Kelly et al., 1987). They mainly have passive uptake of pollutants with little influence of biotic factors, accumulation is fast, release of chemicals is medium-slow, and their lack of roots makes it possible to exclude uptake of pollutants from sediments (Censi, 2000; Deben et al., 2017). Bryophytes have the ability to accumulate pollutants effectively, making it possible to detect intermittent pollutions passing downstream rivers (Say et al., 1981). However, they are resistant to stress from pollution and environmental factors, and usually survive even with high concentrations of pollutants (Censi, 2000; Deben et al., 2017).

1.3. Test species *Fontinalis antipyretica*

Fontinalis antipyretica (willow moss) is an aquatic bryophyte found in Europe and North America (Goffinet & Shaw, 2009; Tuba et al., 2011). It is commonly found in streams with intermittent flow (flow during spring, dry or almost dry during summer) or perennial flow (flow during both spring and summer) (Fritz et al., 2009) and occasionally in lakes (Glime & Acton, 1979). The bryophyte grows densely in frequently branched filaments. It can become up to 70 cm long and grows attached to rocks and roots (Lye, 1968) (Figure 2). It is characterised by 4-7 mm long elongated and keeled fronds (leaflike structures) placed evenly in three rows on the stem, making triangular cross sections (Lye, 1968). Reproduction is mainly done vegetatively or occasionally by sporophytes (Glime et al., 1979).

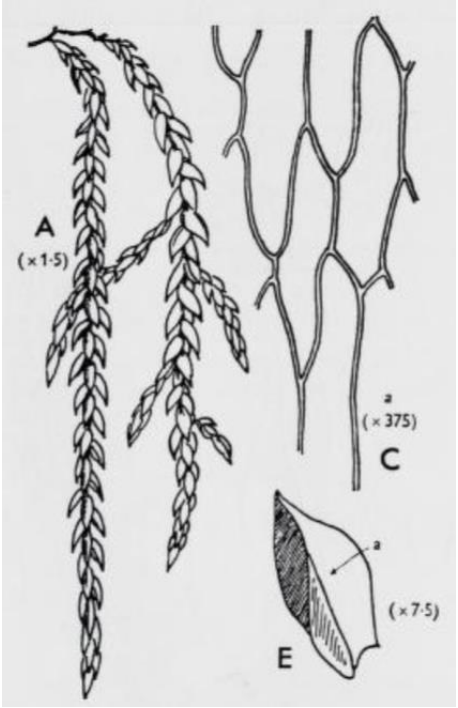

Figure 2: Morphology of *Fontinalis antipyretica*. A = overview of whole plant, E = leaf including the keel (a) and C = cells of the leaves. Source: Lye (1968).

Fontinalis antipyretica has an optimum temperature of 8 - 20°C, varying with the season (Tuba et al., 2011). However, branching was highest at 5-15 °C (Glime & Raeymaekers, 1987), and another species in the *Fontinalis* genus (*F. hypnoides*) had highest growth at 15 °C in laboratorial studies (Glime, 1984a). *Fontinalis antipyretica* thrives in partly shaded locations (Tuba et al., 2011) but is observed to be adapted to variating light conditions (Glime, 1984b).

The life cycle of *Fontinalis* includes new growth in late spring, reproduction in summer/autumn and loss of dead tissue in early spring (Figure 3) (Glime, 2014). New annual growth starts when light intensity and nutrient content is high in the spring flow, and temperature is low. Rhizoids (root-like structures) and gametangia (gamete-producing organs) form during summer. Reproduction happens during autumn, by release of sperm from male

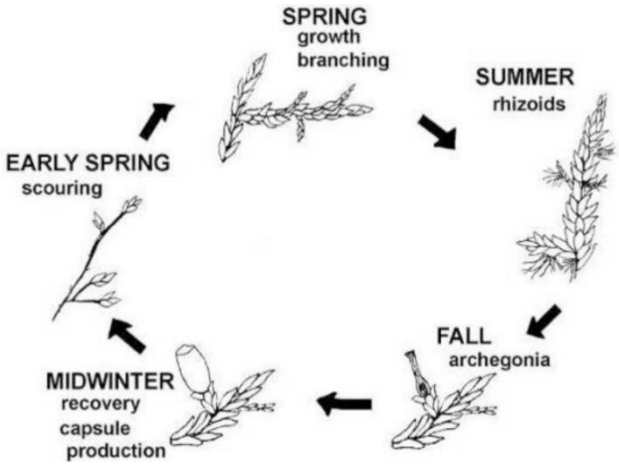

Figure 3: Life cycle of *Fontinalis* species (Upper Peninsula, Michigan) (Glime, 2014).

plants into water, which hopefully finds a female archegonium. Additionally, vegetative growth happens due to fragmenting of branches and attachment to new substrates using the rhizoids. Early spring flows remove old leaves and capsules, making the plants ready for new growth.

1.3.1. Ecological role

As a primary producer, *F. antipyretica* is an important key species in its habitat with cycling of nutrients and oxygen production (Aronsson & Ekelund, 2006). It is additionally found to serve as a food source for the aquatic isopod *Asellus militaris*, despite the bryophyte's content of phenolic deterrents against predatory (Glime, 2006).

However, *F. antipyretica* have additional and perhaps more ecologically important roles, such as habitat for insects and egg attachment site for fish (Tuba et al., 2011). The *Fontinalis* genus are found to be important habitats for insects in water such as midges (*Chiromonidae*), spring stoneflies (*Nemoura*) and black flies (*Simuliidae*) (Glime & Clemons, 1972). As *F. antipyretica* often make dense mats on rocks in relatively shallow waters, it might function as a filter catching particles and organisms in the stream water. The insects locate food particles (e.g., particulate organic matter, detritus, algae, and bacteria) that get stuck on the bryophyte or floats by in the stream water (Glime & Clemons, 1972; Vlčková et al., 2002). This ecologically important role makes *F. antipyretica* an important biomonitoring species for the ecosystems health and impacts at the base of the food chain.

1.3.2. Use as a bioindicator/model species

Within bryophytes, *F. antipyretica* is considered as one of the most used and suitable bioindicator species for streams (Deben et al., 2017; Deben et al., 2020). It is widely distributed worldwide and in Norway according to species registry maps (Artsdatabanken, n.d.), and hence an ecologically relevant species for many areas. The exchange kinetics of heavy metals in bryophytes such as *F. antipyretica* is demonstrated to be fast (Martins et al., 2004). *Fontinalis antipyretica* is reported to be a sensitive and effective accumulator of pollution (Aronsson & Ekelund, 2006) and have for this reason been used in multiple studies, mostly including heavy metal accumulation in fresh water. Multiple field studies have measured the accumulation of heavy metals in *F. antipyretica* (Figueira & Ribeiro, 2005; Mersch & Reichard, 1998; Say & Whitton, 1983). A few field studies have assessed effects of organic pollution (Lopez & Carballeira, 1990) and laboratorial studies have analysed absorption or effect of heavy metals in *F. antipyretica* (Bleuel et al., 2005; Rau et al., 2007; Sutter et al., 2002). *Fontinalis antipyretica* is demonstrated to be challenging to use as a test species in laboratorial studies, due to difficulties of obtaining a sterile culture (de Traubenberg & Ah-Peng, 2004). There are

multiple knowledge gaps of using *F. antipyretica* for laboratorial studies, such as characterisation of MoAs, AOs, and concentration or time-dependent responses for different toxicants.

1.4. Aims of the study

Assessing toxic effects to stream dwelling macrophytes and establishing suitable test methods with various species is necessary to increase knowledge of effects in multiple species and ecosystems. The main aim of this thesis is to examine the effect of toxicants to the aquatic bryophyte *Fontinalis antipyretica* through laboratorial and field studies, and to assess suitability of *F. antipyretica* as a study species.

Firstly, effects of the positive controls $CuSO_4$ and 3,5-DCP will be assessed, based on separate, contolled laboratorial bioassays. This includes assessment of concentration and time-dependent responses on AO and MoA endpoints, estimation of the concentration needed for an effect in 50 % of the population (EC_{50}), concentration needed for no observed effect (NOEC) and concentration needed for the lowest observed effect (LOEC). Secondly, effects of environmentally relevant concentration of pollutants and stressors will be assessed, based on a field study in a stream surrounded by agricultural land. This includes comparison to a stream with little pollution, and comparison of deployed and native moss. The endpoints of focus for laboratorial and field studies are growth and colour change as AOs, and maximal PS II efficiency, pigment concentration, and production of reactive oxygen species (ROS) as MoAs. Additionally photosynthetic oxygen evolution as a MoA was assessed for the field study. Lastly, the suitability of using *F. antipyretica* as a test species (including evaluation of methods used for study design and endpoint analysis) for the studies will be assessed, as no standardised protocols for toxicity studies with this species currently exist.

Summed up, these are the objectives of the study:

- Assess effects of $CuSO_4$ and 3,5-DCP (positive controls) in *F. antipyretica* in two separate laboratorial studies.
- Assess the effect of ecologically relevant pollutants and stressors to *F. antipyretica* in a field study.
- Evaluate the suitability of using *F. antipyretica* as a study species for laboratorial and field studies.

2. Materials and methods

To assess effect of pollutants to the aquatic bryophyte *Fontinalis antipyretica*, one field study and two laboratorial exposure studies were done (Figure 4). The field study was done to assess potential toxicity of a combination of chemicals and stressors in a stream with agricultural and road runoff. Two parallel laboratorial studies were done to assess the separate toxicity of copper sulphate ($CuSO_4$) and 3,5-dichlorophenol (3,5-DCP), two toxicants with known negative effects to plants.

The field study was done in September and October 2020 in stream Skuterudbekken and Sandbekken, with endpoint analysis at the NMBU laboratory in Ås (Norway). The laboratorial studies were done in January and February 2021 at the NMBU laboratory. Endpoints of focus for all studies were effects on growth and colour, and sublethal effects including change in pigment concentration, maximal PS II efficiency, production of reactive oxygen species (ROS) and photosynthetic oxygen evolution (field study only).

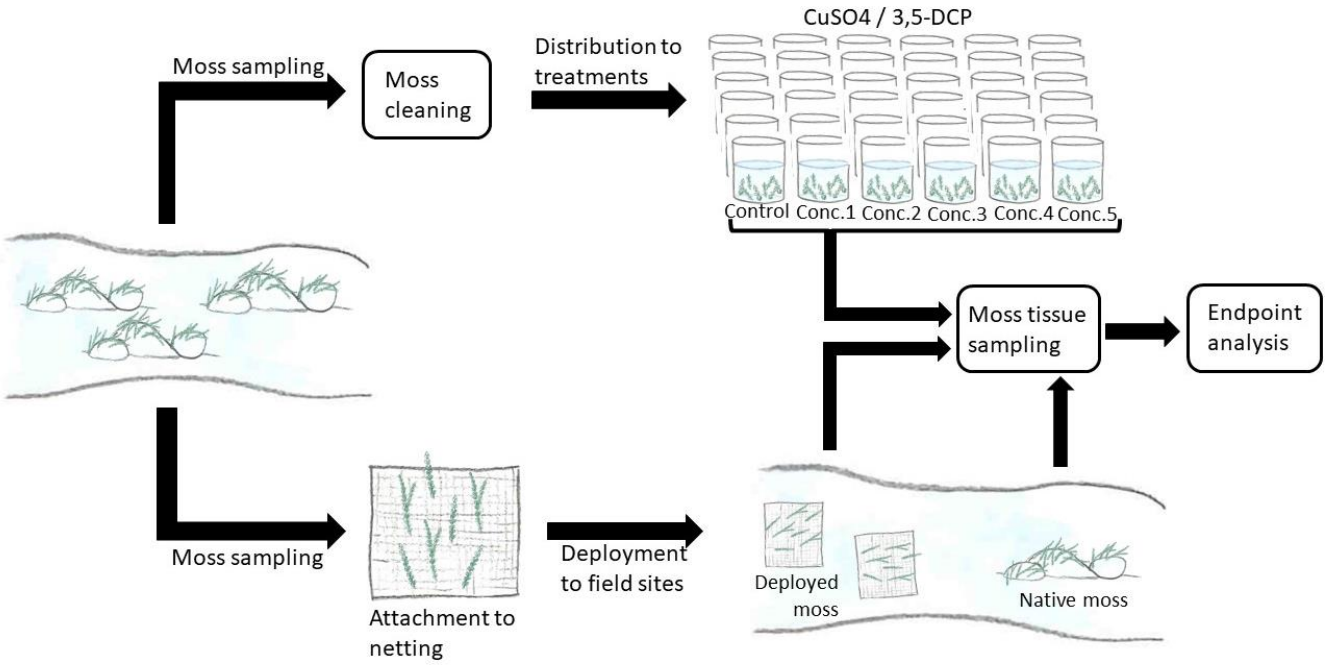

Figure 4: Summary of laboratorial and field study design using *Fontinalis antipyretica* as test organism.

2.1. Sampling and preparation of test species

Fontinalis antipyretica was sampled from Skuterudbekken in Ås municipality (Norway). Sampling for field studies were done at the field locations Skuterud 1 (Skut 1, Lat: 59.682481, Long: 10.830468, WGS1984 UTM Zone 32N) and Skuterud 2 (Skut 2, Lat: 59.685075, Long: 10.831103, WGS1984 UTM Zone 32N), while sampling for lab study was done at a location

in between (Lat: 59.694125, Long: 10.830959, WGS1984 UTM Zone 32N) (Figure 5). In-situ cleaning to remove dead tissue, macrofauna, debris and particles from moss tissue was done using stream water, before putting it in glass bottles with stream water. Bottles with moss were brought directly back to the lab in a Styrofoam box to keep it cool and dark.

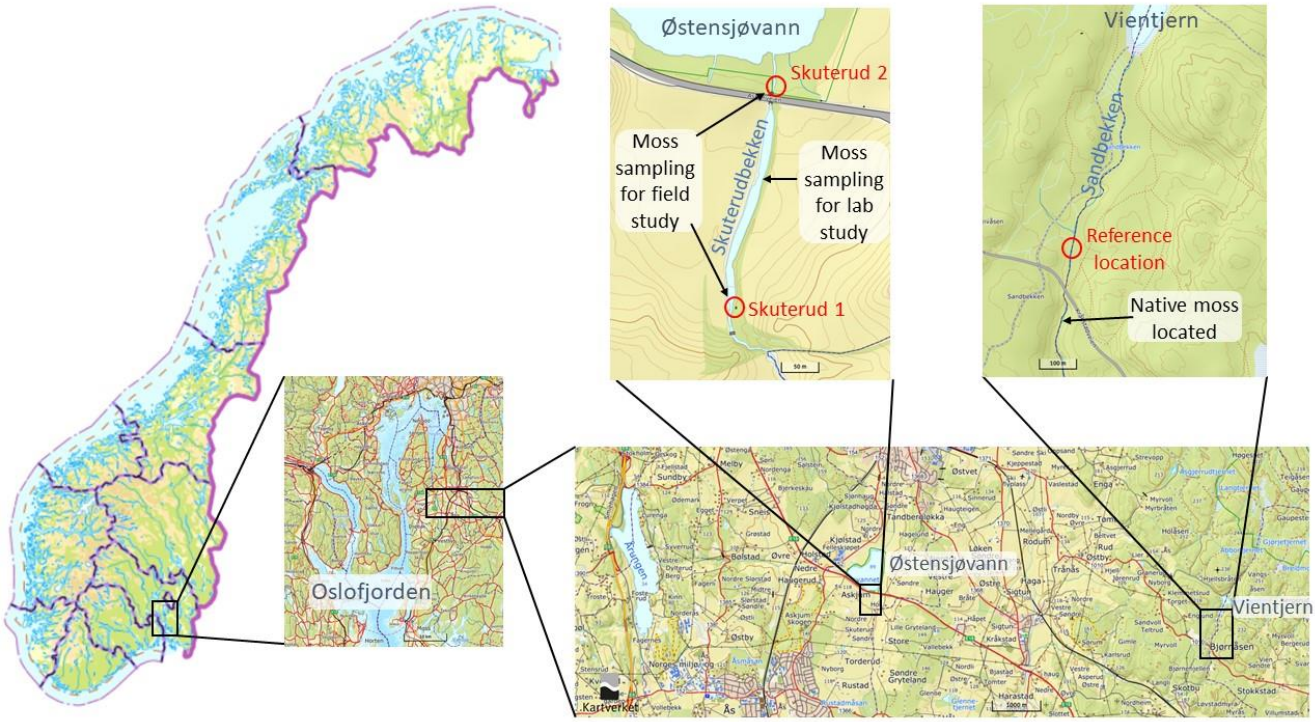

Figure 5: Location of test stream Skuterudbekken (Ås muncipality) and Reference location stream Sandbekken (Nordre Follo/Indre Østfold municipality) in Norway. The aquatic bryophyte *Fontinalis antipyretica* was sampled from Skuterudbekken and used for field and lab study. Skuterud 1, Skuterud 2 and Reference location (red) were used as field study locations. (Norgeskart.no)

2.2. Laboratorial studies

To assess the toxicity of $CuSO_4$ and 3,5-DCP, two separate and parallel laboratorial studies were done. *F. antipyretica* sampled in stream Skuterudbekken was exposed to the chemicals for up to 21 days in a growth cabinet with stable growth conditions. Moss tissue was sampled, and endpoint analysis done at day 0, 7 and 14 (n=5). Chemical and physical factors (including temperature, light intensity, and pH of control and treatment solutions) were monitored during the exposure test.

2.2.1. Preparation and acclimatisation of test species

Moss fragments were firstly rinsed to remove superficial particles, then sterilised to prevent contamination and lastly re-rinsed to remove sterilising agents. Rinsing was done three times using distilled water, with shaking for approx. 10 seconds. Sterilisation was done using sodium dichloro isocyanurate (0.0078 %), with shaking for 5 minutes.

Acclimatisation and storage were done in 1L glass bottles in a SANYO Versatile Environmental Growth Cabinet (MLR-351, SANYO Electric Co., Osaka, Japan) with stable growth conditions. Storage was done in filtered stream water (Stericap PLUS 0.22µm Millipore filter) at 10°C, light intensity of 15 µmol m^{-2} s^{-1} and day/night cycle of 14/10 hours, (as this was closer to field conditions during sampling) until start of acclimatisation. Acclimatisation was done for at least 14 days in tenfold diluted (1:10) KNOP's medium (chap. 2.2.2; Appendix I) at 15 °C, light intensity of 25 µmol m^{-2} s^{-1} and day/night cycle of 14/10 hours (same as for the exposure test). The medium was changed twice each week.

2.2.2. Experimental setup and exposure conditions

The exposure test was done by exposing *F. antipyretica* to five concentrations of one test chemical for up to 21 days. Test chemicals used were $CuSO_4$ (anhydrous, Merck KGaA, Germany) at 3, 10, 30, 100 and 300 µM and 3,5-DCP (Purity 97%, Sigma-Aldrich, USA) at 0.1, 0.3, 1, 3 and 9 mg/L. Tenfold (1:10) dilution of KNOP's medium was used as the control solution and for mixing test treatments, containing macronutrients (nitrogen, phosphorous, potassium, calcium, sulphur and magnesium) (Appendix I). The original working solution of all treatments were adjusted to pH ~7 using NaOH and HCl, before usage at day 0, 7 and 14. 1,5 – 2 cm shoots were put into 50 mL of a test treatment or control solution (n=5). Dimethyl sulfoxide (DMSO; Purity 99.7%, Sigma-Aldrich, USA) was added to all 3,5-DCP treatments to solve the chemical, and an additional solvent control solution with medium and solvent was used for this test. All control and treatment solutions were changed once a week, at the day of moss sampling.

A subgroup of non-exposed moss was sampled and analysed at the day of starting the test (day 0). Sampling and analysis were additionally done at day 1, 7, 14 and 21. All endpoint analyses were done according to chap. 2.4, within two days post sampling. Shoots used for growth analysis were marked using a white thread. Shoots for other analyses were randomly chosen. Total number of shoots needed for the Cu and DCP tests are presented in Appendix II.

Exposure tests were done in SANYO Versatile Environmental Growth Cabinet with temperature of 15 °C, light intensity of approx. 25 µmol m^{-2} s^{-1} (sensor placed horizontally in the middle of the cabinet) and day/night cycle of 14/10 hours. Light source used was cool-white, fluorescent tubes (36 W).

2.2.3. Monitoring of chemical and physical parameters

Light and temperature was controlled in the start and end of the exposure period, using Traceable Refrigerator/Freezer Plus Thermometer (Avantor delivered by VWR, Radnor, PA, USA) (accuracy ± 0.5°C) and LI-250A light meter (LI-COR Biosciences UK Ltd, Cambridge, United Kingdom).

pH of each treatment concentration was measured (n=3) using WTW inoLab 720 pH meter (Avantor delivered by VWR, Radnor, PA, USA), calibrated within the same day. This was done for the start (*On*, before and after pH regulation) and end (*Off*) of solution batch 1 (day 0-7), 2 (day 7-14) and 3 (day 14-21), and additionally before and after adding the moss for batch 1.

2.3. Field study

To assess potential toxicity of stressors and chemicals present in environmentally relevant concentrations, a field study was done using test stream Skuterudbekken (Skut 1 and Skut 2) and reference stream Sandbekken (Ref. loc., Lat: 59.680178, Long: 10.953904, WGS1984 UTM Zone 32N) (Figure 5). *Fontinalis antipyretica* was found growing in both streams. Active and passive biomonitoring was used, including analysis of deployed moss on nets (active), and native moss growing on rocks (passive). The test species was sampled from Skuterudbekken and deployed back into Skuterudbekken and Sandbekken for up to 14 days of exposure (Figure

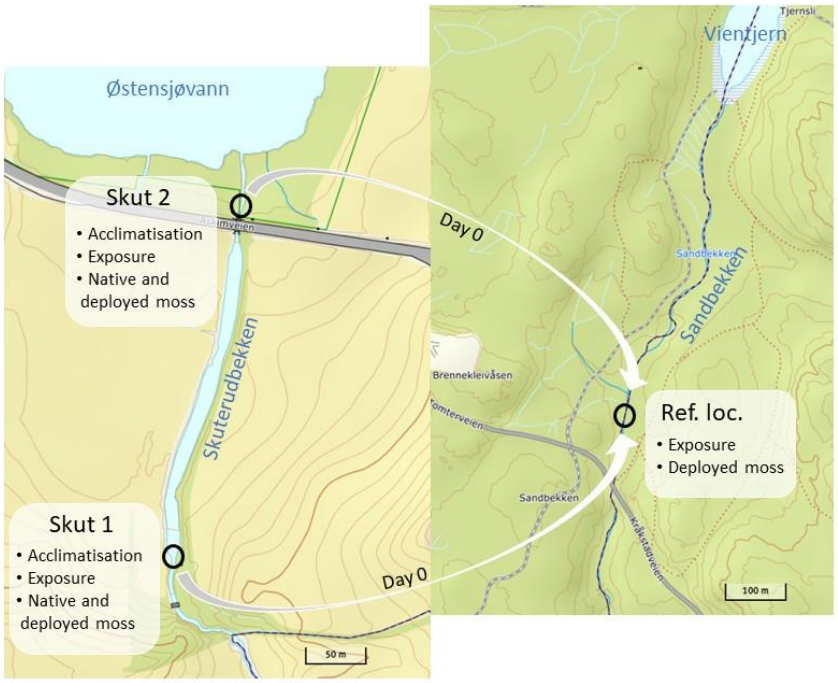

Figure 6: Design of field study using *Fontinalis antipyretica* from stream Skuterudbekken (Skut 1 and Skut 2). Deployed *F. antipyretica* was acclimatised in Skuterudbekken for 7 days and half of it moved to Sandbekken (Ref. loc.) for up to 14 days exposure. Native *F. antipyretica* was additionally analysed. (Norgeskart.no, n.d.)

6). Additionally, native moss in Skuterudbekken was assessed. Deployed and native moss tissue was sampled, and endpoint analysis done at day 0, 7 and 14 (n=6). Chemical and physical factors (including temperature, light intensity, conductivity, pH, stream size, velocity, water discharge, concentration of phosphorous, nitrogen, carbon, elements, and pesticides) of stream sites were monitored during the exposure test.

2.3.1. Field locations

Stream Skuterudbekken, located in Ås municipality (Norway), was used as the test stream. The Skuterud catchment is included in the Norwegian Agricultural Environmental Monitoring Program (JOVA)(Nibio.no/jova) and consists of about 60 % agricultural area dominated by cereal cropping. The agricultural practises of the area include use of pesticides and fertilizers. Analysis of the water quality through JOVA has been recorded since 1992, with pesticides added to the analysis from 1995. In addition to agricultural runoff, the catchment area includes runoff from woodlands and urban areas (Appendix III)(Nevina.nve.no). The European route 18 (E18) crosses Skuterudbekken approximately 60 meters before it flows into the Østensjøvannet lake. The last 300 m upstream of E18 includes a constructed wetland area with one sedimentation pond and two vegetation covered ponds. Skut 1 and Skut 2 were used for the field study (Figure 6). Skut 1 is located directly upstream of the ponds by one of the JOVA water sampling station. Skut 2 is located directly downstream of E18 and the sedimentations ponds, by another JOVA water sampling station.

Stream Sandbekken, located at the municipal boundary between Nordre Follo and Indre Østfold (Norway), was used as the reference location (Ref. loc.). The stream originates from lake Vientjern and is located approx. 7 km east of Skuterudbekken (Norgeskart.no, n.d.) (Figure 6). It's catchment area is dominated by woodlands (Appendix III) (Nevina.nve.no).

2.3.2. Preparation and acclimatisation of test species

At the lab, *F. antipyretica* fragments were attached to nylon nets (approx. 30 x 25 cm, mesh size 16 mm^2) using cotton thread. Each moss fragment included 1-5 shoots and was sewed onto the net approx. 1 cm from the fragment base (Figure 7), with a few centimetres between each fragment. Fragments were placed in the same direction to allow water flow over the moss. Totally 8 nets were used, with 11 to 13 moss fragments (including ≥20 shoots) on each. Total number of shoots needed for the test is presented in Appendix II. Skut 1 nets were marked with a red thread and Skut 2 nets with a white thread. Shoots for growth effects analysis was marked using a white and yellow thread. Nets with moss were put in buckets with river water and

transported back to their originating stream within 24 hours after sampling, for 11 days of acclimatization.

2.3.3. Experimental setup

Moss nettings were attached to the stream bed using 2-3 rocks on each side of the netting, to let stream flow between the rocks and over the moss (Figure 7). Nettings were placed close to native populations where water level was high enough to cover the moss even in dry periods (~10-20 cm above moss nettings). Placement in the middle of the stream was ideal, however at Skut 1 and Skut 2 the high stream velocity or depth did not allow this, and nettings were placed in stream edge zones (~20-30 cm from stream banks).

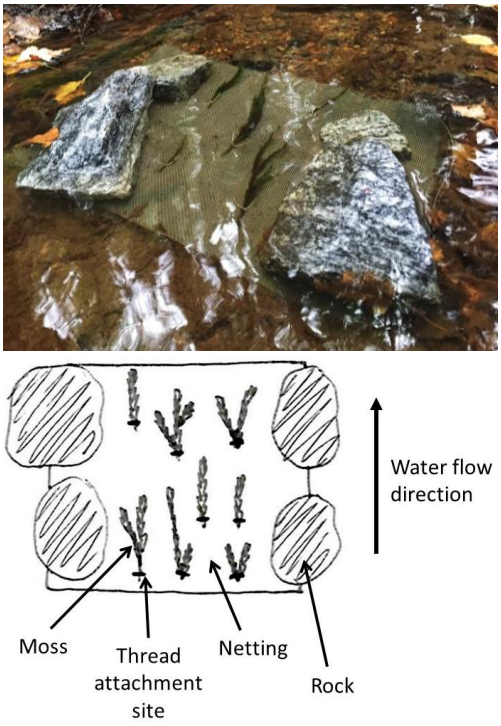

Figure 7: Manner of attaching *Fontinalis antipyretica* to netting and attachment of netting in streams. Photo and illustration: Lina Agneberg Dahl, 2020.

At day 0 of the exposure study, two nettings from each stream were randomly chosen (Dean, 2013) and translocated to Ref. loc. (Figure 6). Transportation (~25 min) was done in separate plastic buckets with stream water.

Six shoots were randomly sampled (Dean, 2013) from both nettings in Skut 1 (day 0, 7, 14), Skut 2 (day 0, 14) and Ref. loc. (day 7, 14). Native moss was sampled from Skut 1 and Skut 2 (day 0, 7, 14). At least 2,5 cm long shoots were sampled (cut using scissors) and directly put into 50ml tubes containing river water. Tubes were stored cool and dark in a Styrofoam box during transport to the lab. Endpoint analyses were done according to chap. 2.4, within two days post sampling. ROS-production was measured on day 14 only, approximately 4-6 hours post sampling, and analysis additionally included one control group (moss grown in KNOP's medium at the lab).

2.3.4. Monitoring of chemical and physical parameters (exposure conditions)

Temperature and light intensity were continually monitored using HOBO Pendant Temperature/Light Data Logger (8/16K) (Onset Computer Corporation, Bourne, MA, USA) and HOBOware software v3.7.21. Two loggers were used at each location, attached using strips downstream of a rock placed on the streambed or a pole hammered into the substrate (Figure

8). Loggers were located close to and at the same depth as deployed moss, positioned horizontally with sensors pointing upward and stabilised with rubber bands. Measurements were done every 10 minutes (accuracy ± 0.43°C at 0°-50°C).

Additionally, temperature (n=1) was measured once a week at the time of moss sampling, using Traceable Refrigerator/Freezer Plus Thermometer (Avantor delivered by VWR, Radnor, PA, USA). The thermometer sensor was left in the stream for at least one hour before registering temperature (accuracy ± 0.5°C).

The light regime at each location was estimated approx. two weeks after the exposure test, to see the difference in sun movement and shading during the exposure period. This was done by taking a horizontal fish-eye picture using Pentax K-5II SLR camera with a Sigma 4.5 mm circular fisheye lens. Pictures were analysed using Hemisfer software (Schleppi & WSL, Zürich, Switzerland).

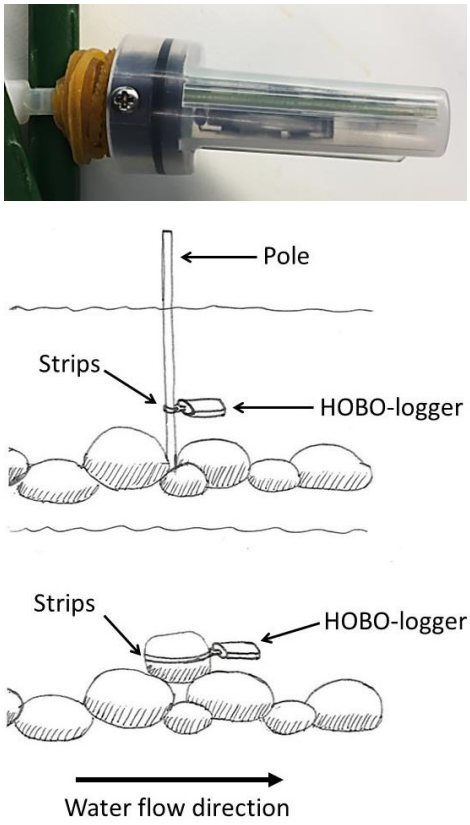

Figure 8: Manner of attaching HOBO Pendant Temperature/Light Data Loggers onto pole or rock in streams Photo and illustration: Lina Agneberg Dahl, 2020.

Conductivity and pH were measured once a week at the time of moss sampling (n=1). Conductivity was measured in situ (directly in stream) at each location using Portable Conductivity Meter (SG3 - SevenGo, Mettler Toledo, Zürich, Switzerland). Water samples were taken and transported to the lab in a Styrofoam box to keep it cool and dark, and pH measured at the lab (n=1) using WTW inoLab 720 pH meter (Avantor delivered by VWR, Radnor, PA, USA), calibrated within the same day.

The approximate width and depth of stream locations were registered at the time of moss sampling (n=1). Stream velocity (m/s) of each location was measured at day 14, by registering the time of a floating device (leaf or small branch) to float 6 m at each location (n=1). Water discharge (Q, m^3/s) was calculated by multiplying stream velocity, width, and depth of the stream location.

Water samples were taken to assess chemical aspects of stream sites, transported to the lab in a Styrofoam box to keep it cool and dark. Concentration of phosphorous (tot-P), nitrogen (tot-N),

carbon (dissolved (DOC) and total content (TOC)) was measured at all locations at day 7 (n=1) and 14 (n=3). Sampling was done in 50 ml plastic bottles. Samples were immediately filtrated (0.45µm) at the lab, stored cool and dark, and analysis done according to Norwegian Standard (NS-EN ISO 6878) at NMBU Faculty of Environmental Sciences and Natural Resource Management. TOC and DOC analysis were done within two days, using TOC-V CPN instrument with ASI-V autosampler (Shimadzu, Tokyo, Japan). tot-P and tot-N samples were added an oxidation agent and autoclaved (121 °C, 30 min) for stabilisation before storage. Tot-N was analysed using Flow Injection Analysis (FIA) and tot-P was spectrophotometrically analysed (Hitachi UH5300).

Concentration of pesticides and elements was measured at all locations at day 0 and 14 (n=1). Sampling was done in 1 L amber glass bottles (pesticides) and 250 ml plastic bottles (glyphosate and elements). Analyses were done at NIBIO (accredited by ISO 17025) Section for Pesticides and Natural Chemistry (pesticides), and Section of Biogeochemistry and Soil Quality (elements). Glass bottle samples were conserved using 2% methanol, stored dark at 4°C, and decanted and filtrated prior to analysis. Analysis was done according to NIBIOs method M101-LC, covering a wide range of pesticides and some degradation products (Appendix IV). Plastic bottle samples for pesticide analysis were stored at -18°C. Analysis was done according to NIBIOs method M59 for the herbicide glyphosate and its major metabolite AMPA. Plastic bottle samples for elemental analysis were conserved using hydrochloric acid (0.084 M HCl) and stored dark at 4°C. Analysis of Al, As, B, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, Pb, S, Se, Si, and Zn were done using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).

2.4. Endpoint analysis

2.4.1. Growth and colour analysis

Growth was measured as change in length of the shoots during the full exposure period, as described by Aronsson and Ekelund (2006), with modifications. A picture of the shoots put on a white and clean background with a measuring scale was taken at the start and end of the exposure test. This was done within approx. 30 minutes and shoots kept moist during the process. For field study, individual shoots were recognised by their location on each net. For lab study, individual shoots were sorted by treatment group and replicate. Length of each shoot was measured to the nearest 0.1 mm using ImageJ software (v.1.53d, Wayne Rasband and Contributors, National Institutes of Health), by calculating the average of 3 measurements per shoot.

Colour analysis was done by measuring amount of red, green, and blue in a picture of 1 cm shoots, using "RGB measure" plugin in Image J software (v.1.53d, Wayne Rasband and Contributors, National Institutes of Health). Greenness index (GI) was calculated using Equation 1, normalized by the white background of shoots (based on PSI Photon Systems Instruments (2018)).

$$100 \times \frac{(G_{moss}/G_{background})}{(R_{moss}/R_{background})} \qquad \textit{(Equation 1)}$$

Where G is the amount of green and R is amount of red, for moss shoots and background.

2.4.2. Maximal PS II efficiency (Fv/Fm)

Maximal photosystem II (PS II) efficiency (Fv/Fm, chlorophyll fluorescence) was assessed as described by Murchie and Lawson (2013) and Rau et al. (2007), with minor modifications. Shoots were briefly rinsed, cut to 1 cm and placed in the dark for 20 minutes to allow all PS II photoreaction centres to open. Shoots were placed in the red LED Imaging-PAM M-series chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) and measurements recorded in ImagingWin software v2.56p. Area of Interest (AOI) were marked over the whole 1 cm shoot, including fronds and stem. Maximum PSII quantum yield was directly recorded by the fluorometer as Fv/Fm. Fm is the maximum fluorescence (when photoreaction centres are closed post a saturating pulse of 5000 µmol m^{-2} s^{-1} for 0.8 seconds) and Fv equals Fm - F_o where F_o is the minimum fluorescence (when photoreaction centres are closed under the weak light of 1 µmol m^{-2} s^{-1}). PS II inhibition percentage was calculated by Equation 2 (modified from OECD (2006)).

$$\%R = \frac{(MeanC - X)}{MeanC} \times 100 \qquad (\textit{Equation 2})$$

Where %R is the inhibition percentage, MeanC is the mean of all replicates from control group and X is each Fv/Fm measurement value.

2.4.3. Pigment concentration

Pigment concentration was assessed spectrophotometrically, as described by Wellburn (1994). 1 cm shoots were carefully dried by blotting on tissue paper (field study) or using Eppendorf 5417C Centrifuge (Marshall Scientific, Hampton, NH, USA) at 5000 rpm for 1 minute (lab study), before weighting. Fresh weight (FW) was measured using XP6 Automated-S microbalance *XP6* (Mettler Toledo, Zürich, Switzerland). Pigments were extracted using

dimethyl sulfoxide (DMSO) solvent saturated with $Mg(CO_3)_2$ in a VWR ultrasonic cleaner at 60°C for 25 minutes.

The solvent was centrifuged using Eppendorf 5417C Centrifuge (Marshall Scientific, Hampton, NH, USA) at 15000 rpm for 5 minutes to remove noise from moss tissue particles. Absorbance of the supernatant was measured at 480, 649, 665 and 750 nm using UV-1800 Spectrophotometer (Shimadzu, Tokyo, Japan). The 750 nm measurement was subtracted from the other measurements to remove remaining noise.

Concentration of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids (Car) was calculated using equation 3-5 (Wellburn, 1994).

$$\text{Chl } a = 12.19 \times A_{665} - 3.45 \times A_{649} \quad (\textit{Equation 3})$$

$$\text{Chl } b = 21.99 \times A_{649} - 5.32 \times A_{665} \quad (\textit{Equation 4})$$

$$\text{Car} = (1000 \times A_{480} - 2.14 \times \text{Chl } a - 70.16 \times \text{Chl } b)/220 \quad (\textit{Equation 5})$$

Where A_x is the measured absorbance at each wavelength (x). Pigment concentration per weight in each moss shoot was calculated by equation 6.

$$RC_x = (C_x \times \text{Vol DMSO}) \div \text{weight} \quad (\textit{Equation 6})$$

Where RC_x is the pigment concentration by weight, C_x is the measured pigment concentration, Vol DMSO is the volume of DMSO added and weight is the weight of each moss sample.

2.4.4. Production of reactive oxygen species (ROS)

Production of reactive oxygen species (ROS; H_2O_2, O_2^- and $^1O_2$) was assessed as described by Razinger et al. (2010), with modifications. 3-4 mm from the outermost 1 cm shoot (including 3-5 fronds) were put into a black Costar 96-well polystyrene microplate with clear bottom (Corning Incorporated, USA) with 200 µL of culture medium and fluorescent ROS probe 2´7´-Dichlorofluorescein diacetate (H_2DCFDA, 100 µM). ROS fluorescence signal was measured using fluorescence plate reader (type 374, Thermo Electron corporations Fluoroskan Ascent, Labsystems, Helsinki, Finland) after 1 h staining, with excitation wavelength 485 nm and emission wavelength 538 nm. ROS signal was calculated using equation 7.

$$FR = FS - \text{blank} \quad (\textit{Equation 7})$$

Where FR is the ROS signal value, FS is the measured value from each well and blank is the mean value of detected signal from well continuing only medium and probe. Moss tissue was

carefully dried by blotting on tissue paper (field study) or using Eppendorf 5417C Centrifuge (Marshall Scientific, Hampton, NH, USA) at 5000 rpm for 1 minute (lab study), before weighting. Fresh weight (FW) was measured using XP6 Automated-S microbalance *XP6* (Mettler Toledo, Zürich, Switzerland). ROS signal was normalised by weight and ROS formation fold change (lab study) was calculated using equation 8.

$$\mathrm{FC} = \mathrm{Vt}/\mathrm{Vc} \qquad (\textit{Equation 8})$$

Where FC is fold change, Vt is value for each treatment and Vt is the mean value for the control.

2.4.5. Photosynthetic oxygen evolution

Photosynthetic oxygen evolution was assessed for the field study as described by Aronsson and Ekelund (2006), with modifications. The test was done approx. one day post sampling (moss was stored dark overnight in distilled water at 15°C). OxyLab+ Control Unit and electrode chamber (Hansatech Instruments Ltd, King's Lynn, United Kingdom) was used for measuring oxygen production, after calibration using the OxyLab software v.1.15 (Hansatech Instruments Ltd, King's Lynn, United Kingdom). Zero oxygen was set using sodiumdithionite and oxygen equilibrium between air and water was set using distilled water. A small piece of plastic was placed above the electrode and magnet stirrer in the test tube to prevent shoots from touching the magnet. 2 mL distilled water (day 0, 7) or medium (day 14) was used and 20-50 µL $NaHCO_3$ added as source of CO_2. Three shoots (1cm) from the same location were measured at once to ensure enough tissue for a visible effect. Shoots were irradiated (PAR approx. 220 µmol m^{-2} s^{-1} (Hansatech LS2 halogen lamp) during the test. Rate of oxygen production (µmol ml^{-1} min^{-1}) was directly registered when stabilised (after 3-10 minutes) and oxygen production by weight per hour (µmol gFW^{-1} h^{-1}) for each group was calculated using equation 9.

$$\mathrm{Ox\ prod} = \frac{(\mathrm{Ox_R} \times \mathrm{added\ liquid})}{(1\mathrm{h}/60\mathrm{sec}) \times \mathrm{weight}} \qquad (\textit{Equation 9})$$

Where Ox prod is oxygen production by weight per hour, $\mathrm{Ox_R}$ is registered rate of oxygen production, added liquid is amount of distilled water/medium used and weight is total weight of shoots used.

2.5. Data analysis

Data calculations were performed using Excel 2016 (Microsoft Office, Redmond, WA, USA). Graphical treatments and statistical analysis were performed using software Jamovi v.1.6.23 (Jamovi Software, Sydney, Australia), R v4.1.0 and scripting in RStudio v1.4.1106 (R Foundation for Statistical Computing, Vienna, Austria). Assumption checks for normality was

done using Shapiro-Wilk test ($p>0.05$) and for homogeneity of variance using Levene's test ($p<0.05$). The optimal exposure time for obtaining a high-quality concentration response curve (CRC) was identified due to low variance, sigmoidal CRC ranging from 0-100 % and responsive at realistic concentrations. Statistically significant difference between time and treatment groups was assessed using One-Way ANOVA ($p<0.05$). For parametric data, Welch's with Tukey (for equal variances) or Games-Howell (for unequal variances) and Post-Hoc tests were used. For non-parametric data, Kruskal-Wallis with Wilcoxon pairwise comparison was used. The concentration needed for an effect in 50 % of the population (EC_{50}), concentration needed for no observed effect (NOEC) and concentration needed for the lowest observed effect (LOEC) was derived for each of the chemicals (lab study). Overview of software used for statistical tests, statistical assumption check results, transformations used, type of ANOVA used and p-value for each data set is given in Appendix V.

3. Results

In the following subsections, results from the laboratorial and field studies are presented, with reference to the endpoints growth and colour change, maximal PSII efficiency, pigment concentration, and production of reactive oxygen species. Raw data from laboratorial and field study are given in Appendix VI.

3.1. Laboratorial study – toxicity of copper

Results from the 21 days exposure study to copper are presented below.

Contamination by algae (and possibly bacteria or fungi) was observed in control and slightly in the 3 µM $CuSO_4$ treatment, gradually increasing from day 7 and throughout the exposure period, growing around moss shoots or in the treatment surface. Increased pH was measured in control *Off* batches at day 14 and 21, with increase from ~ 7.0 in the start (*On*) to 8.6 and 8.3 in the end (*Off*) of batch 2 and 3 respectively. (ref. Appendix VIII for details). Reduced greenness index, maximal PS II efficiency, pigment concentration and increased production of reactive oxygen species was observed for the control shoots during the exposure period (see Appendix VII for details). Details of statistical results are presented in Appendix XI.

3.1.1. Growth and colour change

There was no concentration-dependent response for inhibition of growth, and variation was high. Highest inhibition was observed for 10 µM (92 ± 94 %), and lowest for 3 µM (-227 ± 298 %) (Figure 9 b). No significant difference was observed between treatment groups and control.

Secondary shoots were formed for a few shoots, where multiple shoots were observed to be longer at day 21 compared to day 0 (Figure 9 a).

Colour change, quantified as greenness index (GI) percentage inhibition, was observed for all treatments and control during the exposure study, with a clear concentration-dependent response after 21 days exposure (Figure 9 c). Highest GI inhibition was observed for 300 µM (23.2 ± 3.42 %) and lowest for 3 µM (-10.4 ± 8.96 %). GI was significantly lower for 3 µM and significantly higher for 10 - 300 µM compared to control. NOEC was <3 µM and LOEC was 3 µM.

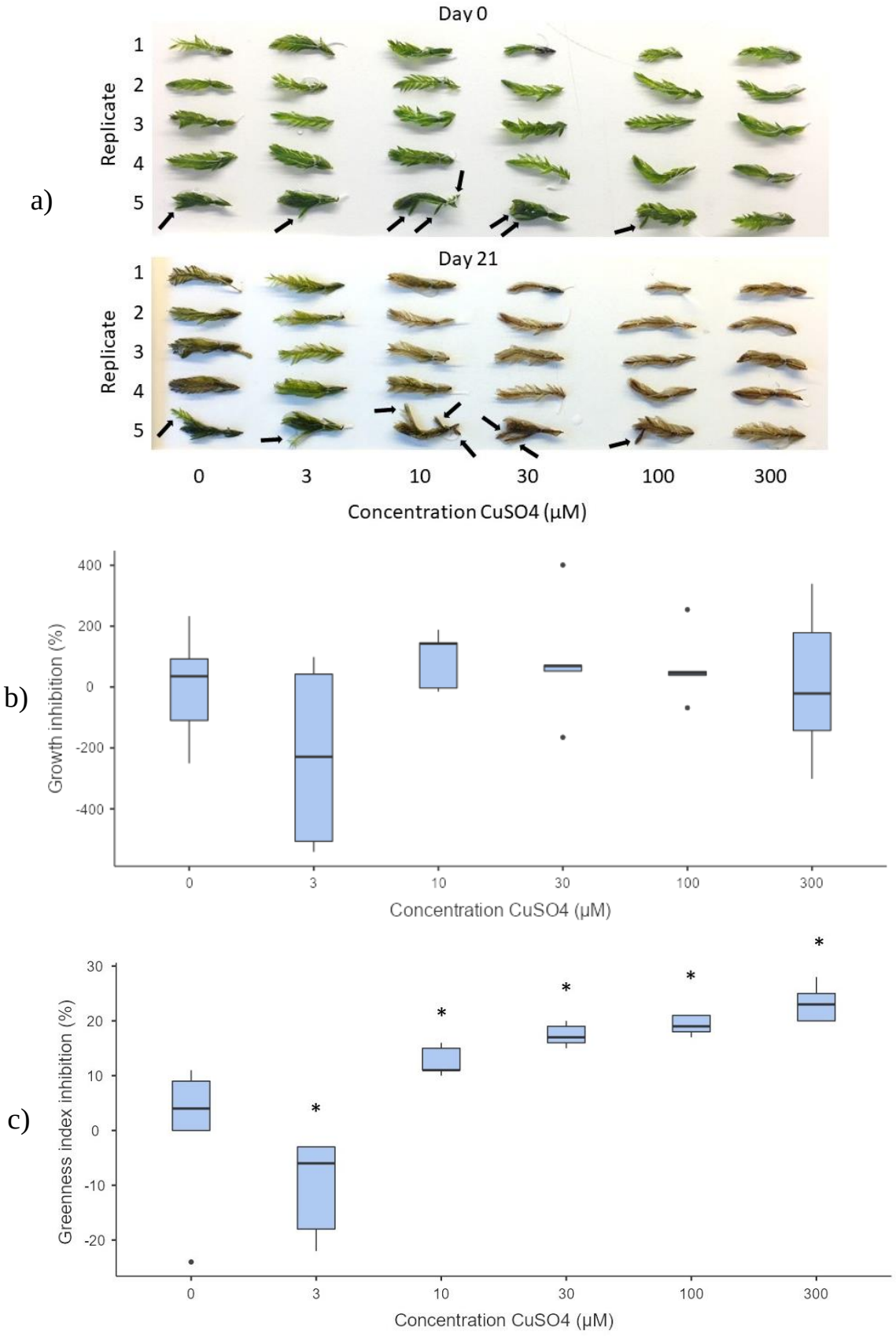

Figure 9: *Fontinalis antipyretica* a) shoots (black arrows indicate secondary shoots); b) inhibition of growth; and c) inhibition of greenness index (asterisks marks statistically significant difference to control (0 µM; * $p<0.05$)) after 21 days exposure to $CuSO_4$

3.1.2. Maximal PSII efficiency (Fv/Fm)

There was a clear concentration and time-dependent response for inhibition of Fv/Fm from day 1 and throughout the exposure period (Figure 10). Highest inhibition was observed for 100-300 µM at day 7-21 (100 %), and lowest for 3-10 µM at day 14 (close to -100 %). Promotion was observed for the single or two lowest concentrations at day 7-21. Fv/Fm inhibition was significantly higher in 100-300 µM (day 1-21) and 30 µM (day 7-14) compared to control. Fv/Fm inhibition was significantly higher in 100-300 µM compared to control at day 1-21, and 30 µM compared to control at day 7-14. Additionally, inhibition was significantly lower in 3 µM (day 7) and 3-10 µM (day 14) compared to control. The CRC for day 7 was most optimal, with NOEC at 10 µM, LOEC at 30 µM and EC_{50} at 28.1 ± 9.5 µM.

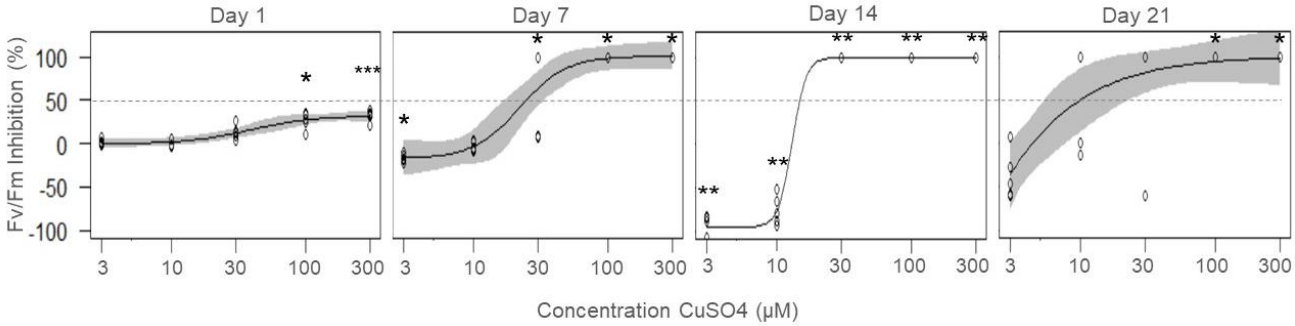

Figure 10: Inhibition of maximal PS II efficiency (Fv/Fm) in *Fontinalis antipyretica* after 1-21 days of exposure to $CuSO_4$. Asterisks marks statistically significant difference to control (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Broken line marks the concentration causing 50 % inhibition.

3.1.3. Pigment concentration

Chlorophyll *a* and chlorophyll *b*

There was a clear concentration and time-dependent response for inhibition of Chl *a* and Chl *b* from day 7 and throughout the exposure period, with similar responses for the two pigments but slightly higher inhibition of Chl *a* compared to Chl *b* (Figure 11). Highest inhibition was observed for 100-300 µM at day 21 (~95 %) for Chl *a* and for 100-300 µM at day 14 (~77 %) for Chl *b*. Lowest inhibition for both pigment was observed for 3 µM at day 14 (-15 % for Chl *a* and -16 % for Chl *b*). At day 1, Chl *a* inhibition was slightly increased for 300 µM, however no significant difference compared to control was detected. At day 7-21, inhibition was significantly higher in 30-300 µM compared to control for both Chl *a* and Chl *b*, and additionally in 10 µM compared to control for Chl *a* at day 7-21 and for Chl *b* at day 21 in. Promotion was observed for 3 µM at day 14-21 and sporadically for multiple concentrations at day 1, for both pigments. The CRC for day 7 was most optimal for both pigments, with NOEC

at 3 µM, LOEC at 10 µM for both pigments and EC_{50} at 13.9 ± 1.9 µM for Chl *a* and 30.4 ± 7.9 µM for Chl *b*.

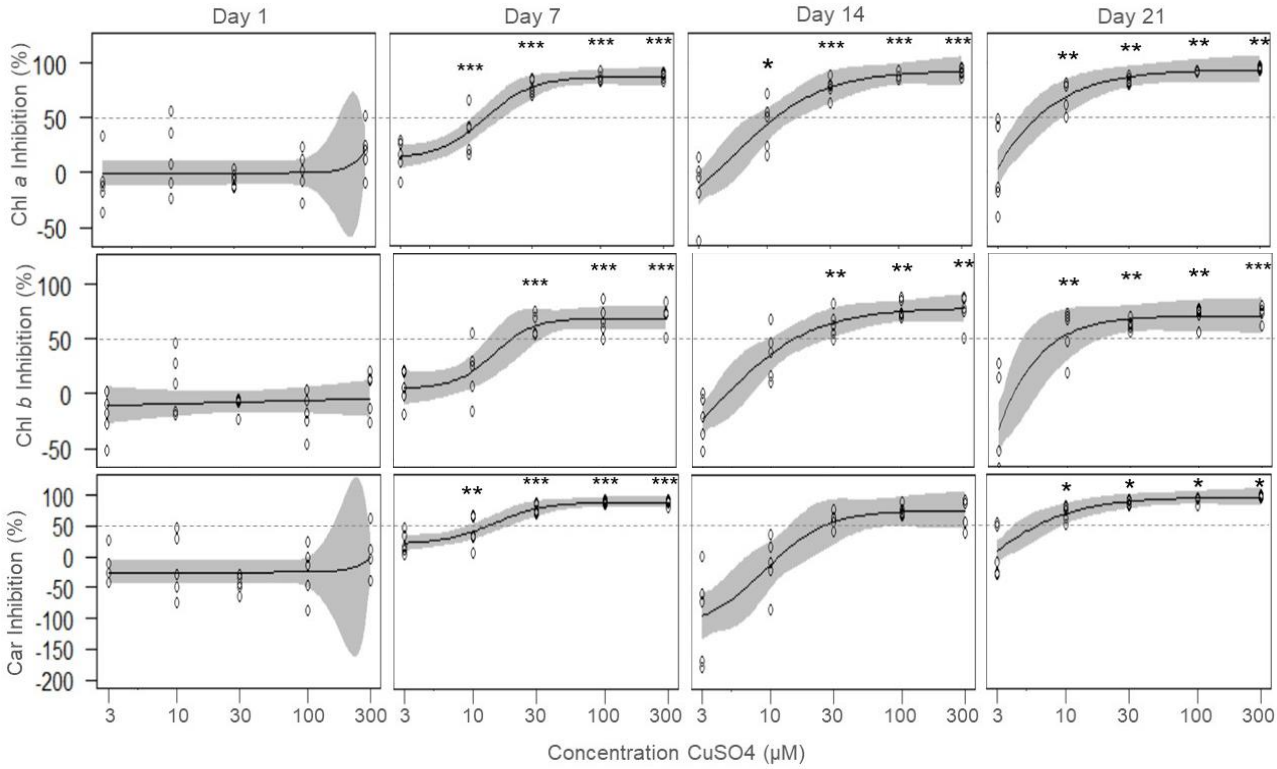

Figure 11: Inhibition of concentration of pigments Chlorophyll (Chl) *a*, Chl *b*, and Carotenoids (Car) in *Fontinalis antipyretica* after 1-21 days of exposure to $CuSO_4$. Asterisks marks statistically significant difference to control (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Broken line marks the concentration causing 50 % inhibition.

Carotenoids

There was a clear concentration and time-dependent response for inhibition of Car from day 7 and throughout the exposure period (Figure 11). Highest inhibition was observed for 300 µM at day 21 (100 %) and lowest for 3 µM at day 14 (-95 %). At day 1, the inhibition was slightly increased for 300 µM, however no significant difference compared to control was detected. At day 7 and 21 the inhibition was significantly higher for 10-300 µM compared to control. At day 14 no statistical difference was observed due to high standard deviation. Promotion was observed for 3 µM at day 14 and sporadically for multiple concentrations at day 1. The CRC for day 7 was most optimal, with NOEC at < 3 µM, NOEC at 3 µM and EC_{50} at 11.7 ± 2.0 µM.

Chlorophyll *a*/*b* ratio

There was a clear concentration and time-dependent response for increased Chl *a*/*b* relative change from day 7 and throughout the exposure period (Figure 12). Highest relative change

was observed for 300 µM at day 21 (82 %) and lowest change for 3 µM (day 1-14) and 3-30 µM (day 1) (0 %). At day 1, the relative change was slightly increased for 100 - 300 µM, however no significant difference compared to control was detected. At day 7 – 21, the relative change was significantly higher in 30-300 µM compared to control. Additionally, significantly higher relative change was observed for 10 µM compared to control at day 7, and for 3-10 µM compared to control at day 21. The CRC for day 14 was most optimal, with NOEC at 10 µM, LOEC at 30 µM and EC_{50} at 104.2 ± 18.7 µM (See Appendix IX for Chl *a/b* ratio plots).

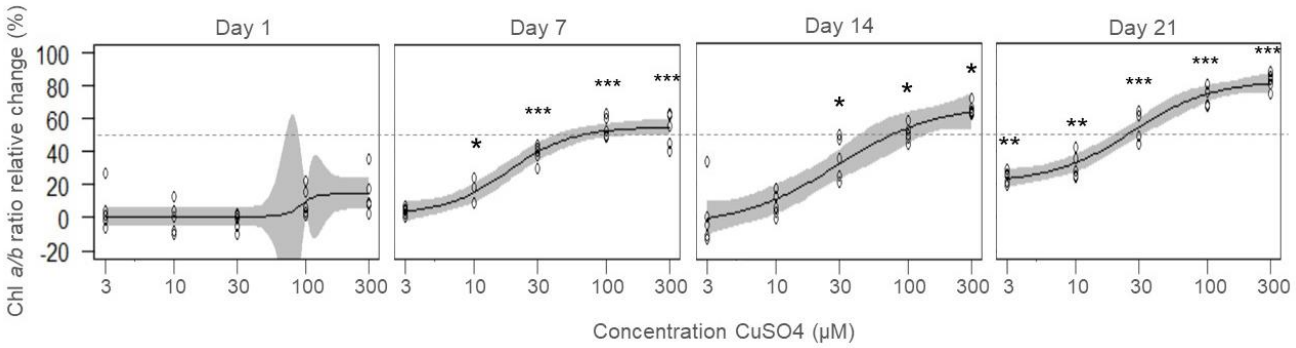

Figure 12: Relative change of Chlorophyll *a/b* ratio in *Fontinalis antipyretica* after 1-21 days of exposure to $CuSO_4$. Asterisks marks statistically significant difference to control (* p<0.05, ** p<0.01, *** p<0.001). Broken line marks the concentration causing 50 % inhibition.

3.1.4. Reactive oxygen species

There was a concentration and time-dependent response for increased ROS formation from day 7 and throughout the exposure period (Figure 13). Highest fold increase compared to control was observed for 300 µM at day 1, 30-300 µM at day 7 and 10-30 µM at day 14 (~3-3.5), and lowest for 3 µM at day 1-21 (~1). At day 1, there was a concentration-dependent response, however no significant difference compared to control was detected. ROS formation fold increase was significantly higher for 10-300 µM compared to control at day 7, and additionally for 10-30 µM compared to control at day 14, however with high variation. At day 14 and 21,

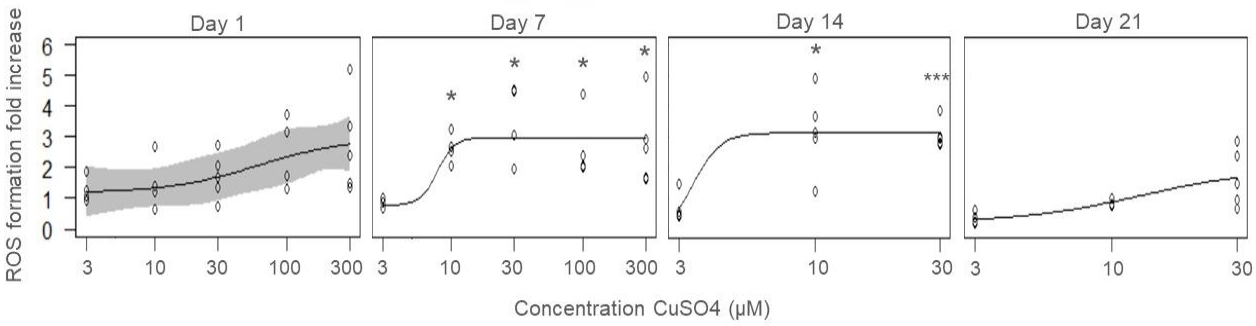

Figure 13: Fold increase of reactive oxygen species (ROS) production in *Fontinalis antipyretica* after 1-21 days of exposure to $CuSO_4$. Asterisks marks statistically significant difference to control (* p<0.05, ** p<0.001, *** p<0.001).

ROS production was reduced (and highly unstable) for 100 and 300 µM. For this reason, the concentration-response model was made for 3-30 µM only for these days. The CRC for day 7 was most optimal, with NOEC at 3 µM and LOEC at 10 µM.

3.2. Laboratorial study – toxicity of 3,5-dichlorophenol (3,5-DCP)

Results from the 21 days exposure study to 3,5-DCP are presented below.

Contamination by algae (and possibly bacteria or fungi) was observed in medium and solvent control and the three lowest 3,5-DCP concentration (0.1-1 mg/L) from day 7 and throughout the exposure period. The contamination was gradually more visible towards the end of the exposure period, mostly growing around moss shoots or in the treatment surface. pH measurements of treatments indicated increased pH in controls and 0 - 1 mg/L treatments *Off* batches at day 14 and 21, with increase from ~ 7.0 in the start (*On*) to ranging between 8.2 and 9.1 for 0 - 1 mg/L treatments in the end (*Off*) of batch 2 and 3 respectively (details are found in Appendix VIII). Reduced greenness index, maximal PS II efficiency, pigment concentration and increased production of reactive oxygen species was observed for the control shoots during the exposure period (see Appendix VII for details). Details of statistical results are presented in Appendix XI.

3.2.1. Growth and colour change

There was a concentration-dependent response for inhibition of growth. Growth inhibition was observed for all controls and treatment groups, with lowest inhibition in medium control (0 ± 52 %) and highest in the 9 mg/L treatment (276 ± 131 %) (Figure 14 b). Shoots in 0.3, 3 and 9 mg/L were significantly shorter than in medium control.

Secondary shoots were formed for a few shoots, where a few shoots were observed to be longer at day 21 compared to day 0 (Figure 14 a).

Colour change, quantified as greenness index (GI) percentage inhibition, was observed for all treatments and control during the exposure study, with a concentration-dependent response for the highest concentrations only (Figure 14 c). Highest inhibition was observed for 9 mg/L (16.6 ± 4.8 %), and lowest for 3 mg/L (-6.7 ± 3.8 %). Additionally, promotion was observed for 0.1-1.0 mg/L compared to control. GI inhibition was significantly higher for 9 mg/L compared to control. NOEC was 3 mg/L and LOEC was 9 mg/L.

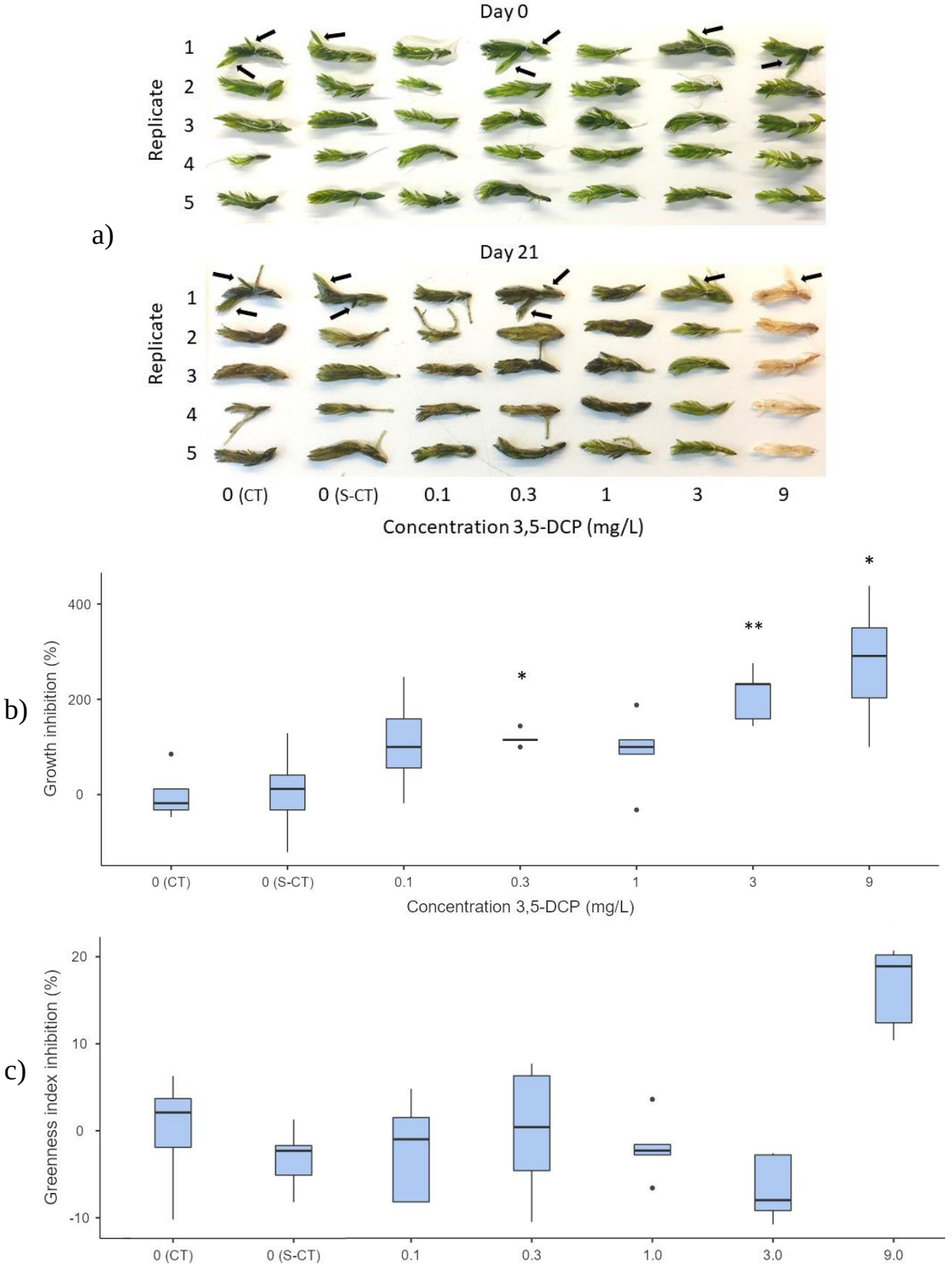

Figure 14: *Fontinalis antipyretica* a) shoots (black arrows indicate secondary shoots); b) inhibition of growth, and c) inhibition of greenness index (asterisks marks statistically significant difference to medium control, 0 (CT) (* $p<0.05$, ** $p<0.01$) after 21 days exposure to 3,5-dichlorophenol (DCP).

3.2.2. Maximal PSII efficiency

There was a clear concentration and time-dependent response for inhibition of Fv/Fm from day 1 and throughout the exposure period (Figure 15). Highest inhibition was observed for 9 mg/L at day 1 (80 %) and day 7-21 (100 %), and lowest for 0.1-1 mg/L at day 14 (-4 - -20 %) where promotion was observed, however with high variation. Fv/Fm inhibition was significantly higher in 3-9 mg/L compared to medium control at day 1, 7 and 21, and in 9 mg/L compared to medium control at day 14. The CRC for day 7 was most optimal, with NOEC at 1 mg/L, LOEC at 3 mg/L and EC_{50} at 3.1 ± 6.7 mg/L.

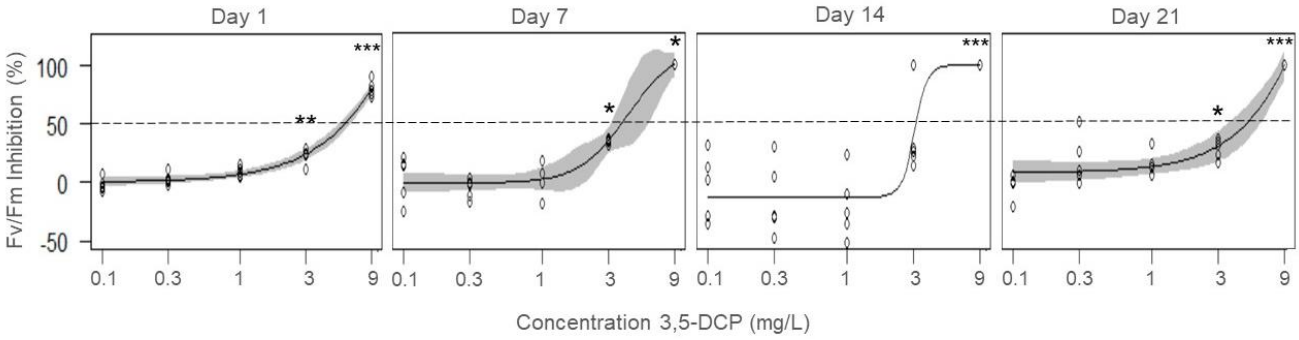

Figure 15: Inhibition of maximal PSII efficiency (Fv/Fm) in *Fontinalis antipyretica* after 1-21 days of exposure to 3,5-dichlorophenol (3,5-DCP). Asterisks marks statistically significant difference to control (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Broken line marks the concentration causing 50 % inhibition.

3.2.3. Pigment concentration

Chlorophyll *a* and chlorophyll *b*

There was a concentration and time-dependent response for inhibition of Chl *a* and Chl *b* for the highest concentrations, from day 7 and throughout the exposure period, with similar responses for the two pigments (Figure 16). Highest inhibition was observed for 9 mg/L at day 14-21 (~100 %) and lowest for 0.1 mg/L at day 14 and 0.3-3 mg/L at day 21 (high variance, varying from 0 to -200 %) for both pigments. At day 1, there was a slight increase of inhibition from 0.1-1 mg/L, with significantly higher inhibition for 1 mg/L compared to medium control. At day 7, inhibition was significantly higher in 9 mg/L compared to medium control for Chl *b*, and additionally at day 14-21 in 9 mg/L compared to medium control for both Chl *a* and Chl *b*. Sporadically promotion was observed for multiple concentrations from 0.1-3 mg/L at day 7-21 for both pigments, with significant promotion in 3 mg/L compared to control at day 21. The CRC for day 14 was most optimal for both pigments, with NOEC at 3 mg/L and LOEC at 9 mg/L for both pigments and EC_{50} at 3.4 ± NaN mg/L for Chl *a* and 358.4 ± 10.0 mg/L for Chl *b*.

Carotenoids

There was a concentration and time-dependent response for inhibition of Car for the highest concentrations from day 7 and throughout the exposure period (Figure 16). Highest inhibition was observed for 9 mg/L at day 14-21 (~100 %) and lowest for 0.1 mg/L at day 14 and 0.3-3 mg/L at day 21 (high variance, varying from 0 to -150 %). At day 1 there was a slight increase of inhibition from 0.1-1 mg/L, however no significant difference compared to medium control was detected. At day 7-21, inhibition was significantly higher in 9 mg/L compared to medium control. Sporadically promotion was observed for multiple concentrations from 0.1-3 mg/L for the whole exposure period. The CRC for day 14 was most optimal, with NOEC at 3 mg/L, LOEC at 9 mg/L and EC_{50} at 3.1 ± 0.9 mg/L.

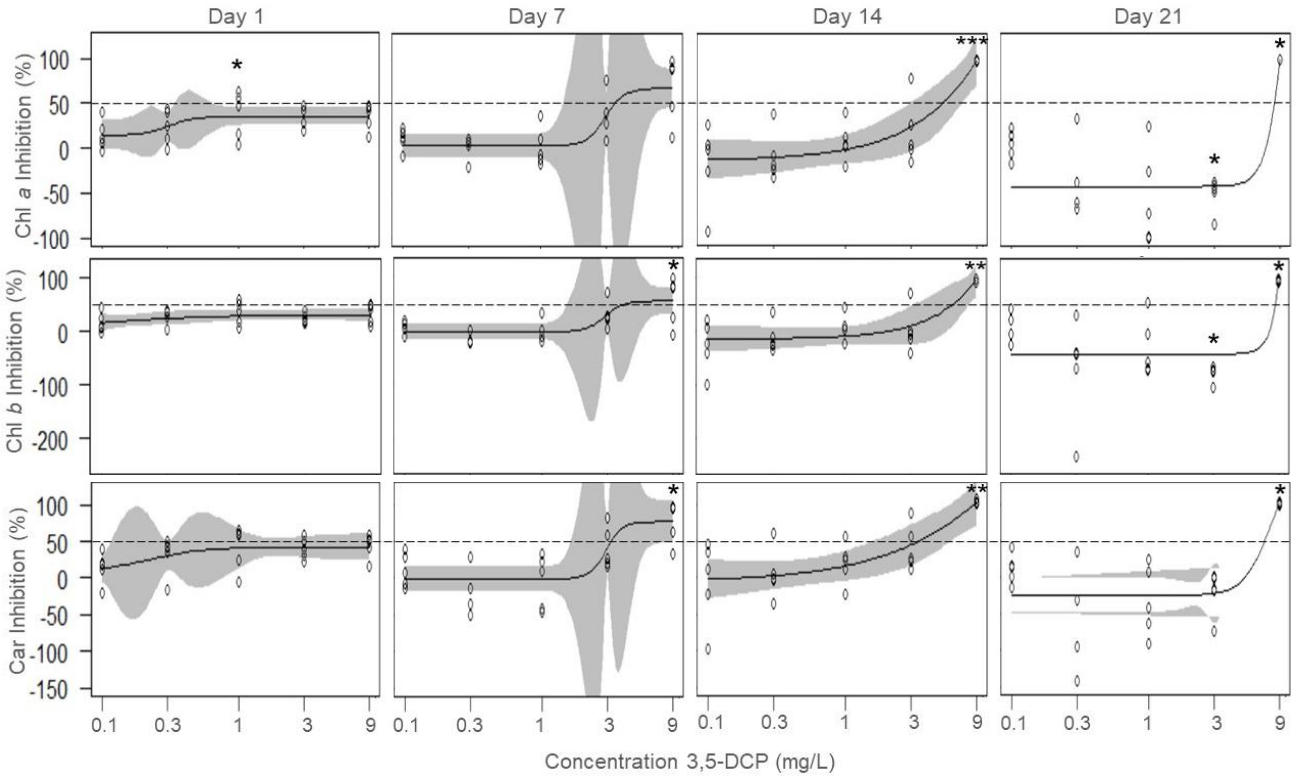

Figure 16: Inhibition of the concentration of pigments Chlorophyll (Chl) *a*, Chl *b*, and Carotenoids (Car) in *Fontinalis antipyretica* after 1-21 days of exposure to 3,5-dichlorophenol (3,5-DCP). Asterisks marks statistically significant difference to control (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Broken line marks the concentration causing 50 % inhibition.

Chlorophyll *a*/*b* ratio

There was a concentration and time-dependent response of increased Chl *a*/*b* relative change for the highest concentrations from day 7 and throughout the exposure period (Figure 17). Highest relative change was observed for 9 mg/L at day 14-21 (~90 %) and lowest for 1 mg/L at day 21 (-23 %). The relative change was significantly higher than medium control for 9 mg/L

at day 7-21. Sporadically promotion was observed for multiple concentrations from 0.1-1 mg/L at day 14-21. The CRC for day 14 was most optimal, with NOEC at 1 mg/L, LOEC at 3 mg/L and EC_{50} at 3.3 ± NaN mg/L (See Appendix IX for Chl *a/b* ratio plots).

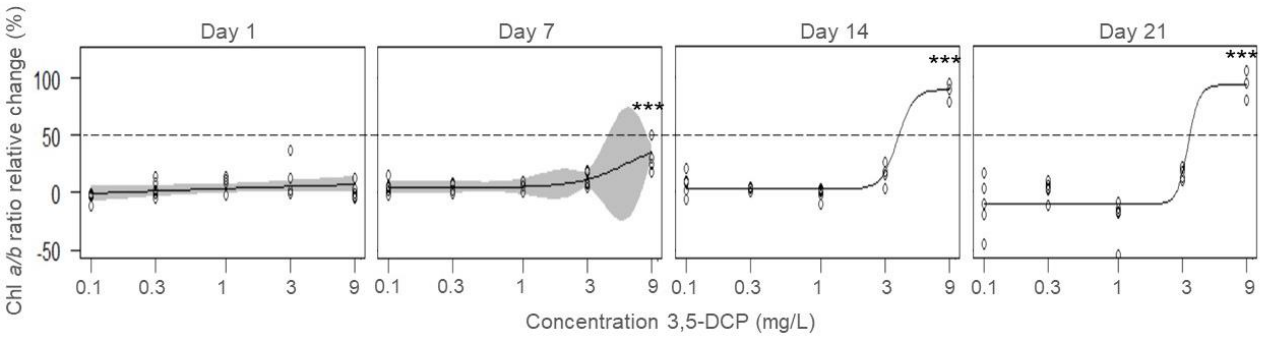

Figure 17: Relative change of Chlorophyll *a/b* ratio in *Fontinalis antipyretica* after 1-21 days of exposure to 3,5-dichlorophenol (3,5-DCP). Asterisks marks statistically significant difference to control (* p<0.05, ** p<0.01, *** p<0.001). Broken line marks the concentration causing 50 % inhibition.

3.2.4. Reactive oxygen species (ROS)

ROS formation fold increase was generally low and stable, without a concentration and time-dependent response (Figure 18). Small variations were observed between concentrations, including increased response for 9 mg/L at day 7, however without significant difference between treatments and medium control. No optimal CRC was generated, and NOEC and LOEC were similar for all days at <0.1 and 0.1 mg/L, respectively.

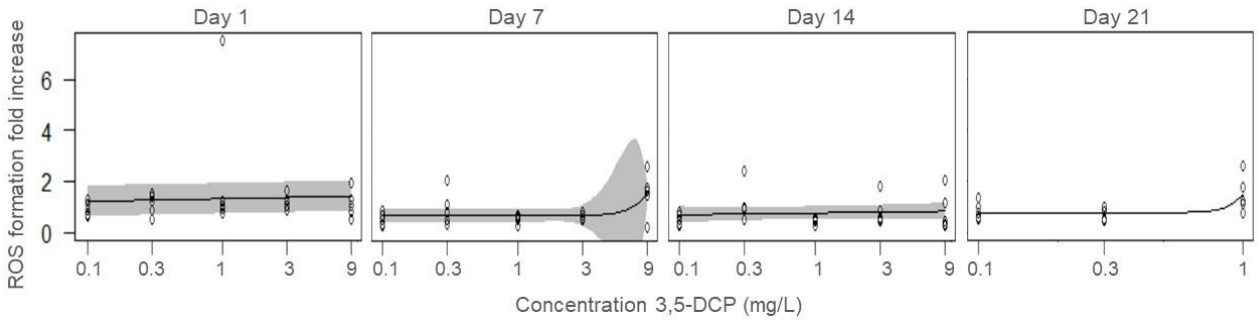

Figure 18: Fold increase of reactive oxygen species (ROS) production in *Fontinalis antipyretica* after 1-21 days of exposure to 3,5-dichlorophenol (3,5-DCP). Asterisks marks statistically significant difference to control (* p<0.05, ** p<0.001, *** p<0.001).

3.3. Field study

Results from the 14 days reversed field study are presented below, where *F. antipyretica* from the sites Skut 1 and Skut 2 in Skuterudbekken were deployed back into their respective sites and into reference stream Sandbekken (Ref. (Skut 1) and Ref. (Skut 2), respectively). Both deployed (dep.) and native (nat.) moss in Skut 1 and Skut 2 was assessed.

Slight differences in chemical and physical conditions of stream sites were observed. Fairly similar light condition was registered in Skuterud 1 (Skut 1; upstream of sedimentation ponds), Skuterud 2 (Skut 2; downstream of sedimentation ponds and European route 18) and reference stream Sandbekken, with slightly lower light intensity at day 4-7 for all locations. Temperature of all locations decreased from approximately 12 to 3°C during the exposure period. Increased water flow was observed in all locations at day 7, with water discharge at ~1-2 times higher in Skut 2 compared to Skut 1 and Ref. loc. at day 14. pH, conductivity, concentration of tot-N, tot-P, pesticides and most elements were higher in Skut 1 and Skut 2 compared to Ref. loc, where pesticides were slightly higher in Skut 1 and elements were slightly higher in Skut 2. Concentration of TOC and DOC was slightly higher in Ref. loc. compared to Skut 1 and Skut 2. One moss netting was lost in Skut 2. At Ref. loc. edges of moss nettings and hence a few shoots were observed to be partly covered by the sandy streambed substrate at day 7 and 14. Details of chemical and physical conditions of streams are found in Appendix X, and discussed in Appendix XVII.

3.3.1. Growth effects

There was a slight site-dependent response for reduced growth of Skut 2 dep. (only negative values), with significantly lower growth compared to Ref. (Skut 2) (Figure 19). No significant difference in length of shoots was detected between day 0 and 14. The growth was lowest in Skut 2 (-2.2 ± 2.1 mm) and highest in Ref (Skut 1) (2.3 ± 4.4 mm).

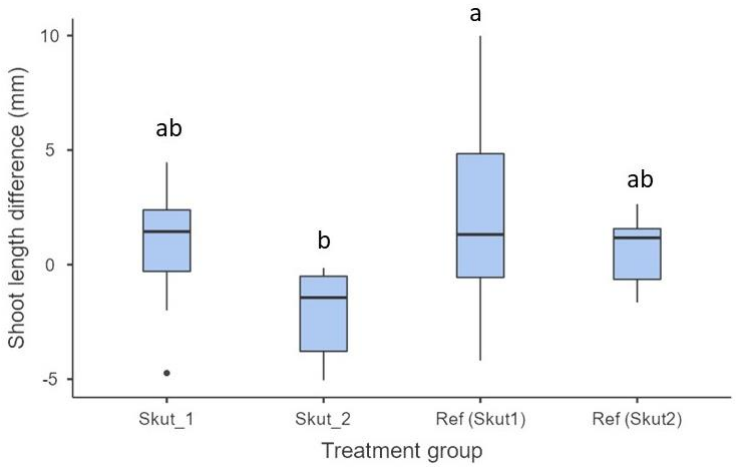

Figure 19: Difference in shoot length (growth from day 0 to 14) for deployed *Fontinalis antipyretica* after 14 days exposure in Skuterud stream site 1 (Skut_1) and 2 (Skut_2), and moss tissue deployed into reference stream from Skut 1 (Ref (Skut 1)) and 2 (Ref (Skut 2)). Significant difference ($p<0.05$) is encoded by letters.

3.3.2. Maximal PS II efficiency

There was no clear site and time-dependent response of Fv/Fm (Figure 20). At the start of the test (day 0) Fv/Fm was significantly higher in Skut 2 nat. compared to Skut 1 dep. and Skut 1 nat. At day 7 Fv/Fm was significantly higher in Skut 1 nat. compared to Skut 1 dep. and Ref. loc. (Skut 1). At day 14 Fv/Fm was significantly higher in Skut 1 nat. compared to Skut 2 dep. and Ref. (Skut 2). Lowest Fv/Fm and highest variation was observed in deployed and native moss in Skut 2.

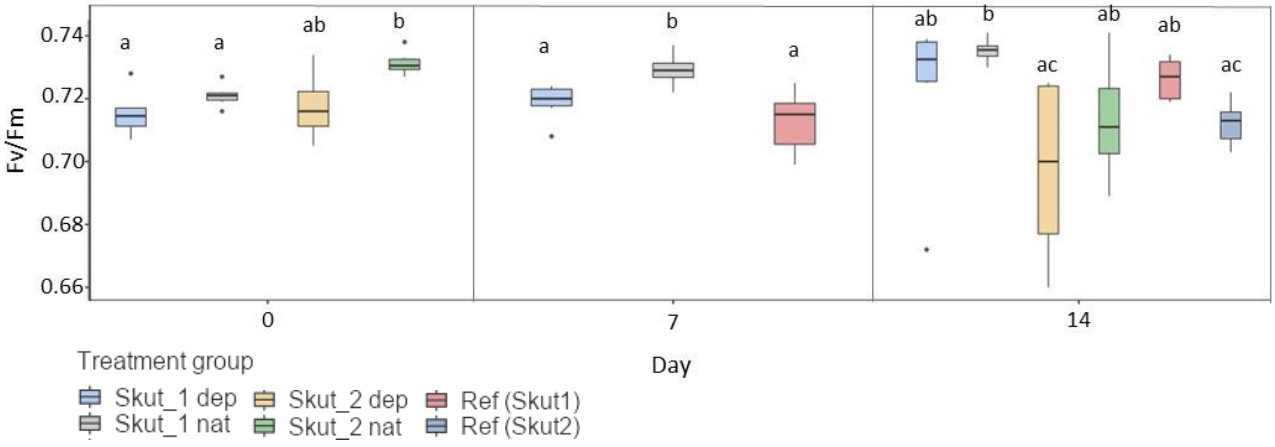

Figure 20: Maximal PSII efficiency (Fv/Fm) at 0-14 days into the field exposure study, for deployed (dep) and native (nat) moss *Fontinalis antipyretica* in Skuterud stream site 1 (Skut_1) and 2 (Skut_2), and moss tissue deployed into reference stream from Skut 1 (Ref (Skut 1)) and 2 (Ref (Skut 2)). Significant difference ($p<0.05$) is encoded by letters.

3.3.3. Pigment concentration

Due to executional errors at day 0, there was a mix of triplicate shoots within each treatment group (i.e., rep 1-3 and 4-6), between execution of weighting and pigment analysis. For this reason, the weight and pigment concentration for triplicates were used (n=2), and consequently no statistical analysis of variance (ANOVA) could be done.

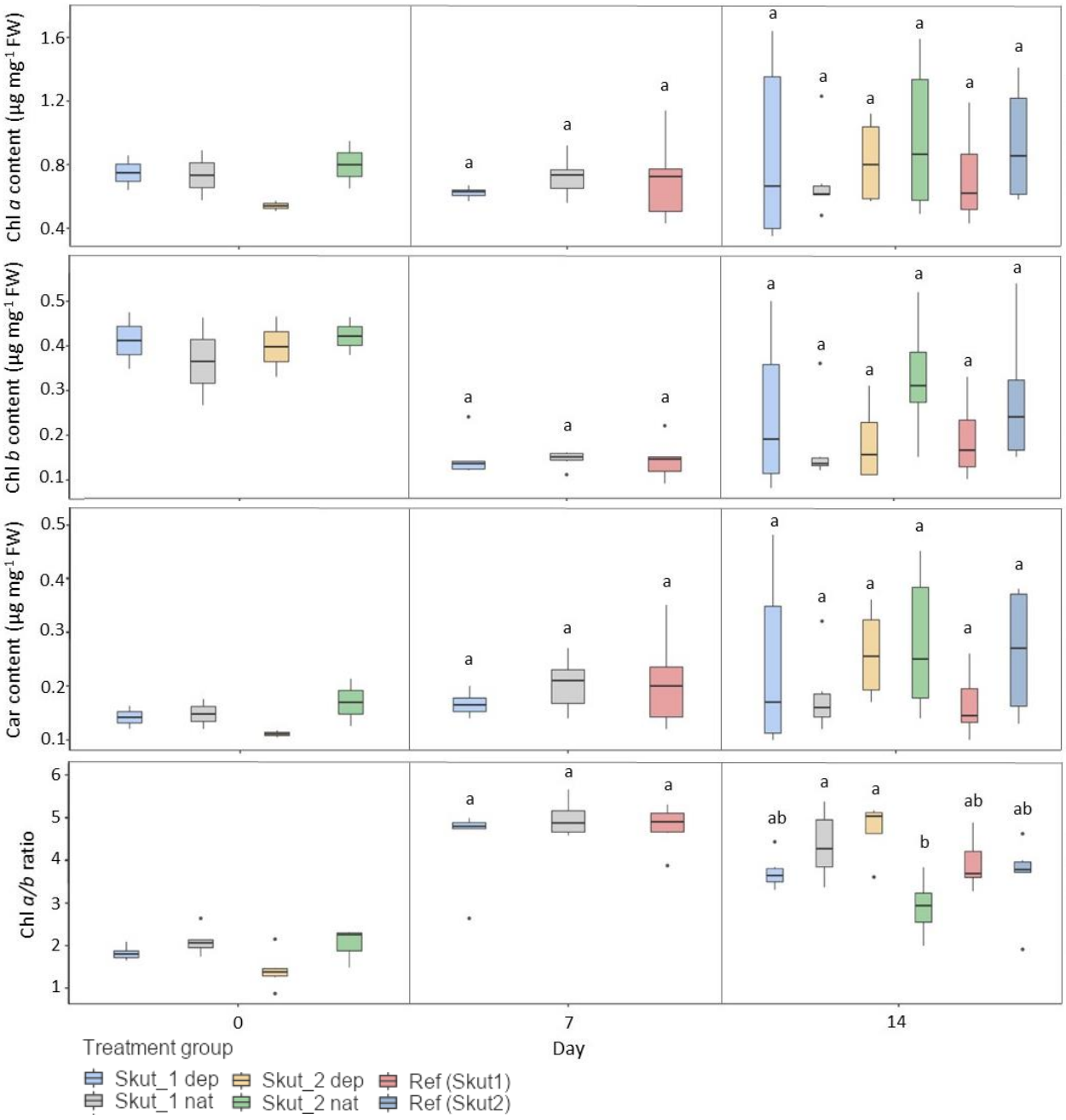

Figure 21: Concentration of pigments chlorophyll (Chl) *a*, Chl *b* and carotenoids (Car), and Chl *a/b* ratio at 0-14 days into the field exposure study, for deployed (dep) and native (nat) moss *Fontinalis antipyretica* in Skuterud stream site 1 (Skut_1) and 2 (Skut_2), and moss tissue deployed into reference stream from Skut 1 (Ref (Skut 1)) and 2 (Ref (Skut 2)). Significant difference ($p<0.05$) is encoded by letters (no statistical significance could be calculated at day 0 where n=2).

Generally, there was no clear site and time-dependent response of pigment concentration (Figure 21). There was no significant difference between exposure sites for Chl *a*, Chl *b* or carotenoids. However, Chl *b* concentration was higher at day 0 compared to day 7-14, leading to lower Chl *a/b* ratio at day 0 (avg. 1.78) compared to day 7-14 (avg. 4.75). The variation was high at day 14 for concentration of all pigments and Chl *a/b* ratio. The only significant difference between exposure sites was observed for Chl *a/b* ratio at day 14, with significantly lower ratio in Skut 2 nat. compared to Skut 1 nat. and Skut 2 dep.

3.3.4. Photosynthetic oxygen evolution

There was a site-dependent response of lower oxygen production for Skut 2 compared to Skut 1 and for native compared to deployed moss at day 0, and for Skut 1 nat. compared to Skut 1 dep. and Ref (Skut 1) (Figure 22). However, no statistical significance could be calculated (as n=2). The oxygen production was generally highest and with largest variation at day 7.

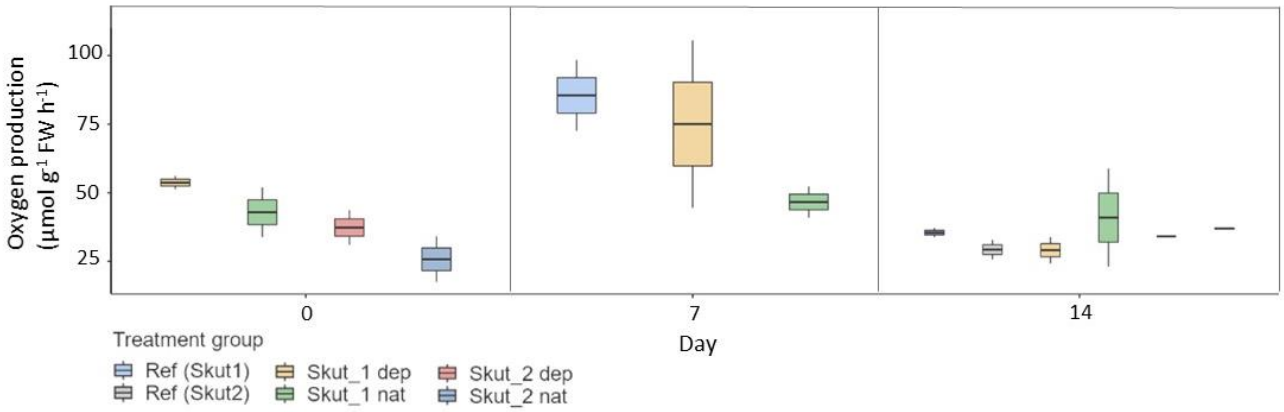

Figure 22: Oxygen production at 0-14 days into the field exposure study, for deployed (dep) and native (nat) moss *Fontinalis antipyretica* in Skuterud stream site 1 (Skut_1) and 2 (Skut_2), and moss tissue deployed into reference stream from Skut 1 (Ref (Skut 1)) and 2 (Ref (Skut 2)). No statistical significance could be calculated as n=2.

3.3.5. Reactive oxygen species (ROS)

There was no clear site and time-dependent response of ROS production. Highest ROS production was observed for deployed moss in Skut 1 and Skut 2, and lowest for Skut 1 nat., Ref. (Skut 1) and Ref. (Skut 2) (Figure 24), where production was significantly lower compared to all other exposure sites and control (lab-grown moss).

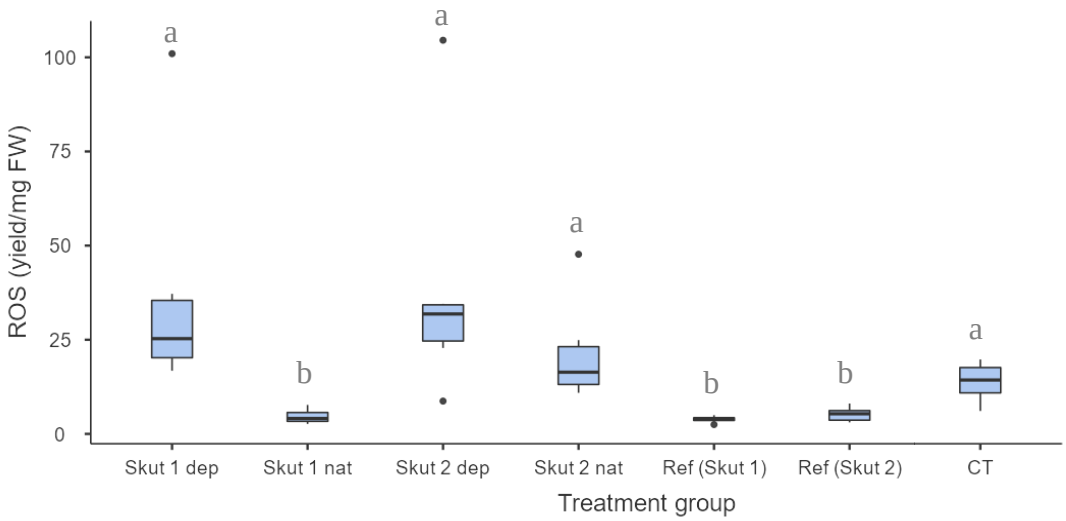

Figure 24: ROS yield for deployed (dep) and native (nat) moss *Fontinalis antipyretica* in Skuterud stream site 1 (Skut_1) and 2 (Skut_2), and moss deployed into reference stream from Skut 1 (Ref (Skut 1)) and 2 (Ref (Skut 2)) after 14 days exposure. CT is *F. antipyretica* grown at the lab in medium during the exposure period. Significant difference ($p<0.05$) is encoded by letters.

4. Discussion

In the following subsections, results from the laboratorial and field study are presented and discussed with reference to quality assessment, results from the laboratory studies, results from the field studies and finally an overall assessment of the suitability of *Fontinalis antipyretica* as an experimental species, including suggestions for improving the studies.

4.1. Quality assessment

Sources of errors occurred for the laboratorial and field studies, possibly resulting in reduced accuracy and increased variation (see Appendix XIII for detailed discussion). This included adaption to agricultural runoff by test species, unideal time of year for studies using the test species, poor randomisation of shoots, lack of repetition of the tests and possible combined effects. *Fontinalis antipyretica* was the best option of test species (according to Deben et al. (2017)), however moss tissue obtained from Skuterudbekken was possibly adapted to agricultural runoff. The test species naturally stops growing and starts fragmenting at the time of year when studies were executed (Glime, 2014), with effects on growth and possibly other endpoint analysis of the present study.

For laboratorial studies, additional sources of errors include lack of micronutrients in growth medium and non-successful sterilisation of test species, leading to reduced health of shoots (visualised through endpoint analysis). Lack of micronutrients was indicated by time-dependent negative response of endpoints for control and treatments except for 3 µM $CuSO_4$, as copper was a limiting micronutrient and low supply was beneficial. *Fontinalis antipyretica* is was unsuccessfully sterilised, as reported to be a common problem for this test species (de Traubenberg & Ah-Peng, 2004), resulting in contamination, and consequently increased pH (from 7 to maximum 9.3) in control and lower treatment concentrations. However, observed levels of pH are reported to have little or no effect on *F. antipyretica* (Aronsson & Ekelund, 2006). Reduced health of control groups was observed through decreased GI, Fv/Fm, pigment concentration, and increased ROS production throughout the exposure period (see Appendix XIV for details).

For the field study, additional sources of errors include loss and partly burial of moss nettings and HOBO loggers (light measurements), and statistical issues. Loss of moss nettings due to high stream velocity led to lack of endpoint results at day 7. Partly burial of moss nettings due to sandy streambed substrate could reduce health of shoots, however results of endpoint analysis

did not indicate this. The acclimatisation period was slightly shorter than ideal, however not considered to have significant impact on results. Stream Sandbekken was chosen as the most ideal reference location, although native *F. antipyretica* could not be assessed and chemical and physical conditions were slightly different but considered non-significant, compared to stream Skuterudbekken (see Appendix XV for details).

4.2. Laboratorial study – Toxicity of copper

Copper (Cu) caused a clear concentration and time-dependent response for greenness index (GI), maximal PS II efficiency (Fv/Fm), pigment concentration and production of reactive oxygen species (ROS), typically from 10 µM with maximal effect at 100-300 µM after 7-21 days exposure. This indicates higher sensitivity for increased concentrations and exposure length. Optimal exposure time were typically 7 days for Fv/Fm inhibition, pigment concentration inhibition, and ROS fold increase, where typical values of NOEC was < 3-10 µM, LOEC was 3-30 µM and EC_{50} was 11.7 – 30.4 µM (Table 1), however variations were seen between different endpoint analyses. Highest inhibition was observed for Fv/Fm at 100 % after 7-21 days exposure, and lowest inhibition was observed for GI at 20 % after 21 days exposure. Fv/Fm was the only endpoint significantly affected at day 1. These results indicate that Fv/Fm was the most sensitive and GI the least sensitive endpoint. However, a slight concentration-response trend was observed for pigment concentration and ROS at day 1, indicating weak early effects on these endpoints as well.

Table 1: NOEC, LOEC and EC_{50} (est. ± std dev) in *Fontinalis antipyretica* after 7-21 days (parenthesis) exposure to $CuSO_4$.

	GI inhibition (d21)	**Fv/Fm inhibition (d7)**	**Chl *a* inhibition (d7)**	**Chl *b* inhibition (d7)**	**Car inhibition (d7)**	**Chl *a/b* ratio (d14)**	**ROS prod fold increase (d7)**
NOEC	< 3 µM	10 µM	3 µM	3 µM	< 3 µM	10 µM	3 µM
LOEC	3 µM	30 µM	10 µM	10 µM	3 µM	30 µM	10 µM
EC_{50}	NA	28.1 ± 9.5 µM	13.9 ± 1.9 µM	30.4 ± 7.9 µM	11.7 ± 2.0 µM	104.2 ± 18.7 µM	NA

These results generally correspond to other studies, indicating negative effects of exposure by heavy metals including Cu in *F. antipyretica*. Rau et al. (2007) observed time and concentration-specific responses in *F. antipyretica* after 7 days exposure to Cu, with chlorophyll fluorescence (Fv/Fm) reduced from 100 % to ~70 % in 25 µM and to ~52 % in 100

µM. This corresponds to the Fv/Fm inhibition of the present study; however, inhibition was even higher here, at 100 % after 7 days exposure to 100 µM Cu. The explanation for this discrepancy is not immediately evident, but might include additional inhibition due to micronutrient depletion, contamination, or unideal time of year for studies using *F. antiyretica* (see Appendix XIII for details). Exposure to heavy metals including Cu, Cd, Pb and Zn in *F. antypyretica* and other macrophytes indicated production of ROS (Choudhury & Panda, 2005), damaged cell walls and affected membrane permeability (Vazquez et al., 2000), and decreased concentration of nitrogen (essential for amino acids) through disrupted nitrogen metabolism (Sutter et al., 2002). Cu is additionally demonstrated to cause reduced concentrations of pigments and Chl *a/b* ratio in terrestrial bryophytes (Shakya et al., 2008; Tremper et al., 2004), corresponding to results of the present study. Shakya et al. (2008) explains the reduced Chl *a/b* ratio by conversion of Chl *a* to Chl *b*, induced by Cu, and Tremper et al. (2004) indicates that chlorophyll inhibition in mosses is more sensitive to Cu compared to other metals. Dumont et al. (2019a) reported dose-dependent responses of Cu toxicity in three other species of macrophytes, wiht EC_{50} values of 0.044 – 0.9 µM for relative growth rate (7 – 14 days exposure), and 3.21 – 13.7 µM for Fv/Fm (96 h exposure). These results indicate varying sensitivity among aquatic species and endpoints. It additionally indicates that *F. antipyretica* is less sensitive compared to other macrophytes regarding the endpoint Fv/Fm, however the EC_{50} value of the present study would probably be lower after 96h exposure, due to the time-dependent response.

Interestingly, no clear concentration or time-dependent response was found for the endpoint growth during the exposure period. This observation contradicts to the study by Davies (2007) with twice as high growth and concentration dependent responses in sulphate exposed *F. antipyretica*. Additionally, a low and non-significant number of secondary shoots were observed without a clear concentration or time-dependent response, contradicts to the study by Aronsson and Ekelund (2006), with concentration dependent response on growth of secondary shoots in wood ash exposed *F. antipyretica.* The explanation for these growth discrepancies are not immediately evident, but factors such as the unideal time of year for studies using *F. antipyretica* (Glime, 2014) and nutrient depletion may provide some explanation (see Appendix XIII (2), (6), and Appendix XVI 0(1) for details).

Inhibition of GI (day 21) and Fv/Fm (day 7-14) was significantly lower in the 3 µM treatment compared to control, indicating improved conditions for *F. antipyretica* with low supply of Cu. The explanation for these discrepancies is not evident, but possibly include lack of

micronutrients of growth medium, where the 3 µM supply prevents deficiency of Cu, which is an essential micronutrient for all organism (Amdur et al., 1993) (see Appendix XIII (6) for details). Chlorosis was not observed for any treatment groups after 21 days exposure. This observation corresponds to the study of Chen et al. (2015), where no apparent chlorosis was observed after exposure of up to 50 µM Cu in two terrestrial mosses.

An exposure time of 7 days was needed for a clear concentration-dependent response of Fv/Fm, Chl *a*, Chl *b* and Car inhibition, and ROS increase, whereas 14 days was needed for Chl *a*/*b* ratio. Hence, exposure length of up to 14 days is suggested as most ideal, however 21 days is needed for growth effects when *F. antipyretica* is in a growing state (spring/summer; Appendix XIII (2). This result corresponds to exposure times used by other studies with heavy metal exposure in *F. antipyretica* (Rau et al., 2007; Vazquez et al., 2000).

The variation was generally high, probably caused by sources of errors including possible adaption of test species to agricultural runoff, poor randomisation or possible combined effects with toxicants produced by contaminating algae (see Appendix XIII for details). Increased inhibition of Fv/Fm and Chl *a/b* ratio after 21 days exposure could be explained by the time-dependent increase in toxicity, possibly in combination with micronutrient depletion, contamination, or unideal time of year for studies using *F. antiyretica* (see Appendix XIII for details). The concentration and time-dependent reduction of ROS production for the highest concentrations was possibly caused by highly reduced health or death of shoots, and hence reduced capacity for ROS production.

$CuSO_4$ was earlier used as an herbicide, however substituted with others due to its toxicity (Amdur et al., 1993). Additionally, $CuSO_4$ mixed with lime was previously used as a fungicide (Amdur et al., 1993). However, as bioavailability of metals generally are dependent of pH with increased bioavailability at lower pH, which is relatively unusual in aquatic systems, Cu originating from $CuSO_4$ is not considered to be a major environmental problem. Nevertheless, heavy metals such as Cu originating from other natural and anthropogenic sources are demonstrated to be an environmental problem in some areas (Bruns et al., 1995; Camizuli et al., 2014; Chen et al., 2015).

4.3. Laboratorial study – Toxicity of 3,5-dichlorophenol (3,5-DCP)

3,5-DCP caused a concentration and time-dependent response for greenness index (GI), maximal PS II efficiency (Fv/Fm) and pigment concentration, typically from 3 mg/L with

maximal effect at 9 mg/L after 7-21 days exposure. This indicates higher sensitivity for increased concentrations and exposure length, with negative effects at the two highest concentrations only. Optimal exposure time were typically 14 days for Fv/Fm inhibition and pigment concentration inhibition, where typical values of NOEC was 1-3 mg/L, LOEC was 3-9 mg/L and EC_{50} was 3.1 – 3.4 mg/L (Table 2), however variations were seen between different endpoint analyses. Highest inhibition was observed for Fv/Fm at 100 % after 7-21 days exposure, and lowest inhibition was observed for GI at 18 % after 21 days exposure. Fv/Fm was the only endpoint significantly affected at day 1. These results indicate that Fv/Fm was the most sensitive and GI the least sensitive endpoint. However, a slight concentration-response trend was observed for pigment concentration at day 1, indicating weak early effects on this endpoint as well.

Table 2: NOEC, LOEC and EC_{50} (est. ± std dev) in *Fontinalis antipyretica* after 7 - 21 days (parenthesis) exposure to 3,5-dichlorophenol (3,5-DCP).

	GI inhibition (d21)	**Fv/Fm inhibition (d7)**	**Chl *a* inhibition (d14)**	**Chl *b* inhibition (d14)**	**Car inhibition (d14)**	**Chl *a/b* ratio (d14)**	**ROS prod fold increase (d7-21)**
NOEC	3 mg/L	1 mg/L	3 mg/L	3 mg/L	3 mg/L	1 mg/L	< 0.1 mg/L
LOEC	9 mg/L	3 mg/L	9 mg/L	9 mg/L	9 mg/L	3 mg/L	0.1 mg/L
EC_{50}	NA	3.1 ± 6.7 mg/L	3.4 ± NaN mg/L	358.4 ± 10.0 mg/L	3.1 ± 0.9 mg/L	3.3 ± NaN mg/L	NA

Previous laboratorial studies with exposure of 3,5-DCP to *F. antipyretica* is not found, however studies with other aquatic macrophytes are done. The concentrations needed for an effect in the present study were higher than for the aquatic still-water floating macrophyte *Lemna minor*, where concentration-dependent responses with significant negative effects on endpoints growth, Fv/Fm, pigment concentration, and ROS production was observed after 7 days exposure (EC_{50} 1.12 - 2.60 mg/L; NOEC 0.5 - 1.5 mg/L; LOEC 1 - 2 mg/L) (Xie et al., 2018). However, these concentrations are lower than observed for a negative effect on growth after 7 days exposure in the aquatic still-water floating macrophyte *Lemna paucicostata* (EC_{50} 4.9 mg/L) (Michel et al., 2004). Additionally, 48 hour exposure studies of 3,5-DCP to different species of aquatic fungi had EC_{50} for growth at 1.1 - 5.7 mg/L (Nagai, 2018). This indicates varying sensitivity among aquatic species, and that *F. antipyretica* is more sensitive than some and more resistant than others.

Interestingly, only slight concentration dependent responses were found for the endpoint growth during the exposure period, where all treatments reduced growth. As for the Cu toxicity study, this observation contradicts to the study by Davies (2007) with growth that was three times higher in sulphate exposed *F. antipyretica*, compared to medium control of the present study. Additionally, as for the Cu toxicity study, a low and non-significant number of secondary shoots were observed, contradicting to the study by Aronsson and Ekelund (2006) with growth of secondary shoots after wood ash exposure to *F. antipyretica*. The explanation for these growth discrepancies are not immediately evident, but factors such as the unideal time of year for studies using *F. antipyretica* (Glime, 2014) and nutrient depletion may provide some explanation (see Appendix XIII (2), (6), and Appendix XVI 0(1) for details).

Chlorosis and significantly increased GI inhibition was observed in the highest concentration (9 mg/L) compared to medium control after 21 days exposure, indicating highly damaged or dead shoots. GI inhibition was rapidly increased from 3 to 9 mg/L, indicating that multiple intermediate concentrations should be assessed to see a gradual concentration dependent response.

Surprisingly, no significant impact on ROS-production was observed, indicating that exposure to 3,5-DCP did not influence ROS production in *F. antipyretica*. However, this is contradictory to results from similar studies on other macrophytes, demonstrating significant effect by 3,5-DCP on ROS production (Xie et al., 2018), and the fact that phenols in general cause formation of free radicals (Michalowicz & Duda, 2007). Part of the explanation for this discrepancy may be that higher concentrations of 3,5-DCP is needed for an effect on ROS, supported by the fact that only the two highest concentrations had effect on other endpoints. However, this indicated that ROS is not the most sensitive endpoint to 3,5-DCP and hence higher concentrations might be needed for an effect.

Exposure concentrations used in the present study were in the lower range of concentrations causing significant effects on *F. antipyretica*. This resulted in suboptimal CRC's that did not stabilise at higher concentrations, high variation, and poor statistical results, such as for estimated EC_{50} values of inhibition of pigment concentration. Additionally, it resulted in no effect on ROS production. To improve results, concentrations of ≥1 mg/L including multiple concentrations between 3 and 9 mg/L are recommended. An exposure time of 7 days was needed for a clear trend for Fv/Fm, Chl *a*, Chl *b* and Car inhibition, whereas 14 days was needed for Chl *a*/*b* ratio. Hence, exposure length of up to 14 days is suggested as most ideal, however 21 days is needed for growth effects when *F. antipyretica* is in a growing state (spring/summer;

Appendix XIII (2)). This exposure length is longer than reported for other studies with macrophytes exposed to 3,5-DCP (Xie et al., 2018)(Michel et al., 2004).

The variation was generally high, probably caused by sources of errors including possible adaption of test species to agricultural runoff, poor randomisation or possible combined effects toxicants produced by contaminating algae (see Appendix XIII for details). The variation was higher at the end of the exposure period, additionally caused by larger variations in response after longer exposure time. Growth was significantly reduced for 0.3 and 3-9 mg/L but not 1 mg/L treatments compared to control, and inhibition of Chl *a* was significantly increased for 1 mg/L compared to control at day 1. Promotion of GI, Chl *a* and Chl *b* concentration was observed in 3 mg/L at day 21. The explanation for these discrepancies is not immediately evident, but factors such as increased variation and reduced accuracy of results due to errors explained in Appendix XIII may provide some explanation. However, an additional explanation could possibly be that the contaminating algae are more sensitive and inhibited by 1-3 mg/L of 3,5-DCP, without simultaneous significant negative effects on the moss, resulting in improved conditions.

3,5-DCP is classified as toxic, with the possibility of long-term adverse effects to aquatic organisms (Nagai, 2018; Zagorc-Koncan et al., 2002), and 3,5-DCP serves as a model compound for other chlorophenols. Chlorophenols are environmentally relevant persistent organic pollutants (POP's) (Koba Ucun et al., 2021), that are persistent to degradation in nature, bioaccumulating in the food web and cause toxic effects to organisms. Chlorophenols originate from sources including previous use as pesticides, disinfectants or as a degradation product from other complex chlorinated hydrocarbons (Igbinosa et al., 2013). For this reason, laboratorial bioassays with 3,5-DCP could provide relevant information for ecotoxicological effects of chlorophenols.

4.4. Field studies

There was no clear deployment status or time-specific response for endpoints of the field studies. The explanation for this is not immediately evident, but factors such as low concentrations of pesticides and heavy metals, possible adaption of test species to agricultural runoff and other sources of errors discussed in Appendix XIII may provide some explanation. The present study did not indicate significant negative effect of pesticides and metals to *F. antipyretica* during autumn. However, water quality of Skuterudbekken during summer have

been classified as very bad the past years, and some pesticides have been detected above the limit of what is harmful for the environment (MF-limit; Appendix XII). The study was done after the major season for use of pesticides, resulting in reduced risk of chronic toxicity for macrophytes. For this reason, field studies with *F. antipyretica* during summer could possibly provide important additional information. The lack of a trend in significant difference between native and deployed moss indicates that the handling of moss tissue did not significantly affect the moss.

There were slight site-specific responses for inhibition of growth, maximal PS II efficiency (Fv/Fm), and production of ROS in Skut 2 compared to Skut 1 or reference location after 14-21 days exposure. The explanation for this is not evident but could possibly include toxicity of metals (as *F. antipyretica* is an effective accumulator of metals) or mixed toxicity of metals, pesticides, and environmental factors. However, chemical, and physical measurements (Appendix IX) generally indicate low concentrations at all sites, but higher presence of pesticides in Skut 1 and higher presence of elements in Skut 2. Pb was the only element present at concentrations above the limit of a good ecological status in Skut 2, at maximum 4 µg/L (moderate ecological status, ref. Appendix XVII). Laboratorial studies assessing effects of Pb have used higher concentrations (Pb of 4 µg/L equals 0.0193 µM), where concentrations of at least 20 µM caused decreased chlorophyll concentration (Yayintas et al., 2019) or nitrogen concentration in *F. antipyretica* (Sutter et al., 2002), or increased ROS production in three terrestrial mosses (Sun et al., 2011). 25-100 µM Pb indicated no effect on Fv/Fm in *F. antipyretica* (Rau et al., 2007). Hence, the Pb concentrations detected in Skut 2 are considered to have no significant toxic effects on *F. antipyretica.* Another possible explanation for the observed effects is damage by the high water discharge in Skut 2 (Appendix X) combined with unideal time of year for bioassays using *F. antipyretica* (Appendix XIII (2)) as it naturally starts fragmenting at this time of year, resulting in reduced growth (shortening) with possible impacts on other endpoints (Appendix XVI). This is supported by the lack of a clear site-specific response, high variation, and the fact that either native or deployed moss in Skut 2, not both, were significantly affected.

Multiple field studies have assessed accumulation of heavy metals in *F. antipyretica* and other aquatic bryophytes (Censi, 2000; Divis et al., 2012; Figueira & Ribeiro, 2005), while fewer have assessed the effects of pollution. However, a few studies have assessed effects of heavy metals. Vazquez et al. (2000) demonstrated that *F. antipyretica* transplanted to a heavy metal contaminated and acidic field site had lower content of essential cations K, Mg and Ca, and

indicated damaged cell wall. Mersch and Reichard (1998) observed concentration and time dependent bioavailability of heavy metals in industrial effluents, with effects including light green and yellow colouring of shoots and some dead tissue after exposure. The author additionally Maresca et al. (2018) demonstrated that the aquatic moss *Leptodictyinn riparium* transplanted to a heavy metal contaminated stream suffered ultra-structural damage, increased ROS production, activity of antioxidant enzymes and DNA damage. Lopez and Carballeira (1990) indicated reduced chlorophyll concentration and pigment ratio of *F. antipyretica* and other aquatic mosses growing in streams contaminated by organic and metal pollution. Mersch and Reichard (1998) observed higher sensitivity of *F. antipyretica* compared to two other aquatic mosses to heavy metals, while Lopez and Carballeira (1990) observed lower sensitivity of *F. antipyretica* compared to four other aquatic mosses to organic and heavy metal pollution. This indicates varying sensitivity among species and types of pollution.

Surprisingly, the low pH of reference location (Appendix X) did not have significant impact on the endpoint results. The explanation for this is not immediately evident, but might include the fact that mosses generally are reported to thrive in acidic aquatic systems (Aarnes, 2016) and that a major effect of low pH is increased bioavailability of metals. However, the concentration of metal was low in reference location (Appendix XV), resulting in reduced risk of metal toxicity. Additionally, the low temperature in all stream sites at the end of the exposure period (Appendix X) did not have significant impact on the endpoint results. The explanation for this is not immediately evident but might include factors such as adaption of *F. antipyretica* to varying temperatures. See Appendix XVII for additional and more detailed discussion of chemical and physical conditions of streams.

The variation was generally high, probably caused by sources of errors including possible adaption of test species to agricultural runoff, poor randomisation, and possible combined effects with environmental factors or toxicants produced by other organisms (Appendix XIII). Chl *a/b* ratio was highly increased at day 7 and 14 (~ 3-5) compared to day 0 (~ 1-2). The explanation for this discrepancy is not immediately evident, but possibly includes methodological errors. This is supported by the fact that no other endpoint results demonstrate the same time-dependent response, and laboratorial results did not indicate the same range of Chl *a/b* ratio. There was a weak deployment status and site-specific response of lower oxygen production in Skut 2 compared to Skut 1 and in native compared to deployed moss in the start of the exposure period. However, due to sub-ideal method development resulting in low statistical strength (Appendix XVI (2); Appendix XIII (11)), this is considered negligible and

will not be further discussed. Additionally, results are considered slightly less accurate than ideal due to drying on tissue paper instead of centrifugal drying prior to endpoint analysis (Appendix XVI (4)).

4.5. Review on using *Fontinalis antipyretica* as a study species

Fontinalis antipyretica demonstrated to be a suitable test species for the laboratorial bioassays and field studies, including successful determination of mode of actions (MoA) and adverse outcomes (AO). Clear concentration and time-dependent responses were observed for the MoAs maximal PS II efficiency (Fv/Fm), pigment concentration and production of reactive oxygen species, and the AO greenness index (GI) of laboratorial studies. No clear site, deployment status or time-specific responses caused by ecologically relevant pollution were observed for the field studies, however vague site-specific responses were observed, probably caused by environmental stressors. Sampling and preparation of test species generally worked

Methods used for sampling and preparation of test species, experimental setups for laboratorial and field studies, methods used for endpoint analysis, and chemical and physical monitoring generally worked well. However, some (minor) limitations occurred, including: 1) unsuccessful sterilisation of test species, growth of contamination, lack of micronutrients in growth medium and suboptimal concentrations of 3,5-DCP for laboratorial studies; 2) too short acclimatisation, suboptimal deployment, attachment and retrieving of moss nettings and suboptimal monitoring of some chemical and physical parameters for field studies; 3) suboptimal growth analysis (including secondary shoots), unsuccessful assessment of photosynthetic oxygen evolution and suboptimal normalisation by weight for endpoint analysis; and 4) the time of year for executing studies using *F. antipyretica*, poor randomisation and lack of repetition of studies as general limitations (chap. 4.1; Appendix XIII; Appendix XVI). Suggestions for improving these factors are provided in the following subchapter.

4.5.1. Suggestions for improving studies

Due to technical limitations and suboptimal methods of the studies, suggestions for improving the bioassays based on the discussion in chap. 0, Appendix XIII, Appendix XVI and Appendix XVII are given below.

Laboratorial study

Improvements of laboratorial bioassays include, but is not limited to, 1) improving methods for successful sterilisation of moss tissue; 2) in case of contamination, more frequent change of growth medium to limit the amount and effect of contamination; 3) adding micronutrients essential for *F. antipyretica* to the growth medium; and 4) for the 3,5-DCP study, including screening of proper concentrations to use (≥ 3 mg/L) to obtain optimal CRCs.

Field study

Improvements of field bioassays include, but is not limited to, 1) using a longer acclimatisation period (≥ 14 days) to ensure stabilisation of moss tissue priory to exposure tests; 2) use alternative technical methods for exposure test in streams with high water current or sandy stream bed substrate, such as moss bags; 3) attaching a small floating device to each moss netting for easier location in periods with high or little transparent water; 4) monitoring of all chemical and physical conditions throughout the study with minimum 3 replicates, if possible using more accurate methods such as ideal measuring equipment and attach HOBO-loggers to rocks instead of poles on the stream bed surface to increase accuracy of measurements.

Endpoint analysis

Improvements of endpoint analysis include, but is not limited to, 1) assessment of growth of secondary shoots for growth parameters; 2) assessment of chlorophyll/pheophytin ratio (D665/D665a) for pigment concentration parameters; 3) running a test assay for photosynthetic oxygen evolution to establish a more suitable method for this endpoint; and 4) using centrifugal drying for normalisation by fresh weight and if possible, use normalisation to dry weight to increase accuracy.

General improvements

General improvements of laboratorial and field bioassays additionally include, but is not limited to, 1) executing experiments during the active growing season (normally spring/summer) of *F. antipyretica* to allow inclusion of growth parameters after chronic exposure; and 2) repeat each study minimum three times for higher accuracy, eliminating executional errors and verify results.

5. Conclusions

Fontinalis antipyretica demonstrated to be a suitable test species for laboratorial bioassays with exposure to copper and 3,5-dichlorophenol (3,5-DCP), and determination of mode of actions (MoA) and adverse outcomes (AO). Both toxicants caused clear concentration and time-dependent responses for greenness index (GI) as an AO, and maximal PS II efficiency (Fv/Fm) and pigment concentration as MoAs. Copper additionally caused a clear concentration and time-dependent response for production of reactive oxygen species (ROS) as a MoA. Optimal exposure time was typically 7-14 days for most endpoints; however, 21 days is needed for growth assay. The observed sensitivity of *F. antipyretica* to copper coheres well with observations by other studies, however sensitivity to both copper and 3,5-DCP have demonstrated to vary among different aquatic macrophytes. 3,5-DCP concentrations used for this bioassay was in the lower range of concentrations needed for a clear concentration-response relationship. Only slight concentration and time-dependent response were found for the AO endpoint growth, possibly caused by unideal time of year for studies using *F. antipyretica*, as growth normally stops, and fragmenting starts during winter. Some technical limitations were identified, including lack of added micronutrients to growth media, non-successful sterilisation of test species and poor randomisation, and solutions to improve this was proposed.

The present field study indicated no clear deployment status or time-dependent responses. However, site-specific negative responses on growth, Fv/Fm, and production of ROS were observed in Skuterud 2, located downstream of sedimentations ponds and European route 18, possibly caused by high water discharge combined with fragmenting of *F. antipyretica* due to the time of year. *F. antipyretica* have demonstrated to be a suitable bioindicator for detecting pollution in previous field studies. However, the present field study demonstrated no clear effects of pollution from agricultural and road runoff to *F. antipyretica* in stream Skuterudbekken, and no clear effect of handling deployed moss. Some technical limitations were identified, including too short acclimatisation, and challenges regarding deployment and attachment of moss nettings. Solutions to improve this were proposed.

Although *F. antipyretica* overall demonstrated to be a suitable test species, some solutions to improve general limitation are proposed. This includes executing studies in spring/summer to obtain effects on the AO growth, use moss tissue with little chance of being adapted to agricultural runoff, improve methods for randomisation, and repeat studies minimum three times, as these errors reduced accuracy and increased variation of the present study. As *F. antipyretica* is an ecologically important species, it is recommended to focus future studies on

effects of pollutants such as pesticides and heavy metals, and combinations of these, to assess ecologically relevant effects.

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Appendix I KNOP's medium

Knop's Solution

Introduction

Knop's solution is an ideal media for culturing *Oscillatoria*, *Oedogonium* and *Volvox*.

Materials

Calcium nitrate, $Ca(NO_3)_2$, 3 g
Magnesium sulfate, $MgSO_4$, 1 g
Potassium nitrate, KNO_3, 1 g
Potassium phosphate, dibasic, K_2HPO_4, 1 g
Sucrose, 50 g (optional)
Water, distilled or deionized (DI)
Balance, 1-g
Beaker, 1-L

Safety Precautions

Calcium nitrate is a strong oxidizer; a potential fire risk when in contact with organic material; and may explode when shocked or heated. Potassium nitrate is a strong oxidant; fire and explosion risk when heated or when in contact with organic material as well as a skin irritant. Wear chemical splash goggles, chemical-resistant gloves and a chemical-resistant apron whenever working with chemicals, heat or glassware. Wash hands thoroughly with soap and water before leaving the laboratory. Follow all laboratory safety guidelines. Please review current Material Safety Data Sheets for additional safety, handling and disposal information.

Procedure

1. Measure 1-L of distilled or deionized water into a 1-L beaker.
2. Mass 3 g of calcium nitrate. Add the calcium nitrate to the water.
3. Mass 1 g of each of the following chemicals—magnesium sulfate, potassium nitrate and potassium phosphate and add to the solution.
4. For immediate use add 5-L of DI water to the original stock solution. *Note:* This 1% solution may need to be shaken before use to mix undissolved salts.
5. Pour solution into desired containers and autoclave.
6. (Optional) Add 50 g of sucrose to 500 mL of Knop's solution to stimulate the formation of zoospores.

Disposal

Please consult your current *Flinn Scientific Catalog/Reference Manual* for general guidelines and specific procedures, and review all federal, state and local regulations that may apply. Any unused chemicals may be stored for future use. Excess prepared media may be disposed of down the drain with excess water according to Flinn Suggested Disposal Method #26b.

The materials needed to make *Knop's Solution* are available from Flinn Scientific, Inc.

Catalog No.	Description
C0350	Calcium Nitrate, 100 g
M0115	Magnesium Sulfate, 100 g
P0070	Potassium Nitrate, 100 g
P0142	Potassium Phosphate, dibasic, 100 g
K0003	Knop's Solution, 500 mL

Consult your *Flinn Scientific Catalog/Reference Manual* for current prices.

Publication No. 11021
061616

Appendix II Calculation of moss shoots needed

Field study:

Table III-1: Number of *Fontinalis antipyretica* shoots needed for the field study at Skuterud 1 and Skuterud 2, individually. Calculation: Total = (Day 0 analysis) + (Day 7 and 14 analysis: #Shoot replicates x #Locations (test stream + ref. stream) x #Time points)

		Day 7 and 14 analysis			
Endpoint analysis	**Day 0 analysis**	**#Shoot replicates**	**#Locations**	**#Time points**	**Total**
Growth change	-	6	2	-	6x2 = 12
Maximal PS II efficiency + Pigment concentration	6	6	2	2	6 + (6x2x2) = 30
Photosynthetic oxygen evolution + ROS production (day 14)	6	6	2	2	6 + (6x2x2) = 30
Total:					**72**

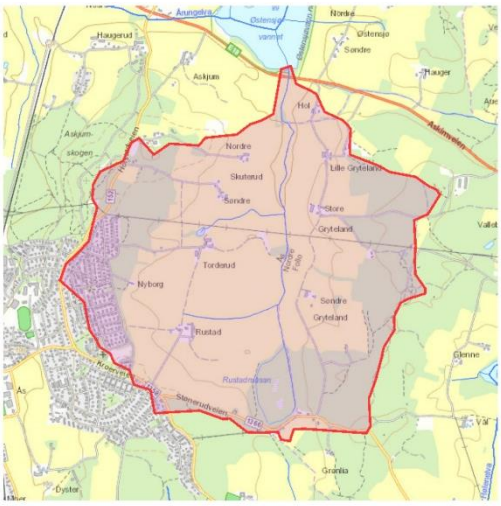
Lab study:

Table III-2: Number of *Fontinalis antipyretica* shoots needed for the lab study using copper sulphate ($CuSO_4$) and 3,5-dichlorophenol (3,5-DCP). Calculation: Total = (Day 0 analysis) + (Day 1, 7, 14 and 21 analysis #Shoot replicates x #Concentrations x #Time points)

		Day 1, 7, 14 and 21 analysis			
Endpoint analysis	**Day 0 analysis**	**#Shoot replicates**	**#Concentrations $CuSO_4$/3,5-DCP**	**#Time points**	**Total $CuSO_4$ / 3,5-DCP**
Growth change	-	5	6/7	-	5x6 = 30 / 5x7 = 35
Maximal PS II efficiency + Pigment concentration	5	5	6/7	4	5+(5x6x4) = 125 / 5+(5x7x4) = 145
ROS production	5	5	6/7	4	5+(5x6x4) = 125 / 5+(5x7x4) = 145
Total:					**280 / 325**

Appendix III Skuterudbekken and Sandbekken catchment areas

Skuterudbekken catchment area:



Norges vassdrags- og energidirektorat

Kartbakgrunn:	Statens Kartverk
Kartdatum:	EUREF89 WGS84
Projeksjon:	UTM 33N
Beregn.punkt:	265366 E 6623729 N

Nedbørfeltgrenser og feltparametere er automatisk generert og kan inneholde feil. Resultatene må kvalitetssikres.

Nedbørfeltparametere

Vassdragsnr.:	**005.3B**
Kommune.:	**Ås**
Fylke.:	**Viken**
Vassdrag.:	**Årungelva**

Feltparametere		
Areal (A)	4.7	km²
Effektiv sjø (A_{SE})	21.79	%
Elvleengde (E_L)	2.6	km
Elvegradient (E_G)	9.9	m/km
Elvegradient $_{1085}$ ($E_{G,1085}$)	10.8	m/km
Helning	2.4	°
Dreneringstetthet (D_T)	0.9	km^{-1}
Feltlengde (F_L)	2.7	km

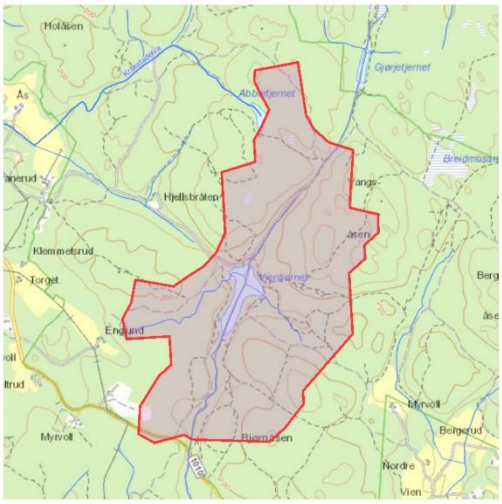
Arealklasse		
Bre (A_{BRE})	0	%
Dyrket mark (A_{JORD})	59.1	%
Myr (A_{MYR})	0.1	%
Leire (A_{LEIRE})	52.1	%
Skog (A_{SKOG})	32.3	%
Sjø (A_{SJO})	0.1	%
Snaufjell (A_{SF})	0	%
Urban (A_U)	5.7	%
Uklassifisert areal (A_{REST})	2.7	%

Hypsografisk kurve		
Høyde $_{MIN}$	89	m
Høyde $_{10}$	109	m
Høyde $_{20}$	116	m
Høyde $_{30}$	118	m
Høyde $_{40}$	119	m
Høyde $_{50}$	120	m
Høyde $_{60}$	125	m
Høyde $_{70}$	129	m
Høyde $_{80}$	132	m
Høyde $_{90}$	138	m
Høyde $_{MAX}$	149	m

Klima- /hydrologiske parametere		
Avrenning 1961-90 (Q_N)	17.2	l/s*km²
Sommernedbør	380	mm
Vinternedbør	391	mm
Årstemperatur	5.2	°C
Sommertemperatur	13.1	°C
Vintertemperatur	-0.5	°C

Rapportdato: 13.4.2021 © nevina.nve.no

Sandbekken catchment area:



Norges vassdrags- og energidirektorat

Kartbakgrunn:	Statens Kartverk
Kartdatum:	EUREF89 WGS84
Projeksjon:	UTM 33N
Beregn.punkt:	272232 E 6622699 N

Nedbørfeltgrenser og feltparametere er automatisk generert og kan inneholde feil. Resultatene må kvalitetssikres.

Nedbørfeltparametere

Vassdragsnr.:	**003.CZ**
Kommune.:	**Nordre Follo**
Fylke.:	**Viken**
Vassdrag.:	**Kråkstadelva**

Feltparametere		
Areal (A)	1.6	km²
Effektiv sjø (A_{SE})	1.73	%
Elvleengde (E_L)	1.9	km
Elvegradient (E_G)	3.8	m/km
Elvegradient $_{1085}$ ($E_{G,1085}$)	11.6	m/km
Helning	6.1	°
Dreneringstetthet (D_T)	1.8	km^{-1}
Feltlengde (F_L)	2.2	km

Arealklasse		
Bre (A_{BRE})	0	%
Dyrket mark (A_{JORD})	0	%
Myr (A_{MYR})	0.7	%
Leire (A_{LEIRE})	33.6	%
Skog (A_{SKOG})	96.9	%
Sjø (A_{SJO})	2.2	%
Snaufjell (A_{SF})	0	%
Urban (A_U)	0.3	%
Uklassifisert areal (A_{REST})	0	%

Hypsografisk kurve		
Høyde $_{MIN}$	178	m
Høyde $_{10}$	180	m
Høyde $_{20}$	183	m
Høyde $_{30}$	187	m
Høyde $_{40}$	193	m
Høyde $_{50}$	199	m
Høyde $_{60}$	207	m
Høyde $_{70}$	218	m
Høyde $_{80}$	226	m
Høyde $_{90}$	233	m
Høyde $_{MAX}$	248	m

Klima- /hydrologiske parametere		
Avrenning 1961-90 (Q_N)	18.4	l/s*km²
Sommernedbør	380	mm
Vinternedbør	408	mm
Årstemperatur	4.6	°C
Sommertemperatur	12.4	°C
Vintertemperatur	-0.9	°C

Rapportdato: 13.4.2021 © nevina.nve.no

Appendix IV Pesticide analysis by NIBIO

NORWEGIAN ACCREDITATION TEST 035

NIBIO NORSK INSTITUTT FOR BIOØKONOMI

Gjelder fra: 03.03.2021

Søkespekter for multimetoder vann M15 og M101

Monitoring programme multi-methods water M15 and M101

Pesticid	Pesticide	Class	LOQ µg/L	Method	Comments
2,4-D	2,4-D	H	0,01	M15	
Abamektin	Abamectin	I	0,05	M101/ LC	
Acetamiprid	Acetamiprid	I	0,01	M101/ LC	Ikke akkreditert
Aklonifen	Aclonifen	H	0,01	M101/ GC	
Aldrin	Aldrin	I	0,05	M101/ GC	Ikke akkreditert
Alfacypermetrin	Alpha-cypermethrin	I	0,05	M101/ GC	
Atrazin	Atrazine	H	0,01	M101/ LC	
Atrazin desetyl	Atrazine-desethyl	M	0,05	M101/ LC	
Atrazin desisopropyl	Atrazine-desisopropyl	M	0,05	M101/ LC	
Azinfosmetyl	Azinphos-methyl	I	0,01	M101/ LC	
Azoksystrobin	Azoxystrobin	F	0,01	M101/ LC	
BAM (2,6-diklorbenzamid)	BAM (2,6-dichlorobenzamide)	M	0,01	M101/ LC	Metabolitt av diklobenil og fluopikolid
Bentazon	Bentazone	H	0,01	M15	
Benzovindiflupyr	Benzovindiflupyr	F	0,01	M101/ LC	Ikke akkreditert
Bifenazat	Bifenazate	I	0,01	M101/ LC	Ikke akkreditert
Biksafen	Bixafen	F	0,01	M101/ LC	
Bitertanol	Bitertanol	F	0,01	M101/ LC	
Boskalid	Boscalid	F	0,01	M101/ GC	
Cyazofamid	Cyazofamid	F	0,01	M101/ LC	
Cyflutrin beta	Cyfluthrin beta	I	0,01	M101/ GC	
Cymoksanil	Cymoxanil	F	0,01	M101/ LC	Ikke akkreditert
Cyprodinil	Cyprodinil	F	0,01	M101/ GC	
Cyprokonazol	Cyproconazole	F	0,01	M101/ LC	
DDD-o,p'	DDD-o,p'	M	0,01	M101/ GC	
DDD-p,p'	DDD-p,p'	M	0,01	M101/ GC	
DDE-o,p'	DDE-o,p'	M	0,01	M101/ GC	
DDE-p,p'	DDE-p,p'	M	0,01	M101/ GC	
DDT-o,p'	DDT-o,p'	I	0,01	M101/ GC	
DDT-p,p'	DDT-p,p'	I	0,01	M101/ GC	
Deltametrin	Deltamethrin	I	0,02	M101/ LC	
Diazinon	Diazinon	I	0,01	M101/ GC	
Dieldrin	Dieldrin	I	0,05	M101/ GC	
Difenokonazol	Difenoconazole	F	0,01	M101/ LC	
Difenokonazol metabolitt CGA205375	Difenoconazole metabolite CGA205375	M	0,01	M101/ LC	
Diflubenzuron	Diflubenzuron	I	0,01	M101/ LC	
Diflufenikan	Diflufenican	H	0,01	M101/ LC	
Dikamba	Dicamba	H	0,02	M15	
Diklorprop	Dichlorprop	H	0,01	M15	
Dimetoat	Dimethoate	I	0,01	M101/ LC	
Dimetomorf	Dimethomorph	F	0,01	M101/ LC	
Dodin	Dodine	F	0,01	M101/ LC	Ikke akkreditert
Endosulfan alfa	Endosulfan alpha	I	0,01	M101/ GC	
Endosulfan beta	Endosulfan beta	I	0,05	M101/ GC	
Endosulfan sulfat	Endosulfan-sulfate	M	0,05	M101/ GC	
Fenamidon	Fenamidone	F	0,01	M101/ LC	
Fenheksamid	Fenhexamid	F	0,01	M101/ LC	
Fenitrotion	Fenitrothion	I	0,01	M101/ GC	
Fenmedifam	Phenmedipham	H	0,01	M101/ LC	Ikke akkreditert
Fenpropidin	Fenpropidin	F	0,01	M101/ LC	
Fenpropimorf	Fenpropimorph	F	0,01	M101/ LC	
Fenpyroksimat	Fenpyroximate	I	0,01	M101/ LC	
Fenvalerat	Fenvalerate	I	0,01	M101/ GC	
Flamprop	Flamprop	H	0,1	M15	
Flonikamid	Flonicamid	I	0,01	M101/ LC	Ikke akkreditert

Pesticid	Pesticide	Class	LOQ µg/L	Method	Comments
Florasulam	Florasulam	H	0,01	M101/ LC	Ikke akkreditert
Fluazinam	Fluazinam	F	0,01	M101/ GC	Ikke akkreditert
Fludioksonil	Fludioxonil	F	0,01	M101/ LC	
Flumetrin	Flumethrin	I	0,01	M101/ LC	
Fluopyram	Fluopyram	F	0,01	M101/ LC	Ikke akkreditert
Fluroksypyr	Fluroxypyr	H	0,05	M15	
Halauksifen-metyl	Halauxifen-methyl	H	0,01	M101/ LC	Ikke akkreditert
Heksaflumuron	Hexaflumuron	I	0,01	M101/ LC	
Heksaklorbenzen (HCB)	Hexachlorobenzene (HCB)	F	0,05	M101/ GC	Ikke akkreditert
Heksytiasoks	Hexythiazox	I	0,01	M101/ LC	
Heptaklor	Heptachlor	I	0,05	M101/ GC	
Heptaklor epoksid trans	Heptachlor-epoxide trans	M	0,01	M101/ GC	
Imazalil	Imazalil	F	0,02	M101/ LC	
Imidakloprid	Imidacloprid	I	0,01	M101/ LC	
Indoksakarb	Indoxacarb	I	0,02	M101/ LC	
Iprodion	Iprodione	F	0,02	M101/ LC	
Isofenfos	Isofenphos	I	0,01	M101/ LC	
Isoksaben	Isoxaben	H	0,01	M101/ LC	Ikke akkreditert
Isoproturon	Isoproturon	H	0,01	M101/ LC	
Karbendazim	Carbendazim	F	0,01	M101/ LC	
Karfentrazon-etyl	Carfentrazone-ethyl	H	0,01	M101/ LC	Ikke akkreditert
Klofentezin	Clofentezine	I	0,01	M101/ LC	
Klomazon	Clomazone	H	0,01	M101/ LC	
Klopyralid	Clopyralid	H	0,05	M15	
Klorantraniliprol	Chlorantraniliprole	I	0,01	M101/ LC	
Klorfenvinfos	Chlorfenvinphos	I	0,01	M101/ LC	
Klorprofam	Chlorpropham	G	0,01	M101/ GC	
Kresoksimmetyl	Kresoxim-methyl	F	0,01	M101/ LC	
Lambdacyhalotrin	Lambda-cyhalothrin	I	0,05	M101/ GC	Ikke akkreditert
Lindan (HCH gamma)	Lindane (HCH gamma)	I	0,01	M101/ GC	
Linuron	Linuron	H	0,01	M101/ LC	
MCPA	MCPA	H	0,01	M15	
Mandipropamid	Mandipropamid	F	0,01	M101/ LC	
Mekoprop	Mecoprop	H	0,01	M15	
Mepanipyrim	Mepanipyrim	F	0,01	M101/ LC	
Metalaksyl	Metalaxyl	F	0,01	M101/ GC	
Metamitron	Metamitron	H	0,01	M101/ LC	
Metiokarb	Methiocarb	I	0,01	M101/ LC	Ikke akkreditert
Metiokarb sulfoksid	Methiocarb-sulfoxide	M	0,02	M101/ LC	Ikke akkreditert
Metiokarb sulfon	Methiocarb-sulfone	M	0,01	M101/ LC	Ikke akkreditert
Metribuzin	Metribuzin	H	0,01	M101/ LC	
Paklobutrazol	Paclobutrazol	G	0,01	M101/ LC	
Pencykuron	Pencycuron	F	0,01	M101/ LC	
Penkonazol	Penconazole	F	0,01	M101/ LC	
Permetrin	Permethrin	I	0,05	M101/ LC	Ikke akkreditert
Pikoksystrobin	Picoxystrobin	F	0,01	M101/ GC	
Pinoksaden	Pinoxaden	H	0,01	M101/ LC	Ikke akkreditert
Pirimikarb	Pirimicarb	I	0,01	M101/ LC	
Pirimikarb desmetyl	Pirimicarb desmethyl	M	0,01	M101/ LC	
Pirimikarb desmetyl formamido	Pirimicarb desmethyl formamido	M	0,01	M101/ LC	
Prokloraz	Prochloraz	F	0,01	M101/ LC	
Prokvinazid	Proquinazid	F	0,01	M101/ LC	Ikke akkreditert
Prokvinazid metabolitt	Proquinazid metabolite	M	0,01	M101/ LC	IN MM671
Propaklor	Propachlor	H	0,01	M101/ GC	
Propakvizafop	Propaquizafop	H	0,01	M101/ LC	
Propamokarb	Propamocarb	F	0,01	M101/ LC	
Propikonazol	Propiconazole	F	0,01	M101/ LC	
Propoksykarbazon	Propoxycarbazone	H	0,01	M101/ LC	
Prosulfokarb	Prosulfocarb	H	0,01	M101/ LC	
Protiokonazol-destio	Prothioconazole-desthio	M	0,01	M101/ LC	
Pyraklostrobin	Pyraclostrobin	F	0,01	M101/ LC	
Pyretriner	Pyrethrins	I	0,01	M101/ LC	Ikke akkreditert
Pyridat	Pyridate	H	0,05	M101/ LC	Ikke akkreditert

Pesticid	Pesticide	Class	LOQ µg/L	Method	Comments
Pyridat metabolitt	Pyridate metabolite	M	0,01	M101/ LC	6-klor-4-hydroksy-3-fenylpyridazin
Pyrimetanil	Pyrimethanil	F	0,01	M101/ GC	
Pyriproksyfen	Pyriproxyfen	F	0,01	M101/ GC	
Pyroksulam	Pyroxsulam	H	0,01	M101/ LC	Ikke akkreditert
Simazin	Simazine	H	0,01	M101/ LC	Ikke akkreditert
Spinosad	Spinosad	I	0,01	M101/ LC	
Spirodiklofen	Spirodiclofen	I	0,01	M101/ LC	
Spirotetramat	Spirotetramat	I	0,01	M101/ LC	Ikke akkreditert
Sykloksydim	Cycloxydim	H	0,01	M101/ LC	Ikke akkreditert
Tau-fluvalinat	Tau-fluvalinate	I	0,01	M101/ LC	Ikke akkreditert
Tebukonazol	Tebuconazole	F	0,01	M101/ LC	
Terbutylazin	Terbuthylazine	H	0,01	M101/ GC	
Tiabendazol	Thiabendazole	F	0,02	M101/ LC	
Tiakloprid	Thiacloprid	I	0,01	M101/ LC	
Tiodikarb	Thiodicarb	I	0,01	M101/ LC	Ikke akkreditert
Tolklofosmetyl	Tolclofos-methyl	F	0,01	M101/ GC	
Trifloksystrobin	Trifloxystrobin	F	0,01	M101/ LC	
Trineksapak-etyl	Trinexapac-ethyl	G	0,01	M101/ LC	Ikke akkreditert
Trisyklazol	Tricyclazole	F	0,01	M101/ LC	
Tritikonazol	Triticonazole	F	0,01	M101/ LC	
Vinklozolin	Vinclozolin	F	0,01	M101/ GC	
Zoksamid	Zoxamide	F	0,01	M101/ LC	

M15: 9 stoffer **M101: 130 stoffer**

H: Herbicide F: Fungicide I : Insecticide M: Metabolite G: Growth regulator/ vekstregulator

Prøvene bør tas og oppbevares på glassflasker.

LOQ: Limit of quantification / kvantifiseringsgrense:
Den laveste konsentrasjonen av stoffet som kan bestemmes kvantitativt med metoden. For multimetoder oppgis bare de pesticider som påvises ved analysen. De andre pesticidene som metoden omfatter, er da ikke påvist over kvantifiseringsgrensen. Dersom analyseresultatet er oppgitt som "Ikke påvist" for en metode, betyr det at ingen av stoffene som metoden omfatter er funnet i konsentrasjoner over kvantifiseringsgrensen.

Måleusikkerhet:
Opplysninger om måleusikkerhet kan fås ved henvendelse til laboratoriet.

Appendix V Statistical overview

Software used for statistical tests

Jamovi	Normality test (Shapiro-Wilk)
Jamovi	Homogeneity of variance test (Levene's)
Jamovi	Parametric One-Way ANOVA (Welch's/Fisher's) and Post-Hoc test (Games-Howell/Tukey)
RStudio	Non-parametric One-Way ANOVA (Kruskal-Wallis) and Post-Hoc test (Kruskal-Wallis)
RStudio	Concentration-response modelling
RStudio	LOEC/NOEC derivation
RStudio	EC50 derivation

CT groups $CuSO_4$	**Trans-formation used**	**Normally distributed (Y/N)**	**Homogen variances (Y/N)**	**Type of One-Way ANOVA**	**Type of pairwise comparison**	**p-value (One-Way ANOVA)**
Greenness index inhibition (%)	None	Y	N	Parametric, Welch's	Games-Howell	0.016
PS II inhibition (%)	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
Chl a inhibition (%)	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
Chl b inhibition (%)	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
Car inhibition (%)	None	Y	N	Parametric, Welch's	Games-Howell	0.006
Chl a/b ratio	None	Y	N	Parametric, Welch's	Games-Howell	0.005
ROS fold increase	Log	Y	N	Parametric, Welch's	Games-Howell	0.018
CT groups 3,5-DCP						
Greenness index inhibition (%)	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
PS II inhibition (%)	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
Chl a inhibition (%)	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
Chl b inhibition (%)	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
Car inhibition (%)	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
Chl a/b ratio	None	Y	N	Parametric, Welch's	Games-Howell	0.123
ROS fold increase	Log	Y	N	Parametric, Welch's	Games-Howell	<0.001

Lab study – $CuSO_4$		**Trans-formation used**	**Normally distributed (Y/N)**	**Homogen variances (Y/N)**	**Type of One-Way ANOVA**	**Type of pairwise comparison**	**p-value (One-Way ANOVA)**
Growth inhibition (%)	Day 21	None	Y	N	Parametric, Welch's	Games-Howell	0.482
Greenness index	Day 21	None	N	Y	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	<0.001

inhibition (%)							
Fv/Fm inhibition (%)	Day 1	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
	Day 7	None	N	Y	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	<0.001
	Day 14	None	N	Y	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	<0.001
	Day 21	None	N	Y	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	0.002
Chl a inhibition (%)	Day 1	None	Y	N	Parametric, Welch's	Games-Howell	0.325
	Day 7	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
	Day 14	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
	Day 21	None	N	Y	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	<0.001
Chl b inhibition (%)	Day 1	None	Y	N	Parametric, Welch's	Games-Howell	0.440
	Day 7	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
	Day 14	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
	Day 21	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
Car inhibition (%)	Day 1	None	Y	Y	Parametric, Fisher's	Tukey	0.413
	Day 7	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
	Day 14	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
	Day 21	None	N	Y	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	<0.001
Chl a/b ratio relative change (%)	Day 1	Log	Y	Y	Parametric, Fisher's	Tukey	0.101
	Day 7	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
	Day 14	None	N	N	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	<0.001
	Day 21	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
Chl a/b ratio	Day 1	None	N	N	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	0.082
	Day 7	Log	Y	Y	Parametric, Fisher's	Tukey	<0.001
	Day 14	None	N	N	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	<0.001
	Day 21	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
ROS fold increase	Day 1	None	Y	Y	Parametric, Fisher's	Tukey	0.039
	Day 7	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
	Day 14	Log	Y	N	Parametric, Welch's	Games-Howell	<0.001
	Day 21	None	N	Y	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	0.011

Lab study – 3,5-DCP		Trans-formation used	Normally distributed (Y/N)	Homogen variances (Y/N)	Type of One-Way ANOVA	Type of pairwise comparison	p-value (One-Way ANOVA)
Growth inhibition	Day 21	None	Y	N	Parametric, Welch's	Games-Howell	0.003
Greenness index inhibition (%)	Day 21	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
Fv/Fm inhibition (%)	Day 1	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
	Day 7	None	N	Y	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	<0.001
	Day 14	Sqrt (x+52)	Y	N	Parametric, Welch's	Games-Howell	<0.001
	Day 21	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
Chl a inhibition (%)	Day 1	None	Y	Y	Parametric, Fisher's	Tukey	0.036
	Day 7	None	Y	N	Parametric, Welch's	Games-Howell	0.042
	Day 14	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
	Day 21	None	N	Y	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	0.007
Chl b inhibition (%)	Day 1	None	Y	N	Parametric, Welch's	Games-Howell	0.329
	Day 7	None	Y	Y	Parametric, Fisher's	Tukey	0.002
	Day 14	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
	Day 21	None	N	Y	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	0.001
Car inhibition (%)	Day 1	None	N	N	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	0.066
	Day 7	None	Y	N	Parametric, Welch's	Games-Howell	0.014
	Day 14	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
	Day 21	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
Chl a/b ratio relative change	Day 1	Sqrt (x+12)	Y	N	Parametric, Welch's	Games-Howell	0.330
	Day 7	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
	Day 14	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
	Day 21	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
Chl a/b ratio	Day 1	None	N	N	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	0.302
	Day 7	None	Y	Y	Parametric, Fisher's	Tukey	<0.001
	Day 14	None	Y	N	Parametric, Welch's	Games-Howell	<0.001
	Day 21	None	Y	N	Parametric, Welch's	Games-Howell	<0.001

ROS fold increase	Day 1	None	N	Y	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	0.764
	Day 7	Log	Y	N	Parametric, Welch's	Games-Howell	0.488
	Day 14	Log	Y	N	Parametric, Welch's	Games-Howell	0.123
	Day 21	Log	Y	N	Parametric, Welch's	Games-Howell	0.012

Field study		**Trans-formation used**	**Normally distributed (Y/N)**	**Homogen variances (Y/N)**	**Type of One-Way ANOVA**	**Type of pairwise comparison**	**p-value (One-Way ANOVA)**
Growth (shoot length diff.)	Day 14 - 0 diff.	None	Y	Y	Parametric, Fisher's	Tukey	0.065
Fv/Fm	Day 0	None	Y	N	Parametric, Welch's	Games-Howell	0.001
	Day 7	None	Y	N	Parametric, Welch's	Games-Howell	0.008
	Day 14	None	N	Y	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	0.009
Chl *a* concentration	Day 0	No statistical results due to n=2					
	Day 7	Log	Y	N	Parametric, Welch's	Games-Howell	0.608
	Day 14	Log	Y	N	Parametric, Welch's	Games-Howell	0.848
Chl *b* concentration	Day 0	No statistical results due to n=2					
	Day 7	Log	Y	N	Parametric, Welch's	Games-Howell	0.955
	Day 14	None	Y	N	Parametric, Welch's	Games-Howell	0.240
Car concentration	Day 0	No statistical results due to n=2					
	Day 7	None	Y	N	Parametric, Welch's	Games-Howell	0.434
	Day 14	None	Y	N	Parametric, Welch's	Games-Howell	0.333
Chl *a/b* ratio	Day 0	No statistical results due to n=2					
	Day 7	None	N	N	Nonparametric Kruskal-Wallis	Kruskal-Wallis, wilcox	0.767
	Day 14	None	Y	N	Parametric, Welch's	Games-Howell	0.035
Oxygen production	Day 0-14	No statistical results due to n=2					
ROS fold increase	Day 14	Log	Y	N	Parametric, Welch's	Games-Howell	<0.001

Appendix VI Raw data

Lab study – $CuSO_4$

Growth and colour (GI)

			Colour shoots			Colour background av.		
Conc. (µM)	Day	Growth day21-day0 (mm)	Red	Green	Blue	Red	Green	Blue
0	0		88	106	48	195	196	193
0	0		65	83	32			
0	0		77	89	44			
0	0		74	91	37			
0	0		66	78	34			
0	21	0.36	79	70	36	195	195	188
0	21	1.96	69	67	32			
0	21	1.17	59	55	30			
0	21	-0.74	61	53	29			
0	21	0.04	29	35	23			
3	21	3.38	95	95	35			
3	21	3.58	97	97	37			
3	21	0.32	94	97	35			
3	21	1.84	66	76	24			
3	21	0.01	37	44	19			
10	21	0.58	92	76	42			
10	21	-0.25	119	97	60			
10	21	-0.49	111	97	56			
10	21	-0.24	84	73	38			
10	21	0.64	59	51	28			
30	21	1.48	86	70	44			
30	21	0.17	107	83	50			
30	21	0.27	104	86	51			
30	21	0.16	106	86	51			
30	21	-1.68	60	47	28			
100	21	0.27	120	97	62			
100	21	0.94	111	88	51			
100	21	0.34	93	74	44			
100	21	-0.86	91	70	40			
100	21	0.30	87	67	38			
300	21	0.68	106	79	42			
300	21	-1.33	109	85	51			
300	21	-0.44	90	66	35			
300	21	1.35	88	62	34			
300	21	2.24	99	77	43			

Fv/Fm, pigment concentration, ROS

			Pigment concentration				ROS	
Conc. (µM)	Day	Fv/Fm	Weight (mg)	WL649	WL665	WL480	Weight (mg)	ROS minus blank
0	0	0.722	2.70	0.177	0.409	0.306	0.97	4.046
0	0	0.618	1.11	0.065	0.140	0.103	1.45	10.557
0	0	0.702	1.47	0.085	0.186	0.131	1.31	10.807
0	0	0.720	2.79	0.191	0.441	0.313	1.13	7.162
0	0	0.693	1.76	0.092	0.171	0.153	0.70	9.746
0	1	0.612	4.13	0.156	0.329	0.255	1.62	14.175
0	1	0.615	3.15	0.144	0.312	0.252	0.94	9.925
0	1	0.644	4.76	0.176	0.391	0.310	0.62	4.689
0	1	0.657	3.98	0.157	0.331	0.242	0.55	6.333
0	1	0.605	3.54	0.130	0.290	0.207	0.59	2.507
3	1	0.630	3.53	0.165	0.356	0.284	0.77	6.994
3	1	0.617	2.55	0.111	0.242	0.198	0.50	4.597
3	1	0.631	1.90	0.059	0.110	0.100	0.79	8.462
3	1	0.584	3.10	0.170	0.361	0.288	1.66	26.725
3	1	0.622	4.65	0.192	0.427	0.325	1.51	11.655
10	1	0.596	3.15	0.109	0.245	0.243	0.77	4.130
10	1	0.641	2.97	0.127	0.275	0.232	1.24	12.625
10	1	0.647	7.18	0.184	0.391	0.322	0.70	8.309
10	1	0.649	3.28	0.152	0.344	0.310	0.50	11.545
10	1	0.597	3.91	0.071	0.145	0.130	1.14	6.289
30	1	0.605	2.80	0.126	0.271	0.238	0.80	9.097
30	1	0.547	3.30	0.138	0.312	0.290	0.47	10.915
30	1	0.537	5.16	0.198	0.425	0.394	2.81	17.935
30	1	0.461	3.60	0.144	0.313	0.271	0.45	6.568
30	1	0.570	4.80	0.194	0.431	0.393	0.90	15.945
100	1	0.452	1.06	0.045	0.090	0.075	1.00	14.775
100	1	0.413	2.61	0.136	0.286	0.277	0.54	17.245
100	1	0.477	2.76	0.096	0.185	0.155	0.67	18.105
100	1	0.407	3.84	0.135	0.289	0.258	2.22	24.705
100	1	0.557	2.42	0.104	0.224	0.205	1.65	24.455
300	1	0.388	3.74	0.143	0.283	0.256	0.71	20.295
300	1	0.489	2.91	0.070	0.123	0.102	0.75	9.665
300	1	0.416	5.29	0.164	0.341	0.294	0.38	7.821
300	1	0.404	1.97	0.062	0.133	0.143	1.09	12.745
300	1	0.428	1.76	0.079	0.165	0.148	0.53	23.515
0	7	0.545	3.08	0.119	0.254	0.204	0.46	5.199
0	7	0.515	1.93	0.083	0.182	0.157	1.16	15.543
0	7	0.630	1.49	0.066	0.136	0.112	0.67	5.097
0	7	0.597	2.38	0.094	0.212	0.205	0.16	3.756
0	7	0.535	1.92	0.089	0.198	0.192	0.35	4.254
3	7	0.670	3.71	0.173	0.373	0.288	0.96	9.053
3	7	0.640	1.47	0.058	0.124	0.114	0.88	8.143
3	7	0.689	4.31	0.135	0.293	0.242	1.01	13.763

3	7	0.626	2.39	0.074	0.157	0.162	0.58	6.929
3	7	0.663	5.49	0.201	0.424	0.306	1.32	12.153
10	7	0.557	2.98	0.105	0.219	0.219	NA	22.543
10	7	0.604	2.49	0.040	0.079	0.077	0.96	33.223
10	7	0.589	6.90	0.184	0.384	0.378	0.68	24.743
10	7	0.614	3.95	0.107	0.212	0.218	0.55	24.263
10	7	0.539	4.58	0.187	0.358	0.234	0.23	6.429
30	7	0.520	3.70	0.057	0.105	0.107	NA	11.473
30	7	0.000	2.67	0.025	0.041	0.042	0.45	27.643
30	7	0.000	1.96	0.029	0.049	0.052	1.68	44.563
30	7	0.514	4.22	0.058	0.096	0.112	1.02	42.413
30	7	0.000	3.81	0.030	0.052	0.053	0.48	29.263
100	7	0.000	3.16	0.024	0.037	0.041	NA	15.513
100	7	0.000	4.16	0.057	0.074	0.083	0.61	19.823
100	7	0.000	2.28	0.009	0.014	0.016	0.35	9.838
100	7	0.000	2.46	0.028	0.038	0.042	0.72	43.163
100	7	0.000	3.02	0.031	0.046	0.054	0.50	13.583
300	7	0.000	3.19	0.016	0.027	0.032	0.39	8.995
300	7	0.000	2.43	0.018	0.026	0.030	0.78	31.223
300	7	0.000	5.41	0.045	0.073	0.076	0.14	9.410
300	7	0.000	3.25	0.025	0.033	0.040	0.29	10.313
300	7	0.000	1.48	0.020	0.026	0.036	0.43	9.614
0	14	0.239	3.52	0.114	0.238	0.179	0.69	12.491
0	14	0.172	5.07	0.145	0.249	0.170	0.42	6.246
0	14	0.313	1.72	0.069	0.146	0.130	0.55	6.886
0	14	0.350	2.76	0.106	0.205	0.135	0.26	5.706
0	14	0.389	4.73	0.111	0.228	0.133	1.45	24.031
3	14	0.606	2.56	0.127	0.268	0.243	0.42	10.711
3	14	0.542	2.38	0.077	0.153	0.146	1.36	11.031
3	14	0.536	3.07	0.110	0.178	0.167	0.47	3.667
3	14	0.554	1.64	0.053	0.111	0.130	0.30	3.082
3	14	0.629	2.83	0.107	0.217	0.179	0.45	3.355
10	14	0.443	1.74	0.031	0.056	0.066	0.91	19.011
10	14	0.488	3.37	0.093	0.186	0.201	0.64	39.821
10	14	0.569	2.91	0.028	0.054	0.060	0.84	41.741
10	14	0.527	2.88	0.045	0.084	0.088	0.40	33.071
10	14	0.554	4.55	0.115	0.225	0.207	0.46	24.601
30	14	0.000	1.51	0.014	0.020	0.032	0.83	54.241
30	14	0.000	2.49	0.012	0.019	0.023	0.54	25.741
30	14	0.000	5.14	0.073	0.126	0.128	0.30	15.241
30	14	0.000	2.39	0.026	0.036	0.044	0.89	41.711
30	14	0.000	4.31	0.039	0.069	0.073	0.22	10.901
100	14	0.000	3.00	0.021	0.026	0.043	0.78	29.001
100	14	0.000	1.92	0.012	0.016	0.023	0.64	11.661
100	14	0.000	4.07	0.012	0.017	0.021	0.22	16.711
100	14	0.000	3.51	0.013	0.018	0.029	0.77	18.961
100	14	0.000	2.70	0.019	0.028	0.035	0.65	22.821

300	14	0.000	3.01	0.009	0.010	0.015	0.63	17.591
300	14	0.000	4.40	0.022	0.025	0.036	0.73	16.611
300	14	0.000	1.88	0.011	0.013	0.028	0.74	18.341
300	14	0.000	4.40	0.013	0.015	0.021	0.34	8.928
300	14	0.000	1.44	0.015	0.015	0.035	0.71	9.484
0	21	0.385	3.78	0.091	0.211	0.192	0.57	19.139
0	21	0.452	4.06	0.083	0.205	0.176	0.58	9.531
0	21	0.313	7.13	0.27	0.649	0.552	0.61	8.571
0	21	0.355	5.97	0.185	0.433	0.468	0.24	26.559
0	21	0.412	3.67	0.144	0.331	0.371	1.12	14.129
3	21	0.489	1.11	0.023	0.047	0.046	0.43	6.706
3	21	0.619	3.21	0.152	0.328	0.319	0.70	6.119
3	21	0.357	3.62	0.151	0.313	0.313	0.56	4.157
3	21	0.566	1.93	0.035	0.073	0.071	0.55	5.155
3	21	0.610	2.67	0.104	0.222	0.245	0.25	6.012
10	21	0.000	2.55	0.017	0.035	0.04	0.84	26.779
10	21	0.000	4.15	0.031	0.065	0.074	0.49	19.029
10	21	0.380	4.34	0.082	0.16	0.172	0.68	26.589
10	21	0.000	1.80	0.013	0.024	0.026	0.22	6.482
10	21	0.433	1.73	0.023	0.048	0.051	0.29	8.900
30	21	0.000	2.78	0.016	0.024	0.026	0.27	9.758
30	21	0.000	1.88	0.014	0.02	0.022	0.92	24.549
30	21	0.000	3.93	0.03	0.052	0.06	0.10	8.974
30	21	0.613	3.22	0.026	0.047	0.046	0.32	18.129
30	21	0.000	3.66	0.031	0.046	0.048	0.06	6.496
100	21	0.000	1.99	0.009	0.012	0.014	0.57	13.739
100	21	0.000	2.86	0.012	0.016	0.018	0.26	8.629
100	21	0.000	2.92	0.014	0.016	0.022	1.09	22.239
100	21	0.000	2.10	0.015	0.015	0.035	0.79	19.149
100	21	0.000	2.92	0.014	0.019	0.024	0.32	8.423
300	21	0.000	2.22	0.009	0.007	0.014	0.90	12.549
300	21	0.000	2.26	0.014	0.013	0.021	0.43	4.811
300	21	0.000	3.55	0.012	0.014	0.014	0.30	23.179
300	21	0.000	2.54	0.008	0.007	0.01	0.28	7.165
300	21	0.000	2.01	0.008	0.008	0.009	1.03	14.329

Lab study – 3,5-dichlorophenol

Growth and colour (GI)

			Colour shoots			Colour background av.		
Conc. (mg/L)	Day	Growth day21-day0 (mm)	Red	Green	Blue	Red	Green	Blue
0 (CT)	0		88	104	46	213	213	202
0 (CT)	0		70	78	35			
0 (CT)	0		70	83	32			
0 (CT)	0		64	76	33			
0 (CT)	0		81	94	37			
0 (CT)	21	0.8	59	61	52	202	201	192
0 (CT)	21	0.9	73	66	47			
0 (CT)	21	0.1	83	73	51			
0 (CT)	21	0.6	86	79	54			
0 (CT)	21	1	68	65	48			
0 (S-CT)	21	0.9	66	67	48			
0 (S-CT)	21	0.4	95	88	51			
0 (S-CT)	21	-0.2	71	70	42			
0 (S-CT)	21	0.6	75	72	42			
0 (S-CT)	21	1.5	66	63	39			
0.1	21	-0.4	65	66	40			
0.1	21	-1	77	73	41			
0.1	21	0	66	61	38			
0.1	21	0.3	75	67	40			
0.1	21	0.8	65	66	37			
0.3	21	-0.3	61	57	38			
0.3	21	-0.1	91	80	40			
0.3	21	-0.1	54	53	35			
0.3	21	-0.1	82	71	38			
0.3	21	0	54	56	36			
1	21	-0.1	64	61	35			
1	21	0	63	57	35			
1	21	0.9	56	54	35			
1	21	-0.6	50	48	32			
1	21	0.1	69	69	33			
3	21	-0.9	81	78	38			
3	21	-0.9	86	83	35			
3	21	-0.4	76	79	34			
3	21	-1.2	82	84	32			
3	21	-0.3	76	77	33			
9	21	-0.7	163	122	76			
9	21	-1.7	160	119	72			
9	21	-1.3	159	121	79			
9	21	0	169	139	99			
9	21	-2.3	176	148	103			

Fv/Fm, pigment concentration, ROS

			Pigment concentration				ROS	
Conc. (mg/L)	Day	Fv/Fm	Weight (mg)	WL649	WL665	WL480	Weight (mg)	ROS minus blank
0 (CT)	0	0.597	4.99	0.205	0.446	0.360	0.39	7.068
0 (CT)	0	0.654	1.17	0.044	0.101	0.088	0.53	6.237
0 (CT)	0	0.652	1.85	0.087	0.195	0.167	0.37	4.186
0 (CT)	0	0.653	3.19	0.135	0.281	0.226	0.51	3.239
0 (CT)	0	0.701	1.77	0.070	0.152	0.120	0.33	3.277
0 (CT)	1	0.653	1.28	0.059	0.131	0.103	0.83	7.155
0 (CT)	1	0.595	1.76	0.090	0.180	0.146	0.69	17.789
0 (CT)	1	0.617	2.46	0.105	0.229	0.174	0.36	3.153
0 (CT)	1	0.560	1.83	0.116	0.255	0.219	0.36	2.117
0 (CT)	1	0.642	0.80	0.060	0.134	0.109	0.64	10.069
0 (S-CT)	1	0.610	1.72	0.079	0.174	0.136	0.26	4.392
0 (S-CT)	1	0.654	3.77	0.139	0.315	0.243	0.13	1.525
0 (S-CT)	1	0.598	2.52	0.125	0.270	0.198	0.36	5.205
0 (S-CT)	1	0.566	2.33	0.125	0.279	0.222	0.66	9.056
0 (S-CT)	1	0.561	4.80	0.226	0.481	0.357	0.17	3.174
0.1	1	0.635	1.40	0.073	0.160	0.124	0.45	6.388
0.1	1	0.565	1.91	0.109	0.239	0.210	1.19	19.719
0.1	1	0.617	3.11	0.156	0.339	0.261	0.83	9.009
0.1	1	0.642	2.50	0.109	0.240	0.194	0.20	1.580
0.1	1	0.658	3.01	0.096	0.218	0.172	0.70	6.273
0.3	1	0.547	3.04	0.126	0.275	0.207	0.42	4.655
0.3	1	0.607	3.43	0.177	0.370	0.254	0.60	3.939
0.3	1	0.590	1.26	0.069	0.153	0.133	0.70	11.299
0.3	1	0.625	2.03	0.068	0.146	0.116	0.65	11.519
0.3	1	0.598	3.06	0.104	0.211	0.170	0.58	11.399
1	1	0.546	1.29	0.033	0.068	0.054	0.66	10.339
1	1	0.560	3.38	0.107	0.217	0.165	0.18	17.539
1	1	0.582	2.83	0.130	0.285	0.217	1.10	10.539
1	1	0.519	2.15	0.113	0.248	0.210	0.24	3.270
1	1	0.574	4.92	0.107	0.223	0.180	0.73	8.559
3	1	0.470	2.15	0.093	0.190	0.149	0.35	4.624
3	1	0.435	1.80	0.069	0.120	0.106	0.16	3.392
3	1	0.465	3.69	0.148	0.323	0.256	0.53	8.610
3	1	0.466	1.35	0.060	0.131	0.104	0.69	7.633
3	1	0.540	4.95	0.164	0.352	0.267	1.51	24.219
9	1	0.151	5.03	0.150	0.330	0.251	0.55	7.256
9	1	0.127	2.82	0.091	0.198	0.146	0.28	2.844
9	1	0.110	1.98	0.085	0.173	0.134	0.80	4.965
9	1	0.163	2.71	0.134	0.287	0.229	0.57	14.119
9	1	0.058	3.58	0.102	0.226	0.158	0.17	2.801
0 (CT)	7	0.393	5.39	0.271	0.631	0.571	0.74	13.612
0 (CT)	7	0.340	4.32	0.195	0.440	0.360	0.43	4.168

0 (CT)	7	0.491	2.70	0.089	0.202	0.175	0.08	5.269
0 (CT)	7	0.388	3.29	NA	NA	NA	0.52	12.962
0 (CT)	7	0.375	2.61	0.101	0.229	0.199	0.38	6.360
0 (S-CT)	7	0.401	5.21	0.202	0.453	0.380	0.37	5.879
0 (S-CT)	7	0.339	2.87	0.152	0.332	0.289	0.60	7.505
0 (S-CT)	7	0.380	4.41	0.185	0.423	0.384	0.43	15.232
0 (S-CT)	7	0.486	2.75	0.112	0.229	0.184	0.32	6.703
0 (S-CT)	7	0.453	4.83	0.177	0.405	0.374	0.95	16.092
0.1	7	0.496	1.32	0.060	0.136	0.118	0.34	4.651
0.1	7	0.340	4.02	0.134	0.297	0.251	0.50	4.356
0.1	7	0.334	5.97	0.234	0.523	0.464	0.79	17.862
0.1	7	0.432	3.76	0.140	0.296	0.231	1.10	7.246
0.1	7	0.314	3.28	0.120	0.276	0.282	0.85	15.112
0.3	7	0.444	4.39	0.177	0.390	0.296	0.41	8.678
0.3	7	0.404	3.85	0.158	0.357	0.346	0.54	4.852
0.3	7	0.464	3.90	0.153	0.335	0.263	0.43	6.232
0.3	7	0.387	1.58	0.080	0.183	0.169	0.46	13.912
0.3	7	0.401	2.60	0.132	0.299	0.302	0.23	12.722
1	7	0.325	3.01	0.081	0.180	0.166	0.40	6.182
1	7	0.366	3.78	0.178	0.387	0.316	0.64	4.261
1	7	0.400	3.59	0.167	0.381	0.386	0.60	9.861
1	7	0.471	1.94	0.096	0.218	0.219	0.42	7.952
1	7	0.366	5.29	0.209	0.457	0.374	0.47	6.523
3	7	0.262	2.75	0.108	0.239	0.203	0.35	6.517
3	7	0.255	4.34	0.048	0.100	0.079	0.33	5.256
3	7	0.249	4.04	0.112	0.232	0.178	0.45	10.152
3	7	0.255	3.58	0.111	0.244	0.221	0.65	14.152
3	7	0.273	5.47	0.170	0.380	0.354	0.51	6.822
9	7	0.000	3.05	0.081	0.157	0.127	0.31	12.032
9	7	0.000	4.49	0.029	0.047	0.039	0.29	1.735
9	7	0.000	1.97	0.080	0.167	0.133	0.37	25.692
9	7	0.000	2.00	0.002	0.004	0.004	0.68	32.152
9	7	0.000	1.49	0.008	0.013	0.012	0.46	20.032
0 (CT)	14	0.256	5.79	0.169	0.383	0.340	1.32	35.830
0 (CT)	14	0.331	2.86	0.069	0.157	0.135	0.46	32.910
0 (CT)	14	0.295	4.56	0.148	0.330	0.348	0.62	25.710
0 (CT)	14	0.284	1.52	0.056	0.126	0.117	0.28	3.914
0 (CT)	14	0.280	4.11	0.113	0.261	0.238	0.18	6.279
0 (S-CT)	14	0.402	2.89	0.064	0.141	0.120	0.85	42.340
0 (S-CT)	14	0.285	4.77	0.150	0.333	0.312	0.42	37.890
0 (S-CT)	14	0.439	7.33	0.077	0.179	0.148	1.94	51.040
0 (S-CT)	14	0.243	2.88	0.107	0.240	0.216	0.38	13.000
0 (S-CT)	14	0.390	6.34	0.170	0.392	0.302	0.89	16.280
0.1	14	0.253	5.90	0.235	0.510	0.396	2.09	41.790
0.1	14	0.200	3.40	0.200	0.449	0.425	0.72	7.296
0.1	14	0.283	2.60	0.085	0.174	0.140	0.36	4.691
0.1	14	0.373	3.02	0.089	0.207	0.217	0.60	17.870

0.1	14	0.393	2.45	0.056	0.122	0.098	1.14	29.310
0.3	14	0.203	3.14	0.126	0.285	0.269	0.65	12.490
0.3	14	0.372	2.48	0.090	0.200	0.171	0.38	13.880
0.3	14	0.277	3.55	0.117	0.262	0.225	0.81	73.360
0.3	14	0.429	8.80	0.165	0.368	0.270	1.17	40.410
0.3	14	0.378	3.36	0.125	0.282	0.236	1.94	66.390
1	14	0.364	3.25	0.094	0.215	0.188	0.93	9.920
1	14	0.320	3.57	0.103	0.232	0.182	3.03	53.510
1	14	0.392	6.57	0.174	0.390	0.338	1.13	22.370
1	14	0.438	5.45	0.094	0.222	0.165	1.09	19.770
1	14	0.223	3.41	0.124	0.280	0.264	0.25	5.493
3	14	0.250	3.16	0.099	0.209	0.181	0.65	14.230
3	14	0.227	2.37	0.061	0.121	0.094	0.81	14.590
3	14	0.214	4.88	0.151	0.338	0.290	0.65	21.530
3	14	0.206	2.11	0.081	0.168	0.129	0.22	15.270
3	14	0.000	4.23	0.033	0.065	0.048	0.52	9.566
9	14	0.000	4.71	0.003	0.002	0.000	0.49	5.643
9	14	0.000	4.94	0.003	0.002	0.002	0.44	19.650
9	14	0.000	2.57	0.004	0.004	0.001	0.32	5.059
9	14	0.000	4.62	0.002	0.001	-0.001	0.22	17.290
9	14	0.000	1.62	0.002	0.001	-0.003	0.55	6.103
0 (CT)	21	0.495	3.31	0.048	0.113	0.118	1.02	25.737
0 (CT)	21	0.486	4.37	0.066	0.154	0.132	0.67	17.127
0 (CT)	21	0.318	7.11	0.170	0.385	0.381	0.98	24.567
0 (CT)	21	0.382	2.94	0.050	0.121	0.135	0.22	8.588
0 (CT)	21	0.440	3.13	0.050	0.110	0.098	0.38	29.087
0 (S-CT)	21	0.354	4.14	0.142	0.353	0.352	1.24	35.597
0 (S-CT)	21	0.371	3.43	0.135	0.294	0.243	0.74	26.977
0 (S-CT)	21	0.461	5.97	0.059	0.138	0.104	0.26	8.047
0 (S-CT)	21	0.439	3.42	0.109	0.237	0.277	0.61	26.277
0 (S-CT)	21	0.479	6.21	0.079	0.188	0.159	NA	-0.004
0.1	21	0.425	4.73	0.060	0.160	0.146	0.74	38.077
0.1	21	0.398	3.06	0.059	0.142	0.134	0.26	7.137
0.1	21	0.422	3.37	0.051	0.127	0.123	0.70	15.187
0.1	21	0.397	2.60	0.052	0.111	0.099	0.99	38.217
0.1	21	0.514	6.17	0.084	0.192	0.158	1.52	28.127
0.3	21	0.204	3.76	0.207	0.460	0.400	0.34	5.474
0.3	21	0.427	2.73	0.072	0.174	0.193	1.09	21.007
0.3	21	0.384	7.18	0.084	0.190	0.188	0.68	21.067
0.3	21	0.401	4.00	0.096	0.220	0.210	0.50	19.167
0.3	21	0.316	4.26	0.124	0.285	0.240	NA	-0.010
1	21	0.361	4.11	0.044	0.120	0.109	0.61	26.237
1	21	0.285	3.83	0.110	0.264	0.223	0.21	20.557
1	21	0.370	2.86	0.092	0.225	0.188	0.37	25.227
1	21	0.398	5.46	0.175	0.433	0.398	0.69	19.397
1	21	0.369	5.91	0.119	0.294	0.226	0.44	20.207
3	21	0.289	4.47	0.121	0.251	0.217	1.17	25.237

3	21	0.269	3.89	0.106	0.232	0.206	1.41	14.617
3	21	0.280	2.86	0.077	0.168	0.149	0.59	5.282
3	21	0.324	2.59	0.087	0.193	0.187	0.18	2.555
3	21	0.356	4.46	0.121	0.257	0.216	NA	0.002
9	21	0.000	2.74	0.002	0.001	0.000	0.63	21.187
9	21	0.000	3.18	0.001	0.000	0.000	0.35	2.611
9	21	0.000	3.42	0.002	0.002	0.003	0.37	4.383
9	21	0.000	4.68	0.001	0.000	0.000	0.55	12.637
9	21	0.000	3.90	0.001	0.001	0.001	0.31	2.653

Field study

Growth

Stream	Netting no.	Time (day)	Length (mm)
Skut_1	2	0	30.8
Skut_1	2	0	39.8
Skut_1	2	0	55.5
Skut_1	2	0	57.2
Skut_1	2	0	35.8
Skut_1	2	0	34.7
Skut_1	3	0	22.2
Skut_1	3	0	28.4
Skut_1	3	0	48.3
Skut_1	3	0	42.1
Skut_1	3	0	32.9
Skut_1	3	0	28.9
Skut_2	2-1	0	32.6
Skut_2	2-1	0	39.6
Skut_2	2-1	0	52.4
Skut_2	2-1	0	32.4
Skut_2	2-1	0	63.1
Ref.(Skut_1)	1	0	44.6
Ref.(Skut_1)	1	0	43.1
Ref.(Skut_1)	1	0	58.3
Ref.(Skut_1)	1	0	45.6
Ref.(Skut_1)	1	0	74.1
Ref.(Skut_1)	1	0	28.7
Ref.(Skut_1)	4	0	24.7
Ref.(Skut_1)	4	0	38.5
Ref.(Skut_1)	4	0	36.0
Ref.(Skut_1)	4	0	123.5
Ref.(Skut_1)	4	0	57.7
Ref.(Skut_1)	4	0	53.2
Ref.(Skut_2)	1	0	25.5
Ref.(Skut_2)	1	0	28.1
Ref.(Skut_2)	1	0	24.7
Ref.(Skut_2)	1	0	52.3

Ref.(Skut_2)	1	0	38.8
Ref.(Skut_2)	1	0	40.9
Ref.(Skut_2)	2-2	0	24.8
Ref.(Skut_2)	2-2	0	96.1
Ref.(Skut_2)	2-2	0	23.3
Ref.(Skut_2)	2-2	0	31.1
Ref.(Skut_2)	2-2	0	59.3
Skut_1	2	14	34.0
Skut_1	2	14	35.1
Skut_1	2	14	56.4
Skut_1	2	14	61.6
Skut_1	2	14	38.0
Skut_1	2	14	35.6
Skut_1	3	14	24.6
Skut_1	3	14	26.4
Skut_1	3	14	50.8
Skut_1	3	14	41.6
Skut_1	3	14	34.8
Skut_1	3	14	28.7
Skut_2	2-1	14	32.4
Skut_2	2-1	14	34.6
Skut_2	2-1	14	51.0
Skut_2	2-1	14	31.9
Skut_2	2-1	14	59.3
Ref.(Skut_1)	1	14	51.3
Ref.(Skut_1)	1	14	47.3
Ref.(Skut_1)	1	14	60.7
Ref.(Skut_1)	1	14	47.9
Ref.(Skut_1)	1	14	84.1
Ref.(Skut_1)	1	14	28.3
Ref.(Skut_1)	4	14	23.8
Ref.(Skut_1)	4	14	47.6
Ref.(Skut_1)	4	14	31.8
Ref.(Skut_1)	4	14	123.4
Ref.(Skut_1)	4	14	58.0
Ref.(Skut_1)	4	14	51.3
Ref.(Skut_2)	1	14	27.8
Ref.(Skut_2)	1	14	27.8
Ref.(Skut_2)	1	14	26.4
Ref.(Skut_2)	1	14	51.3
Ref.(Skut_2)	1	14	40.2
Ref.(Skut_2)	1	14	39.2
Ref.(Skut_2)	2-2	14	25.7
Ref.(Skut_2)	2-2	14	94.7
Ref.(Skut_2)	2-2	14	24.4
Ref.(Skut_2)	2-2	14	32.5
Ref.(Skut_2)	2-2	14	61.9

Fv/Fm, pigment concentration, Photosynthetic oxygen evolution (Ox.dev.), ROS.

			Pigment concentration (µg ml-1) *					ROS	
Group_name	Day	FvFm	Weight (mg)	Chl *a*	Chl *b*	Car	Ox.dev.	Weight (mg)	ROS minus blank
Skut_1 dep	0	0.712							
Skut_1 dep	0	0.711					56.150		
Skut_1 dep	0	0.717	14.96	9.574	5.204	1.807			
Skut_1 dep	0	0.717							
Skut_1 dep	0	0.707					51.248		
Skut_1 dep	0	0.728	15.22	13.036	7.236	2.482			
Skut_1 nat	0	0.719							
Skut_1 nat	0	0.727					51.928		
Skut_1 nat	0	0.721	12.71	11.307	5.881	2.235			
Skut_1 nat	0	0.721							
Skut_1 nat	0	0.716					33.866		
Skut_1 nat	0	0.722	21.26	12.239	5.659	2.557			
Skut_2 dep	0	0.705							
Skut_2 dep	0	0.723					43.664		
Skut_2 dep	0	0.720	23.36	13.364	10.862	2.742			
Skut_2 dep	0	0.711							
Skut_2 dep	0	0.712					30.952		
Skut_2 dep	0	0.734	38.77	19.681	12.796	4.064			
Skut_2 nat	0	0.727							
Skut_2 nat	0	0.733					17.436		
Skut_2 nat	0	0.730	27.53	17.884	10.426	3.463			
Skut_2 nat	0	0.729							
Skut_2 nat	0	0.738					34.033		
Skut_2 nat	0	0.731	17.63	16.725	8.187	3.764			
Skut_1 dep	7	0.724	4.15	2.656	0.560	0.750			
Skut_1 dep	7	0.720	5.8	3.319	0.676	0.939	105.553		
Skut_1 dep	7	0.720	8.24	5.085	1.050	1.175			
Skut_1 dep	7	0.708	9.94	5.964	1.194	1.720			
Skut_1 dep	7	0.724	6.58	4.414	0.926	1.289	44.486		
Skut_1 dep	7	0.717	5.06	3.221	1.219	0.766			
Skut_1 nat	7	0.726	8.37	5.260	1.147	1.160			
Skut_1 nat	7	0.737	6.23	4.395	0.954	1.198	40.936		
Skut_1 nat	7	0.732	5.92	5.468	0.964	1.589			
Skut_1 nat	7	0.729	7.28	5.514	1.119	1.639			
Skut_1 nat	7	0.722	6.99	5.403	1.117	1.627	52.333		
Skut_1 nat	7	0.729	8.66	4.830	0.920	1.409			
Ref (Skut1)	7	0.719	5.97	2.550	0.658	0.738			
Ref (Skut1)	7	0.713	8.75	6.548	1.231	1.903	72.551		
Ref (Skut1)	7	0.699	1.82	2.082	0.409	0.636			
Ref (Skut1)	7	0.703	7.85	5.534	1.170	1.414			
Ref (Skut1)	7	0.725	3.23	2.508	0.490	0.765	98.428		
Ref (Skut1)	7	0.717	3.55	1.554	0.334	0.469			

Skut_1 dep	14	0.727	8.7	4.467	1.292	1.331		1.69	28.344
Skut_1 dep	14	0.739	5.84	9.587	2.893	2.784	33.841	1.62	32.984
Skut_1 dep	14	0.738	3.19	4.879	1.266	1.276		1.28	25.834
Skut_1 dep	14	0.672	6.17	2.140	0.482	0.630		2.17	80.694
Skut_1 dep	14	0.738	10.63	3.864	1.048	1.033	24.240	2.5	252.414
Skut_1 dep	14	0.725	7.54	6.208	1.719	1.464		1.21	36.574
Skut_1 nat	14	0.736	7.88	4.903	1.180	1.130		0.81	4.603
Skut_1 nat	14	0.737	6.07	3.709	0.722	1.056	23.044	1.25	9.615
Skut_1 nat	14	0.733	6.88	3.305	0.881	0.791		3.45	11.524
Skut_1 nat	14	0.735	4.88	5.980	1.773	1.563		3.72	15.244
Skut_1 nat	14	0.730	8.68	5.300	1.202	1.292	58.824	NA	-0.001
Skut_1 nat	14	0.741	8.88	6.009	1.114	1.727		3.4	9.021
Skut_2 dep	14	0.700	4.34	4.867	1.348	1.559		0.84	19.174
Skut_2 dep	14	0.724	3.7	2.190	0.423	0.726	34.130	2.16	72.364
Skut_2 dep	14	0.677	3.52	3.538	0.692	1.086		1.44	12.544
Skut_2 dep	14	0.725	6.02	3.406	0.684	1.030		2.89	99.794
Skut_2 dep	14	0.660	NA	NA	NA	NA	NA	1.4	42.254
Skut_2 dep	14	NA	NA	NA	NA	NA	NA	0.53	55.404
Skut_2 nat	14	0.689	3.73	5.922	1.542	1.673		2.92	43.234
Skut_2 nat	14	0.726	3.61	2.787	1.129	0.845	37.201	0.84	15.084
Skut_2 nat	14	0.707	3.95	2.013	1.012	0.631		0.81	10.184
Skut_2 nat	14	0.701	2.19	3.196	1.147	0.930		0.65	31.004
Skut_2 nat	14	0.715	3.55	3.400	1.101	0.952	36.669	0.61	6.658
Skut_2 nat	14	0.741	7.35	3.602	1.100	1.005		0.77	19.174
Ref (Skut1)	14	0.734	10.44	5.131	1.049	1.352		1.41	3.499
Ref (Skut1)	14	0.719	6.51	6.124	1.620	1.347	33.835	2.04	8.373
Ref (Skut1)	14	0.719	9.65	6.129	1.405	1.442		1.65	7.140
Ref (Skut1)	14	0.731	13.27	5.688	1.575	1.370		1.62	6.611
Ref (Skut1)	14	0.723	7.36	4.387	1.337	1.035	37.109	2.67	13.274
Ref (Skut1)	14	0.732	5.24	6.259	1.739	1.380		2.5	8.505
Ref (Skut2)	14	0.703	10.69	6.295	1.571	1.419		1.94	12.344
Ref (Skut2)	14	0.711	4.03	4.132	2.161	1.489	25.723	0.72	2.292
Ref (Skut2)	14	0.715	3.94	5.034	1.311	1.451		0.43	2.435
Ref (Skut2)	14	0.706	6.82	4.638	1.244	1.176		1.24	9.994
Ref (Skut2)	14	0.716	6.41	3.700	0.994	1.033	32.823	0.97	2.967
Ref (Skut2)	14	0.722	5.05	7.101	1.534	1.919		1.6	8.029
Control	14							2.76	47.744
Control	14							1.81	35.774
Control	14							1.81	32.084
Control	14							1.69	10.294
Control	14							1.51	17.094
Control	14							1.6	17.204

***** Day 0: total weight and pigment concentration for triplicates.

Appendix VII Results for control groups (laboratorial studies)

Control groups should ideally be the treatment group with best health, visualised through endpoint analysis. For the laboratorial studies, reduced health of control and solvent control was observed throughout the exposure period. There was a trend of decreased greenness index, maximal PS II efficiency (Fv/Fm), concentration of chlorophyll (Chl) *a*, Chl *b* and carotenoids (CAR), and increased production of reactive oxygen species (ROS) (Figure VII-1).

For the $CuSO_4$ study, Fv/Fm, Chl *a* concentration, Car concentration and Chl *a/b* ratio for control groups was lowest at day 14. Greenness index and Chl *b* concentration was lowest, and ROS production highest, at day 21. Significant difference compared to day 0 was detected for greenness index at day 21, Fv/Fm at day 7-21, Chl *a* and *b* concentration at day 1-21, and Car concentration at day 14. No significant difference was observed for Chl *a*/*b* ratio and ROS production.

For the DCP study, Fv/Fm of control groups was lowest at day 14 while Greenness index, concentration of Chl *a*, *b* and Car was lowest at day 21. Chl *a*/*b* ratio was lowest at day 1 and ROS production highest at day 21. Solvent control (S-CT) was generally similar or slightly lower than control (CT) in the start and similar or slightly higher than CT in the end of the exposure period. A leap was observed for Chl *a*, *b* and Car concentration, increasing from day 0 to day 1 and then gradually decreasing throughout the exposure period. Significant difference compared to day 0 was detected for greenness index, Car concentration and ROS at day 21, Fv/Fm at day 7-21, Chl *a* concentration at day 14-21, and Chl *b* concentration at day 1 and 14-21. No significant difference was observed for Chl *a*/*b* ratio.

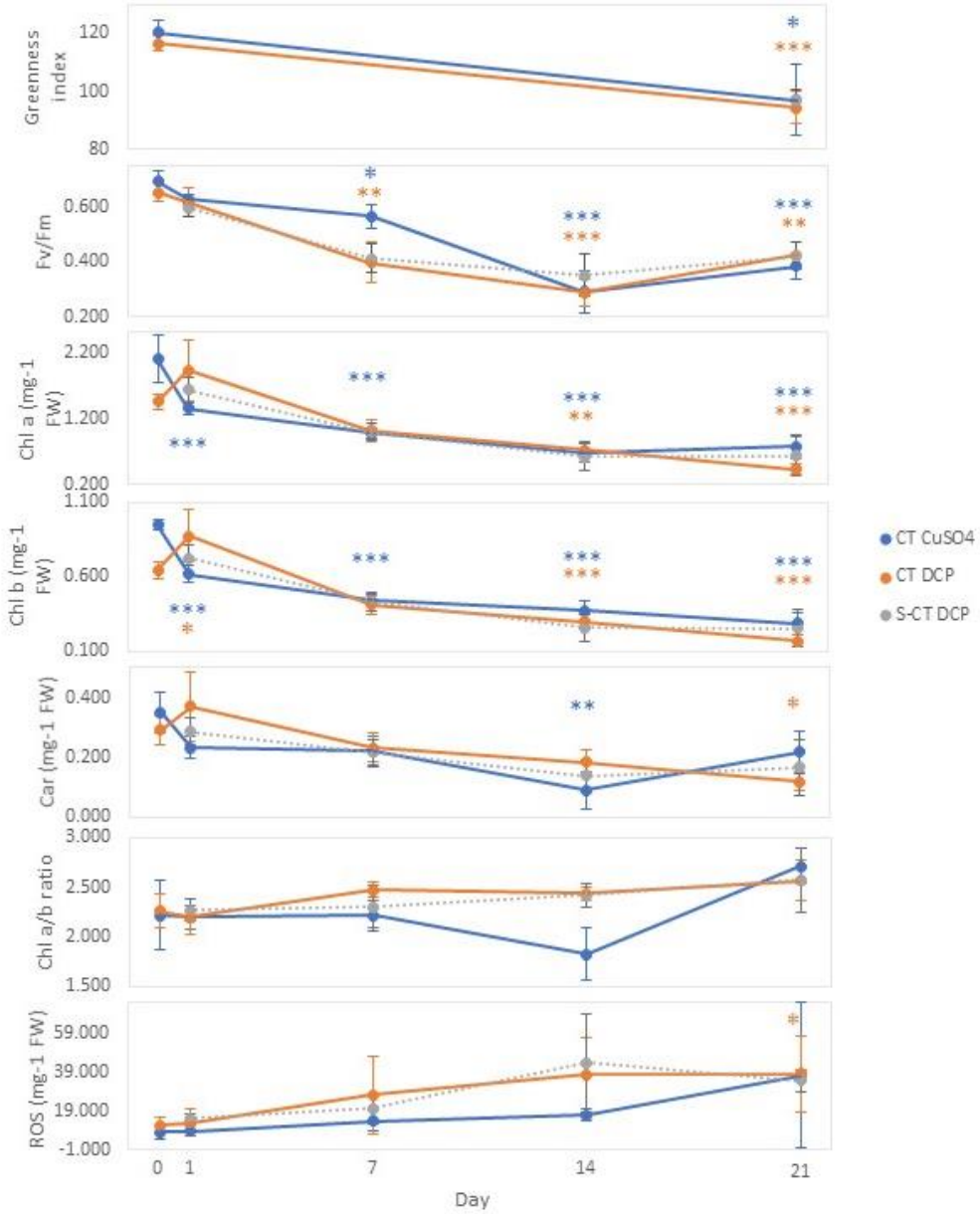


Figure VII-1: Greenness index, PSII maximum quantum efficiency (Fv/Fm), concentration of pigments Chlorophyll (Chl) a and b, Carotenoids (Car), Chl a/b ratio, and production of reactive oxygen species (ROS) in Fontinalis antipyretica after 0-21 days exposure to KNOP's medium (control) in the $CuSO_4$ and 3,5-dichlorophenol (DCP) studies. Both media control (CT) and solvent control using DMSO (S-CT) are used for DCP. Asterisks marks statistically significant difference to day 0 (* $p<0.05$, ** $p<0.001$, *** $p<0.001$).

Appendix VIII pH of solution batches

$CuSO_4$

The pH was generally stable, however with peaks for the control *Off* solutions for Batch 2 and 3 (Figure VIII-1). *On* solutions before adding moss were similar at pH 7.03 ± 0.05 for all batches and concentrations. For Batch 1, *On* solutions after adding moss were fairly similar at pH 7.00 ± 0.07. *Off* solutions were slightly higher (pH 7.2 ± 0.05) for control and slightly lower for 10-300 µM treatments (average pH 6.76 ± 0.01). For Batch 2 and 3, treatments before pH regulation were weakly decreasing from lowest to highest concentrations (pH 6.9 – 6.4). *Off* solutions had a highly increased pH in control solutions, at pH 8.6 ± 0.1 for batch 2 and pH 8.3 ± 0.2 for batch 3.

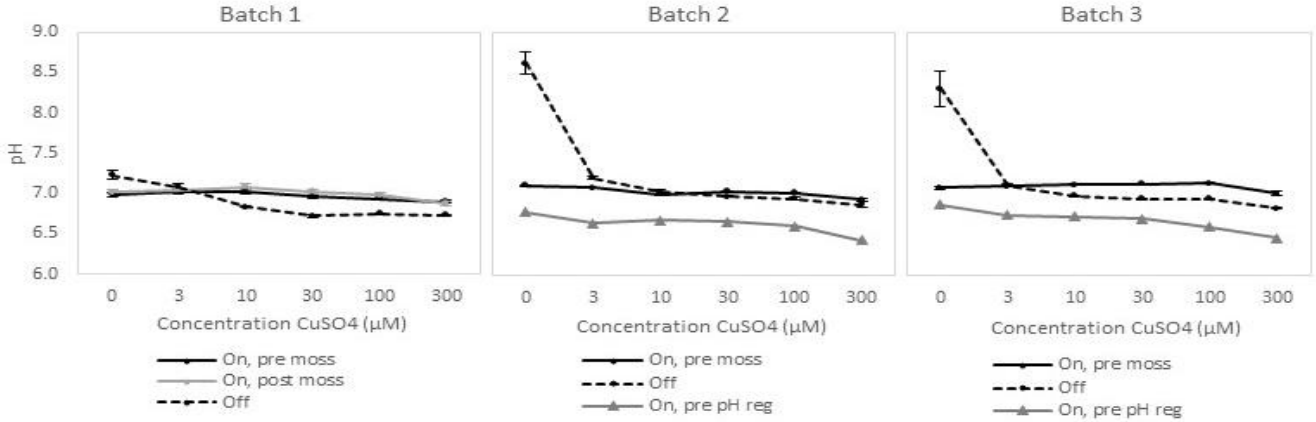


Figure VIII-1: pH of solution batch 1 (day 0-7), 2 (day 7-14) and 3 (day 14-21) of CuSO4 (0-300 µM). "On" indicates start of batch, "Off" indicates end of batch. For batch 1 the pH was measured both before (pre) and after (post) moss was added. For Batch 2 and 3, pH before pH regulation ("On, pre pH reg") is additionally shown.

3,5-DCP

The pH was generally stable for all *On* batches, however highly increased for all *Off* batches for controls and up to 1 mg/L treatments (Figure VIII-2). *On* solutions before adding moss were similar at pH 7.08 ± 0.03 for all batches and concentrations. On batches before pH regulation were slightly higher for control and slightly lower for 9 mg/L treatment for batch 1, and slightly lower for all controls and treatments for batch 2 and 3. For Batch 1, *On* solutions after adding moss were relatively similar at pH 7.13 ± 0.04. *Off* solutions for all batches were highly increased for 0 - 1 mg/L treatments, ranging from 9.1 to 8.2, while being stable at approx. pH 7 for 3-9 mg/L treatments.

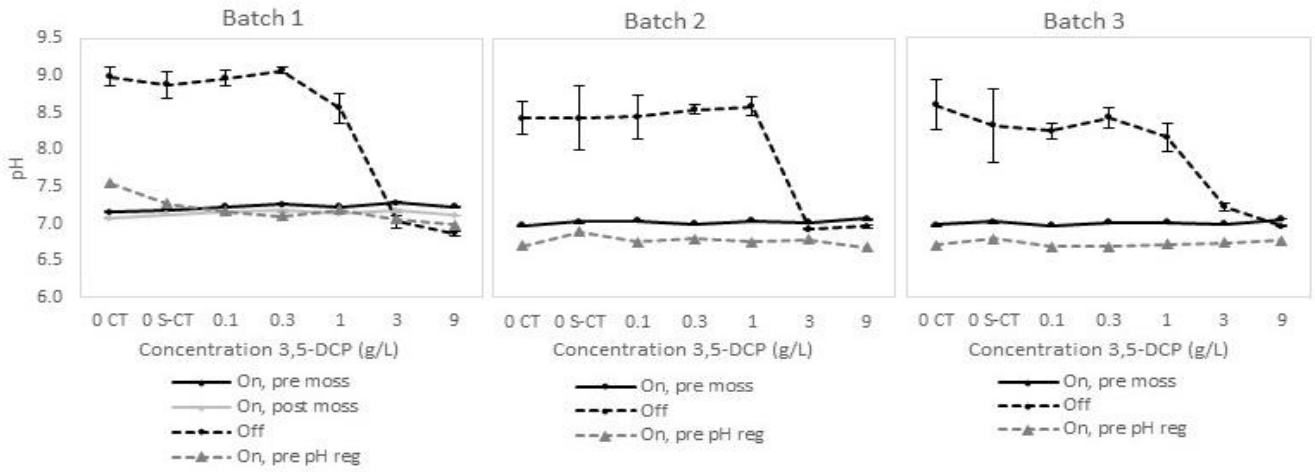


Figure VIII-2: pH of solution batch 1 (day 0-7), 2 (day 7-14) and 3 (day 14-21) of 3,5-dichlorophenol (DCP) (0 - 9 mg/L). "On" indicates start of batch, "Off" indicates end of batch. For batch 1 the pH was measured both before (pre) and after (post) moss was added. For Batch 1, 2 and 3, pH before pH regulation ("On, pre pH reg") is additionally shown.

Appendix IX Chlorophyll *a/b* ratio plots

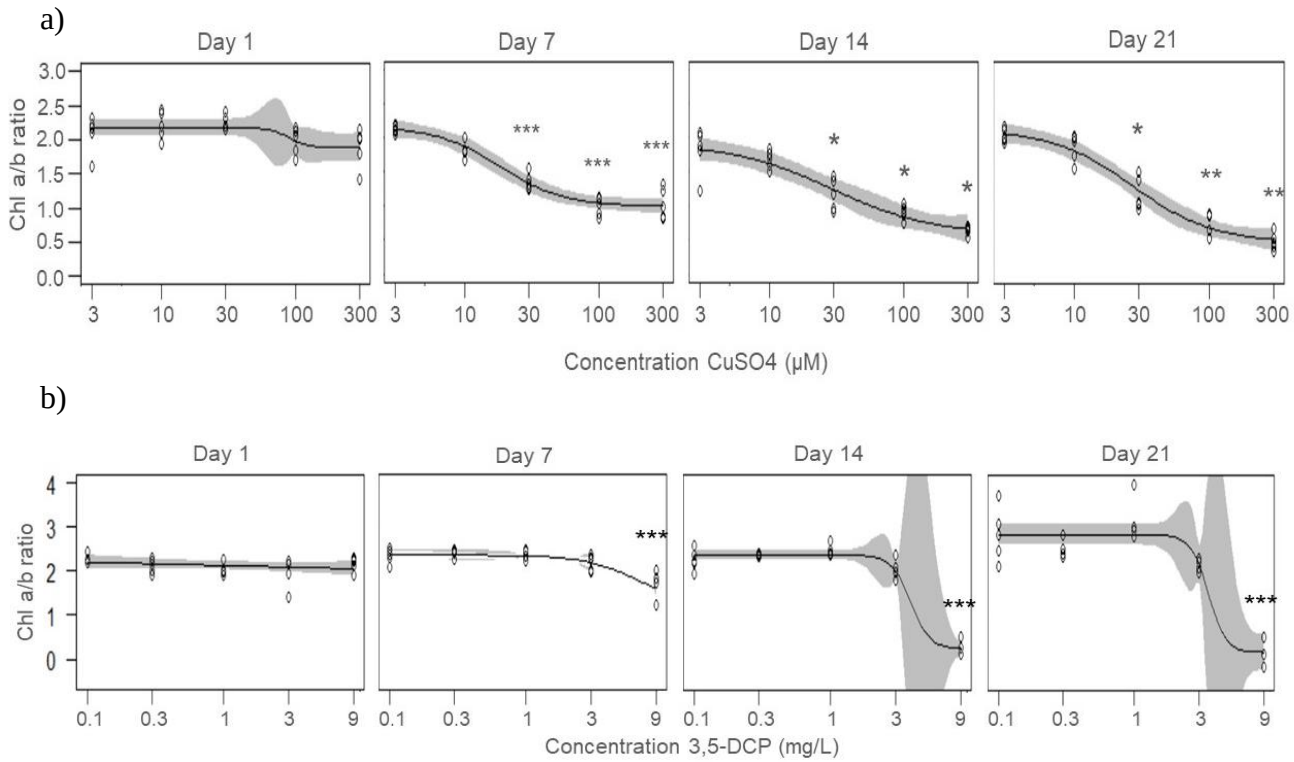


Figure IX-1: Chlorophyll *a/b* ratio in *Fontinalis antipyretica* after 1-21 days of exposure to a) $CuSO_4$ (3-300 µM) and b) 3,5-dichlorophenol (3,5-DCP, 0-9 mg/L). Asterisks marks statistically significant difference to control (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Appendix X Chemical and physical conditions of streams

Light intensity

The light measurements generally indicate lower light intensity for all streams in the middle of the exposure period (day 4-7), however day-to-day variations occurred for all streams (Figure IX-1). For Skut 2 the light intensity was highest the first three days, however for Skut 1 it was highest at day 8 and 9. For Ref. loc. the light intensity was slightly higher at day 11-13. The fish-eye pictures from each stream site indicate that there was more shadowing by trees at the reference location (Figure IX-2).

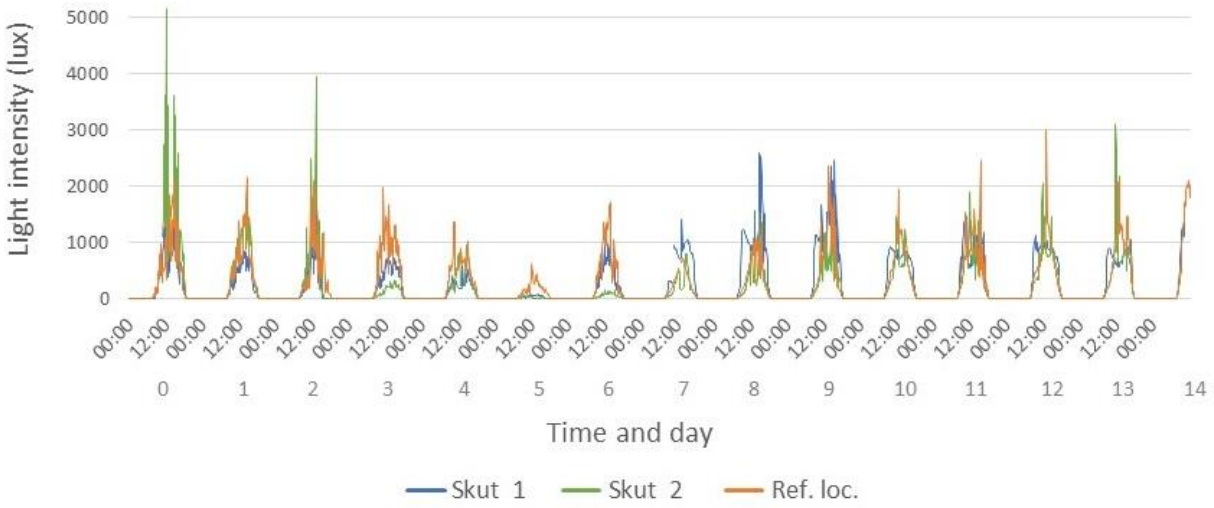


Figure X-1: Light intensity from continuous monitoring in Skuterud test site 1 (Skut 1) and 2 (Skut 2), and reference location stream Sandbekken (Ref. loc.) at exposure day 0-14.

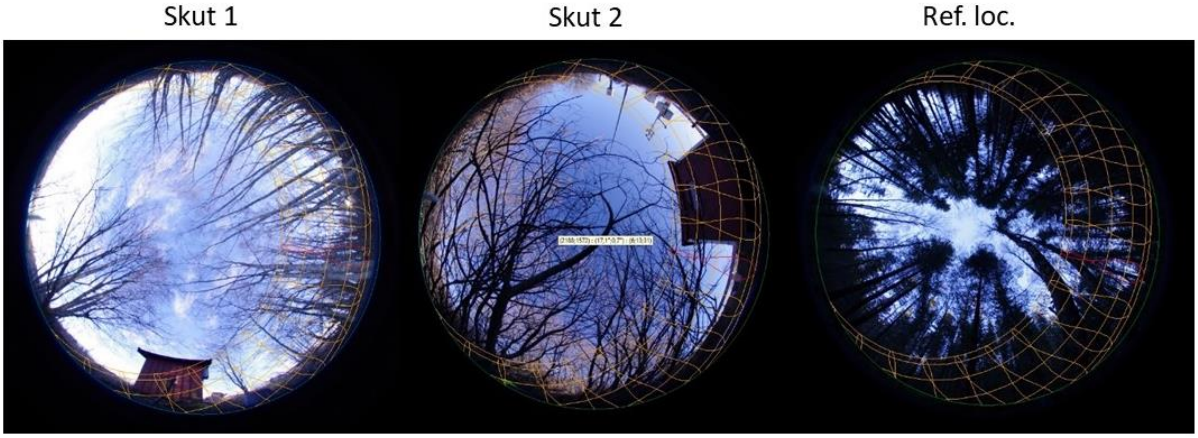


Figure X-2: Light regime at Skuterud test site 1 (Skut 1) and 2 (Skut 2), and reference location stream Sandbekken (Ref. loc.). Photos: Knut Asbjørn Solhaug, 2020.

Temperature

The temperature was relatively similar for the stream locations, with a decreasing trend throughout the exposure period from about 12 to 3°C for all locations (Figure IX-3). There are slight variations between point estimates and continuously logged data (HOBO) data in what stream location has highest or lowest temperature, however the variations are small. HOBO-data indicate that there were small day-night variations during the first seven days and larger variations (approx. 3°C) the last seven days of the exposure period. The variation was highest for the two Skuterud locations. The point estimates at day 7 and 14 indicate slightly lower temperature compared to HOBO-data temperatures (approx. difference of 3-4°C).

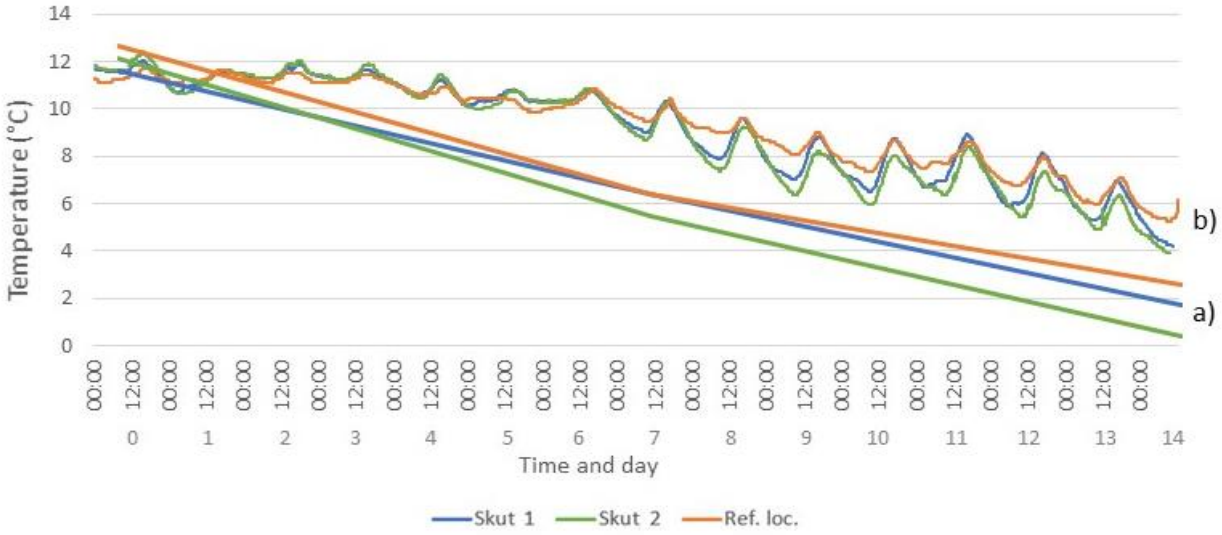


Figure X-3: Temperature from a) point estimates and b) continuous monitoring in Skuterud test site 1 (Skut 1) and 2 (Skut 2), and reference location stream Sandbekken (Ref. loc.) at exposure day 0-14.

Stream size and water discharge (Q)

The amount of water, hence stream size and water discharge, were generally higher on day 7, especially for Skut 2 (Figure IX-4). Both Skuterud locations were larger than Ref. loc., except for Skut 2 having slightly smaller width at day 0 and 14. Water discharge estimated at day 14 was highest for Skut 2 and lowest for Skut 1.

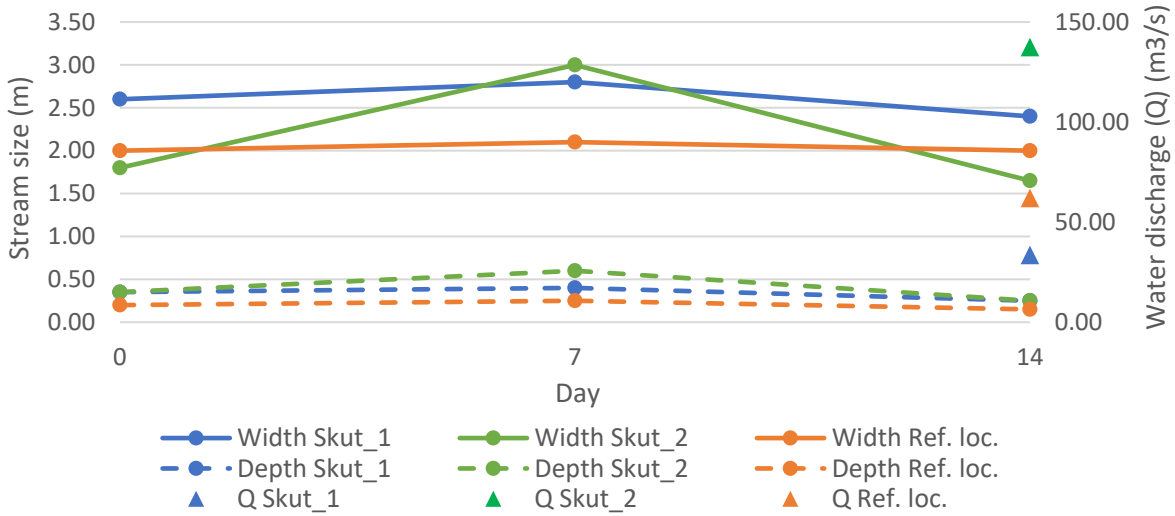


Figure X-4: Stream size (with and depth) and water discharge (Q) in Skuterud test site 1 (Skut 1) and 2 (Skut 2), and reference location stream Sandbekken (Ref. loc.) at exposure day 0-14.

pH and conductivity

Both pH and conductivity were lower in Ref. loc. compared to Skut 1 and Skut 2 (Figure IX-5). pH was stable throughout the exposure period, with slightly increasing values at day 14 for all locations. It was relatively similar for both Skuterud locations (avg. 6.8 for all days). At Ref. loc., it was 1.4 pH-units lower (avg. 5.4 for all days). Conductivity indicates the same trend, with almost identical values at Skuterud locations on (avg. 280 µS/cm for all days) and considerably lower at Ref. loc. (avg. 32 µS/cm for all days).

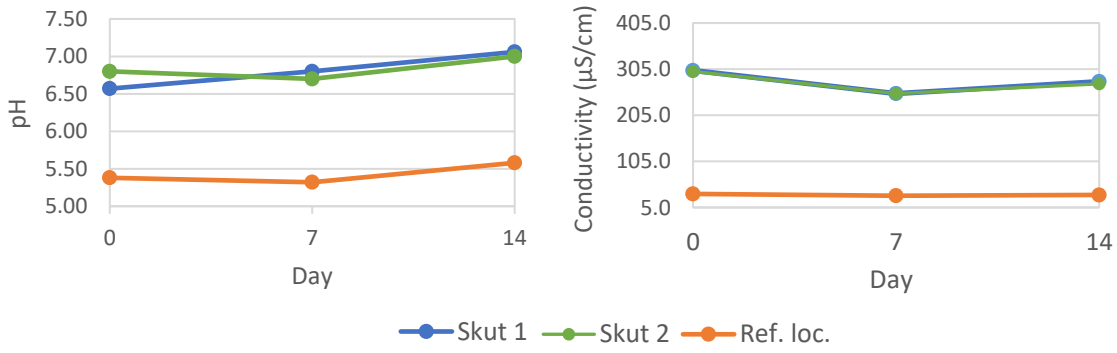


Figure X-5: pH and conductivity in Skuterud test site 1 (Skut 1) and 2 (Skut 2), and reference location stream Sandbekken (Ref. loc.) at exposure day 0-14.

Nitrogen, phosphorous and carbon concentration

Both concentration of total nitrogen (Tot-N) and total phosphorous (Tot-P) were higher in the two Skuterud locations compared to Reference location (Figure IX-6). There was a slight decrease of Tot-N and Tot-P from day 7 to 14 in Skut 1 and Skut 2, while it was constant in

Ref. loc. The concentration of total organic carbon (TOC) and dissolved organic carbon (DOC), however, was slightly higher in Ref. loc. compared to Skut 1 and Skut 2. There was little difference between Skut 1 and Skut 2. TOC and DOC decreased at all locations from day 7 to 14, however it was more reduced in the two Skuterud locations compared to Ref. loc.

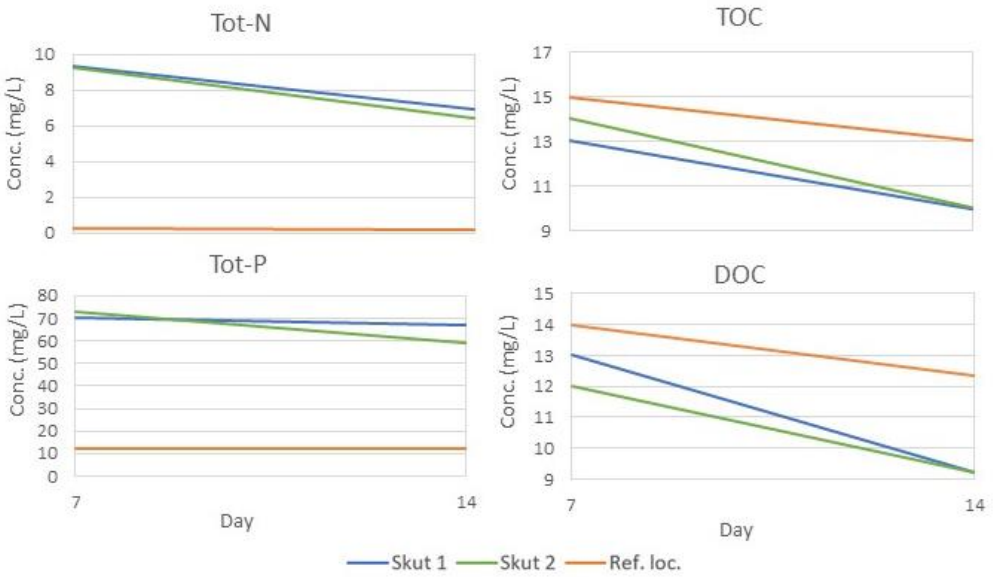


Figure X-6: Concentration of total nitrogen (Tot-N), total phosphorous (Tot-P), total organic carbon (TOC) and dissolved organic carbon (DOC) in Skuterud test site 1 (Skut 1) and 2 (Skut 2), and reference location stream Sandbekken (Ref. loc.) at exposure day 7 and 14.

Elements

Generally, the concentration of elements was low, however highest at day 0 in stream Skuterud, with slightly higher concentrations in Skut 2 compared to Skut 1 (Figure IX-7), with some exceptions. For most metals, the concentration was reduced from day 0 to day 14, and close to or below LOQ at day 14 at all locations.

For Al, B, Fe, K, Mg, Ni, P, S, Si and Pb the concentration was highest for both Skuterud locations with slightly higher values for Skut 2, and close to or below LOQ for Ref. loc. For Be and Cr the concentration was highest for Skut 2 at and close to or below LOQ for Skut 1 and Ref. loc. Na and Mn was the only elements with increasing concentrations from day 0 to day 14, for Skut 1 and Skut 2. Low concentration of multiple elements was detected at Ref. loc., where Cu, Zn and Mn were the only elements registered in significant concentrations. Mn was the only one at higher concentrations in Ref. loc. compared to Skuterud (day 0).

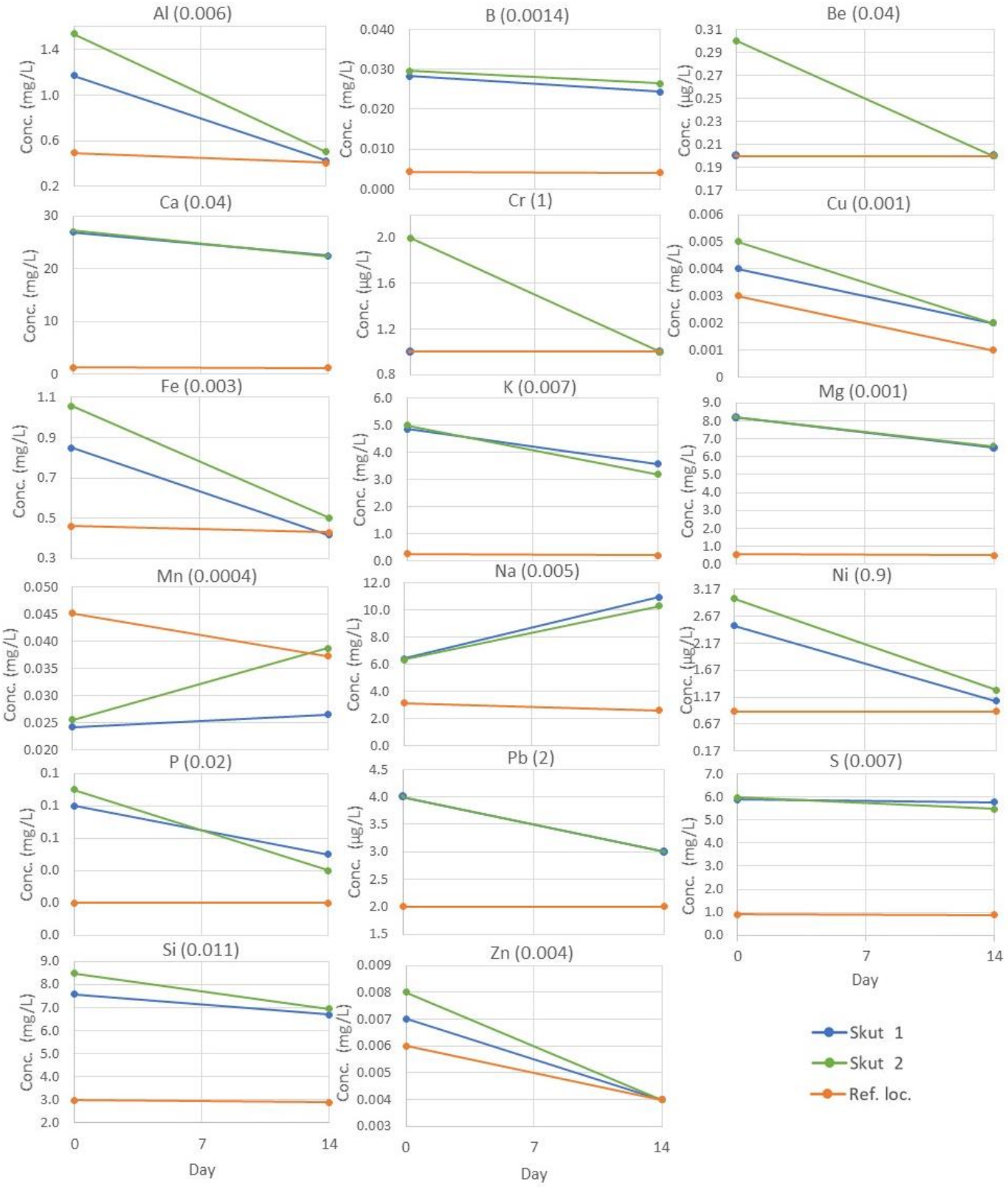


Figure X-7: Concentration of detected elements in Skuterud test site 1 (Skut 1) and 2 (Skut 2), and reference location stream Sandbekken (Ref. loc.) at exposure day 0 and 14. Limit of quantification (LOQ) for each metal is given in parenthesis.

Pesticides (Herbicides and fungicides)

The detection of pesticides was generally low at all locations, and highest concentrations were observed in Skut 1 at day 0 (Figure IX-8). No pesticides were detected at Ref. loc., and for stream Skuterud the concentration was higher in Skut 1 compared to Skut 2 for all pesticides.

The herbicide glyphosate was detected at both Skuterud locations, while its metabolite aminomethylphosphonic acid (AMPA) was detected in Skut 1 at day 0 only. The fungicides bixafen, fluopyram and herbicide prosulfocarb was detected at both Skuterud locations at day 0. The fungicide Prothioconazole was not detected, however its metabolite Protioconazole-desthio was detected at day 0 in Skut 1. Glyphosate was the only pesticide still detected at day 14, in both Skuterud locations.

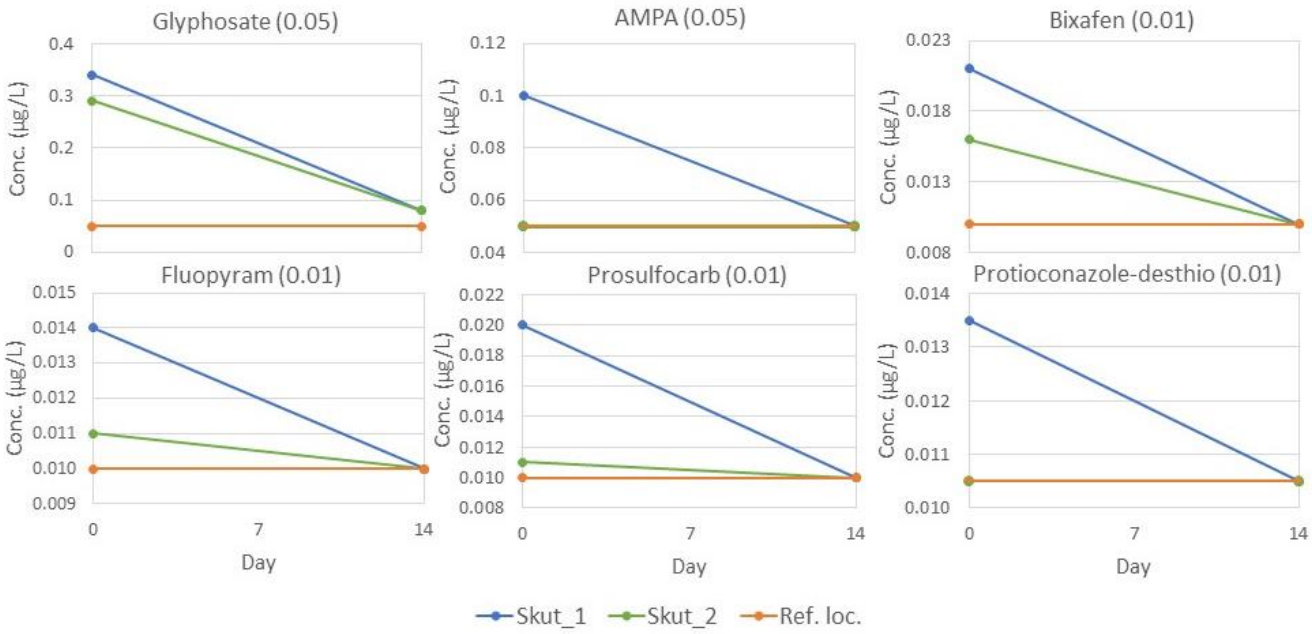


Figure X-8: Concentration of detected pesticides in Skuterud test site 1 (Skut 1) and 2 (Skut 2), and reference location stream Sandbekken (Ref. loc.) at exposure day 0 and 14. Limit of quantification (LOQ) for each pesticide is given in parenthesis.

Appendix XI Details of statistical results

$CuSO_4$

Table XI-1: No Observable Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and concentration needed for an effect in 50 % of the population (EC_{50}) for a) Fv/Fm inhibition, b) Chlorophyll *a*, c) Chlorophyll *b*, d) Carotenoids, e) Chl *a/b* ratio, and f) Reactive oxygen species in *Fontinalis antipyretica* after 1-21 days exposure to $CuSO_4$. The best day for concentration-response curve for each endpoint is highlighted with bold text.

	Day 1	Day 7	Day 14	Day 21
a) Fv/Fm inhibition				
NOEC	10 µM	**10 µM**	> 3 µM	10 µM
LOEC	30 µM	**30 µM**	3 µM	30 µM
EC50	NA	**28.1 ± 9.5 µM**	20.7 ± 46.0 µM	11.5 ± 10.6 µM
b) Chlorophyll *a* inhibition				
NOEC	300 µM	**3 µM**	3 µM	3 µM
LOEC	> 300 µM	**10 µM**	10 µM	10 µM
EC50	NA	**13.9 ± 1.9 µM**	13.7 ± 2.5 µM	7.5 ± 1.0 µM
c) Chlorophyll *b* inhibition				
NOEC	300 µM	**3 µM**	3 µM	3 µM
LOEC	> 300 µM	**10 µM**	10 µM	10 µM
EC50	NA	**30.4 ± 7.9 µM**	22.8 ± 6.3 µM	20.8 ± 9.4 µM
d) Carotenoid inhibition				
NOEC	300 µM	**< 3 µM**	< 3 µM	3 µM
LOEC	> 300 µM	**3 µM**	3 µM	10 µM
EC50	NA	**11.7 ± 2.0 µM**	29.1 ± 413.1 µM	7.3 ± 1.1 µM
e) Chlorophyll a/b ratio relative change				
NOEC	300 µM	3 µM	**10 µM**	< 3 µM
LOEC	>300 µM	10 µM	**30 µM**	3 µM
EC50	NA	123.1 ± 25.4 µM	**104.2 ± 18.7 µM**	24.2 ± 2.6 µM
f) Reactive oxygen species				
NOEC	100 µM	**3 µM**	3 µM	300 µM
LOEC	300 µM	**10 µM**	10 µM	>300 µM

3,5-dichlorophenol (3,5-DCP)

Table XI-2: No Observable Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and concentration needed for an effect in 50 % of the population (EC_{50}) for a) Fv/Fm inhibition, b) Chlorophyll *a*, c) Chlorophyll *b*, d) Carotenoids, e) Chl *a/b* ratio, and f) Reactive oxygen species in *Fontinalis antipyretica* after 1-21 days exposure to 3,5-Dichlorophenol (3,5-DCP). The best day for concentration-response curve for each endpoint is highlighted with bold text.

	Day 1	Day 7	Day 14	Day 21
a) Fv/Fm inhibition				
NOEC	0.3 mg/L	**1 mg/L**	3 mg/L	1 mg/L
LOEC	1 mg/L	**3 mg/L**	9 mg/L	3 mg/L
EC50	NA	**3.1 ± 6.7 mg/L**	3.2 ± 1.1 mg/L	3.7 ± 0.5 mg/L
b) Chlorophyll a inhibition				
NOEC	0.3 mg/L	3 mg/L	**3 mg/L**	3 mg/L
LOEC	1 mg/L	9 mg/L	**9 mg/L**	9 mg/L
EC50	NA	5.0 ± 1.2 mg/L	**3.4 ± NaN mg/L**	2914.7 ± 10.0 mg/L
c) Chlorophyll b inhibition				
NOEC	0.3 mg/L	3 mg/L	**3 mg/L**	3 mg/L
LOEC	1 mg/L	9 mg/L	**9 mg/L**	9 mg/L
EC50	NA	7.2 ± 2.0 mg/L	**358.4 ± 10.0 mg/L**	0.08 ± 0.3 mg/L
d) Carotenoid inhibition				
NOEC	9 mg/L	3 mg/L	**3 mg/L**	3 mg/L
LOEC	> 9 mg/L	9 mg/L	**9 mg/L**	9 mg/L
EC50	NA	4.2 ± 1.1 mg/L	**3.1 ± 0.9 mg/L**	8636.2 ± 10.0 mg/L
e) Chlorophyll a/b ratio relative change				
NOEC	< 0.1 mg/L	3 mg/L	**1 mg/L**	3 mg/L
LOEC	0.1 mg/L	9 mg/L	**3 mg/L**	9 mg/L
EC50	NA	16.5 ± 4.6 mg/L	**3.3 ± NaN mg/L**	8.5 ± NaN mg/L
f) Reactive oxygen species				
NOEC	< 0.1 mg/L	< 0.1 mg/L	< 0.1 mg/L	< 0.1 mg/L
LOEC	0.1 mg/L	0.1 mg/L	0.1 mg/L	0.1 mg/L

Appendix XII Water quality of Skuterudbekken

The condition state of stream Skuterudbekken from 2017 to 2019 was classified as very bad for water chemistry (total phosphorous, total nitrogen and water transparency/Secchi depth), moderate for benthic animals and fouling algae, and moderate for the ecological quality in total (Pettersen et al., 2020). The quality of sediments and chemical state was good.

Out of 10 water samples taken in 2018/2019, pesticides were found in 8 of them, including 10 different types (JOVA, 2020). The herbicide MCPA and insecticide beta-cyfluthrin was detected above the limit of what is harmful for the environment (MF-limit) for one sample. Other herbicides and insecticides, such as prosulfocarb were detected in this thesis, but at low concentrations. The JOVA sampling period ended before autumn pesticide spraying and hence concentrations of these pesticides were not measured. However, the concentrations reported have been highly varying from year to year, due to variation in area treated, weather and runoff. For instance, the concentration of herbicides and fungicides was higher while concentration of insecticides was lower from 2015 to 2017 compared to 2018/2019 (JOVA, 2020).

In 2018/2019 the use of nitrogen and especially phosphorous was relatively high in the Skuterud catchment area (JOVA, 2020). However, the water concentration at Skut 1 was relatively low for phosphorous and high for nitrogen, compared to the main value from 1994 to 2019. The sedimentation ponds were reported to effectively retain suspended matter and total phosphorous, but not nitrogen. In 2019 the metal content of Skut 1 was reported as low and within a good state (Pettersen et al., 2020).

Appendix XIII Discussion – Quality assessment

1) Use of *Fontinalis antipyretica* from stream Skuterudbekken and possible adaption to agricultural runoff

Fontinalis antipyretica was the best choice of aquatic macrophyte for the study area as it was found in both test and reference streams. The populations present in reference stream Sandbekken were small, unhealthy-looking, and located downstream of an old and partly buried landfill. For this reason, *F. antipyretica* tissue was sampled from the test stream Skuterudbekken, where it was present in large quantities and healthy-looking populations. This to ensure that sampling did not threat existence of indigenous populations of test species, as proposed by Deben et al. (2017).

However, *F. antipyretica* in stream Skuterudbekken was probably exposed and adapted to agricultural runoff. The test species should ideally be sampled from a non-polluted stream or upstream of possible pollution, with low concentration of the chemicals of focus for the study (Deben et al., 2017). These goals were not fulfilled and might have increased variation and limited the accuracy. The Skuterud catchment area includes 60 % of agricultural areas and runoff such as pesticides and nutrients, and agricultural practices have been reported in this area for decades (Norkart AS, n.d.). *Fontinalis antipyretica* was registered downstream of lake Østensjøvannet in 2012 in Norwegian species registry maps (Artsdatabanken, n.d.). Due to the large populations present in Skuterudbekken it is assumed to have been there for at least as long. Hence, the *F. antipyretica* population has been exposed to agricultural runoff for multiple years and might be adapted to varying concentrations of agricultural pollution.

Sampling was done during fall after the main spraying season for pesticides (summer), and hence possible exposure to periodically higher concentrations of pollution. However, sampling was done downstream of several sedimentation ponds where less pollution was present, and hence reducing the risk of chronic exposure. On the other hand, periods of heavy rainfall prior to sampling for the field study might have resulted in periodically more pollution from runoff, thus increasing the risk of acute exposure.

Due to the location for sampling upstream of European route 18, there was assumably little to no impact of road runoff (including metals). For this reason, accumulation (which is demonstrated to be effective for *F. antipyretica*, chap. 1.3.2), and toxicity of metals is assumed to be a non-important issue for tissue used.

2) Unideal time of year for studies using *Fontinalis antipyretica*

Due to the natural life cycle of *F. antipyretica*, the moss stops growing and starts asexual reproduction by fragmenting of shoots during autumn and winter when the field study (Sept. – Oct.) and laboratorial studies (December - January) were executed (Glime (2014), chap. 1.3). Hence, the *F. antipyretica* tissue was naturally not in its growing phase of life when the studies were done, and probably was more susceptible to stressors.

3) Poor randomisation of shoots

Executional errors possibly led to poor randomisation of shoots into test solutions/streams, and poorly randomised sampling of shoots for endpoint analysis (e.g., large, or healthy-looking shoots was unintentionally chosen first). This could lead to selection bias with reduced accuracy and increased variation in results, indicating differences between streams, treatments or exposure lengths that were not actually there.

4) Lack of repetition of the tests

Each laboratorial and field experiment was executed once only but should ideally be done at least three times to reveal executional errors and reduce variation. For this reason, the results are considered less accurate.

5) Combined effects

Combined effects with environmental factors (such as nutrient deficiency, water composition, pH, temperature, and light) can lead to additive, antagonistic or synergistic toxicity (chap. 1.2.1). Effects of environmental factors that vary between individual positioning in the chamber/field site are limited by the randomized study design. Additionally, combined effects with toxicants, such as chemicals produced by algae or other organisms can lead to increased toxicity. This may affect the concentration-response studies and statistics, leading to higher variation and reduced accuracy in results.

Laboratorial studies

6) Lack of micronutrients

Micronutrients were not added to the media due to executional errors. As the total length of acclimatisation and exposure tests were 35 ($CuSO_4$) and 42 (3,5-DCP) days, micronutrients were needed. Lack of micronutrient was visible through increased inhibition of GI, Fv/Fm and pigment concentration, and increased ROS production of control groups. Additionally,

inhibition was lower in 3 µM $CuSO_4$ treatments compared to control, indicating that this concentration prevents Cu deficiency and that Cu was a limiting micronutrient for *F. antipyretica*. This included higher but non-significant growth of shoots, significantly lower inhibition of greenness index, PSII efficiency and pigment concentration, and significantly lower formation of reactive oxygen species, most prominent after 14 days exposure. It is additionally assumed that *F. antipyretica* lacked other micronutrients needed for plants, as other bioassays using *F. antipyretica* added multiple micronutrients such as MS microelements (Ares et al., 2014) or Hutner's Metal 49 micronutrient solution (de Traubenberg & Ah-Peng, 2004).

7) Non-successful sterilisation of moss prior to exposure tests

Contamination by algae and possibly bacteria and fungi occurred due to non-successful sterilisation of the moss tissue, despite multiple sterilisation methods including ultrasonic cleaning and concentration ranges of Clorox and sodium dichloro isocyanurate being tested. This corresponds to the fact that *F. antipyretica* and other aquatic bryophytes are relatively hard to successfully sterilise (de Traubenberg & Ah-Peng, 2004). The high growth of contamination in control may have reduced light and nutrients for the moss and produced phytotoxins, causing additional stress for *F. antipyretica*. For this reason, it is suggested to improve methods for successful sterilisation, and in case of contamination use more frequent change of growth medium to limit the amount and effect of contamination.

The contamination was most prominent in control, the lowest $CuSO_4$ concentrations (3 µM) and the three lowest 3,5-DCP concentration (0.1-1 mg/L) from day 7 and throughout the exposure period. This suggests that higher concentrations of $CuSO_4$ and 3,5-DCP prevented growth of contamination. The contamination reappeared after the medium was changed every week and increased with exposure length, indicating that the contamination was transferred with the moss into fresh medium and new test beakers. This result corresponds to the increased pH in off-solutions for control and lower concentrations of $CuSO_4$ and 3,5-DCP, as higher growth of algae results in increased pH.

8) pH of solution batches

Highly increased pH was observed for control in batch 2 and 3 for $CuSO_4$, and all batches for 3,5-DCP, changing from approx. pH 7 to 8.3 ($CuSO_4$) and 9.3 (3,5-DCP). Increased pH was probably a result of algae contamination, as this result in increased level of photosynthesis that uses more CO_2, and hence more HCO_3^- and less H_2CO_3 is present in the water (shifting the CO_2 – H_2CO_3 – HCO_3^- equilibrium to the left), increasing pH. As the $CuSO_4$ study was started one

week before the 3,5-DCP study, contamination and hence increased pH had yet not been build up in batch 1 of the $CuSO_4$ study. Increased pH could possibly have negative effects on *F. antipyretica*, as mosses typically are adapted to growing in acidic aquatic systems (Aarnes, 2016). However, Aronsson and Ekelund (2006) demonstrated that pH of 7.5 - 10 had no or little negative impact on *F. antipyretica*. Additionally, increased pH reduced bioavailability of metals such as Cu, as neutral (~7) and basic (~10) pH leads to formation of less bioavailable Cu complexes, such as $CuOH^+$, $CuHCO_3^+$ and $Cu(CO_3)_2^2$ (VanLoon & Duffy, 2017). However, this is non-relevant as pH did not increase in Cu treatments.

pH was adjusted in the whole storage bottle used for all batches; however, it is shown that the pH decreased from approx. 7 to 6.0 - 6.5 in one week for most batches and hence had to be re-adjusted before use the next week (Appendix VIII). However, *Off* treatments (after 1 week of contact with moss) did not decrease as much, and hence this is considered to have no significant effect on the moss.

Field study

9) Too short acclimatisation period

It is normally suggested to use at least 14 days of acclimatisation in exposure studies using plants, to ensure that the test species is not stressed after handling, cleaning, transportation etc. However, in the field study of this thesis, 11 days was used due to lack of time before the end of the season. For this reason, *F. antipyretica* might have had stress symptoms in the start of the exposure test. The only endpoint indicating this trend was Chl *a/b* ratio, with lower ratio at day 0 compared to the rest of the exposure period. However, there were statistical issues at day 0 resulting in lack of replicates and statistically significant data. Consequently, these results are considered non-significant.

10) Deployment and attachment of moss nettings and HOBO loggers

Due to high stream velocity and turbidity (suspended particles) in Skut 2 at day 7, one moss netting was lost, and the remaining nettings were not possible to locate. For this reason, there were no results at this location at day 7, and hence comparison to Skut 1 and Ref. loc. in the middle of the exposure period could not be done. To make moss nettings easier to locate, it is suggested to attach a small floating device to each netting.

Due to sandy stream bed substrate in Ref. loc., the edges of some nettings (deployed from Skut 1 and 2 into Ref. loc.) were buried in the substrate at day 7 and 14. This resulted in partial burial

of a few moss shoots, leading to altered environmental conditions such as reduced light, gas exchange and susceptibility to uptake of sediment substances. This could possibly have negative impact on the moss, and hence lead to increased variability in endpoint results for these groups. However, the results do not indicate this trend, and effects of partly burial are assumed to be non-significant. To avoid burial of nettings in sandy habitats, it is suggested to use alternative methods for attaching moss nettings or use alternative methods for bioassays of streams with high water current or sandy stream bed substrate, such as use of moss bags (Deben et al., 2018).

Additionally, one HOBO logger was lost due to the high velocity at Skut 2, and hence data from only one logger was used for this location. The attachment of HOBO-loggers to poles was not satisfactory, as forest debris accumulated on poles and was shadowing the loggers, impacting light measurements. Loggers attached to rocks did not get this shading. Consequently, attaching loggers onto rocks is considered the best solution for light measurements. Temperature records by loggers are not significantly affected by shading due to the movement of water and time of year (less warming by solar radiation in autumn).

11) Statistical issues

Two replicates were used for measuring pigment concentration at day 0 and for photosynthetic oxygen evolution, and hence statistically significant difference cannot be calculated. Measurements of pH, conductivity, continuous light/temperature (HOBO), stream size and content of elements and pesticides lacked replicates for calculating statistical significance. For stream velocity in Skut 2, rocks in the stream made it impossible to do multiple measurements. However, these measurements were mainly done to monitor general trends and differences between streams and are not considered to have major impacts on results. Despite this, the errors resulted in reduced accuracy of measurements, and it is suggested to monitor all chemical and physical conditions throughout the study with minimum 3 replicates, and if possible, using more accurate methods such as ideal measuring equipment.

Appendix XIV Discussion laboratorial study – Control groups

There was a general time-dependent response for decreased greenness index (GI), PSII efficiency (Fv/Fm), concentration of pigments Chlorophyll (Chl) *a*, Chl *b* and Carotenoids, and increased ROS for control shoots throughout the exposure period for both lab studies (Appendix VII). Additionally, there was a time-dependent response for reduced GI and Fv/Fm, and increased ROS production in control groups compared to the lowest $CuSO_4$ concentration (3 µM) from day 7 and throughout the exposure period. This is assumed to be caused by the lack of micronutrients (Appendix XIII (6)) and contamination of control groups due to non-successful sterilisation (Appendix XIII (7)), leading to unideal growth conditions. Increased pH due to contamination was observed in all control batches (from ~ 7 to max. 9.3), however considered to not have a major impact, as proposed by Aronsson and Ekelund (2006) (Appendix XIII (8)).

For $CuSO_4$ control shoots, there was a major decrease in pigment concentration from day 0 to day 1 and increase of Fv/Fm, Car and Chl *a*/*b* ratio from day 14 to 21. Additionally, the variation was higher in Chl *a* and Car concentration at day 0, while Greenness index and ROS had higher variation in the end of the exposure period. For 3,5-DCP, however, there was a major increase in pigment concentration from day 0 to 1, with high variation at day 1. As for the $CuSO_4$-study, Fv/Fm and Chl *a/b* ratio increased from day 14 to 21. The explanation for these discrepancies are not immediately evident, but might be caused by poor randomisation of shoots, leading to reduced accuracy and increased variation (Appendix XIII (3)).

Lastly, it is important to note that combined effects of micronutrient depletion and contamination might have occurred.

Appendix XV Discussion field study – Reference stream

A stream within the same catchment as lake Østensjøvann was desirable, although not possible due to anthropogenic impact or not enough water to keep the moss submerged in dry seasons. For this reason, the reference stream Sandbekken was chosen under the following criteria 1) proximity to test location Østensjøvann, 2) similar geology, geography, and climate, 3) stream with already existing *Fontinalis antipyretica* and enough water to keep the moss submerged in dry season, and 4) as little anthropogenic impact (agriculture, infrastructure, and urbanization) as possible to the catchment. Native *F. antipyretia* in the reference stream was not assessed due to small and unhealthy-looking populations, probably caused by contaminated water as it was growing downstream of an old and partly buried landfill. For this reason, the control site used in Sandbekken was placed upstream of this polluted site.

No risk is considered connected to the slightly lower temperature, smaller stream size and lower water discharge of the reference stream compared to test stream Skuterudbekken. The low pH at 5.4, possibly caused by runoff from swamps and low buffer capacity (little Ca, ref. Appendix X), could have direct negative consequences. However, mosses are generally reported to thrive in acidic aquatic systems (Aarnes, 2016). On the other hand, the low pH could possibly increase the bioavailability and hence toxicity of metals. The elements detected at significant concentrations were copper (max. 0.003 mg/L), zinc (max. 0.006 mg/L) and manganese (max. 0.045 mg/L). All metals were assumed to originate from natural sources (bedrock, organic matter etc.) and were within the concentration limit for good ecological status (Miljødirektoratet (Norwegian Environment Agency), 2016). Cu is available in its most bioavailable form (Cu^{2+}) at pH 4, while at pH 7 it becomes deprotonated and forms less bioavailable complexes (VanLoon & Duffy, 2017). For this reason, significant amounts of the Cu present are bioavailable, however due to the low concentration it is considered little risk of Cu toxicity. No risk is connected to the low concentrations of other elements.

The low conductivity indicates lower concentration of ions and possibly macro- and micronutrient, corresponding to the low concentrations of elements and total nitrogen and phosphorous. If resulting in too low nutrient concentration for *F. antipyretica*, this could possibly have negative effects. However, the results did not indicate this and mosses are generally reported to thrive in soft-watered (low concentration of ions) aquatic systems (Wetzel, 2001).

Appendix XVI Discussion – Endpoint analysis

1) Growth analysis

Growth was generally small, at maximum 3.5 mm ($CuSO_4$ study), 1.0 mm (3,5-DCP study) and 2.3 mm (field study). This is contradictory to the study of Davies (2007), observing concentration-dependent growth at maximum 6 mm after 21 days exposure of sulphate in *F. antipyretica*. For this reason, the slow growth in the present study was possibly caused by the unideal time of year for using *F. antipyretica* (Appendix XIII (2)), as it normally stops growing and starts fragmenting during winter (Glime, 2014).

Additionally, results demonstrate growth of secondary shoots (could not be used as an adverse outcome indicator due to lack of replicates within treatments). Growth of secondary shoots was used by Aronsson and Ekelund (2006), where secondary branches emerged after 21 days exposure. *Fontinalis* spp. is reported to frequently have secondary branching (Glime & Raeymaekers, 1987) which is relatively common and contribute to much of the total growth of multiple moss species (Rowntree et al., 2003). For this reason, it is recommended to use growth of secondary shoots as an additional AO endpoint.

2) Pigment content

Chlorophyll/pheophytin ratio (D665/D665a) is additionally suggested to use as an endpoint for aquatic bryophytes, as physiological stress results in degradation of chlorophyll to pheophytin due to the loss of a magnesium atom (Ah-Peng & De Traubenberg, 2004; Lopez & Carballeira, 1990).

3) Photosynthetic oxygen evolution

The method used for photosynthetic oxygen evolution was not ideal due to non-satisfactory development of the methods, resulting in low statistical strength. As 6 cm of shoots were needed for each measurement, and hence only two replicates were used for each test group, no statistical calculations could be done (need ≥ 3 replicates). The time before measurement stabilisation varied a lot, from ~ 3 to 10 minutes, and some did not stabilise at all within the time available. For these cases the mean of multiple values were used. It is suggested to use medium instead of water for storage of moss tissue and analysis in test tube, as use of water will lead to disrupted osmotic balance. As photosynthetic oxygen evolution has previously been successfully studied in *F. antipyretica* (Aronsson & Ekelund, 2006), it is suggested to run test assays to establish better analysis methods for this endpoint.

4) Normalisation by weight and drying of shoots

For endpoints where shoots are not reused for other endpoint analysis, and where complete drying of shoots does not disrupt endpoint analysis, normalisation by dry weight is recommended for increased accuracy. For fresh weight, centrifugal drying (used for laboratorial studies) is considered more accurate than drying on tissue paper (used for field studies) prior to endpoint analysis, and hence centrifugal drying is the recommended method.

Appendix XVII Discussion field study – Chemical and physical conditions of streams

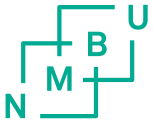
Parameters with highest difference between stream sites were pH, conductivity, water discharge and concentration of nitrogen and phosphorous. The low pH in Ref. loc. might have an impact of bioavailability of metals, as discussed in Appendix XV. The conductivity difference indicates different content of ions including macro- and micronutrients, however considered to be above concentrations needed by *F. antipyretica* at all locations since it was native to all streams. Differences in water discharge might have a negative impact where it is high (Skut 2), inducing fragmenting of moss shoots. The high content of nitrogen and phosphorous in stream Skuterudbekken is assumed to originate from agricultural fertilisation in the catchment area. Concentration of nitrogen was the same for Skut 1 and 2, whereas phosphorous was lower in Skut 2. This indicated that only phosphorous was retained in the sedimentation ponds, corresponding to the JOVA results (Appendix XII). The slightly different light intensity of sites is considered to have no significant effect, as *F. antipyretica* tolerate varying light conditions.

The parameter with highest difference between exposure time was temperature. The low temperature in the end of the exposure period (0 – 6 °C) might have negative effect on growth and possibly other endpoints for *F. antipyretica*, as it is below its optimum temperature. At the end of the period the difference between point estimates and continuous measurements was higher due to lower accuracy for the equipment at lower temperatures.

Parameters with high differences between both location and exposure time were contents of elements, pesticides, and carbon. The content was generally highest at the start of the exposure period, due to less rain and hence less land runoff, and less use of pesticide in the Skuterud catchment area. Content of pesticides was highest in Skut 1, due to agricultural runoff which was partly sedimented before reaching Skut 2. Metal content was highest in Skut 2, probably originating from road runoff (European route 18). The concentration of most elements was within the limit for good ecological status. However concentration of lead (max. 4 µg/L for Skut 1 and 2), was within a moderate ecological status (1.2 – 14 µg/L) (Miljødirektoratet (Norwegian Environment Agency), 2016). According to Sutter et al. (2002), Pb toxicity cause decreased concentration of nitrogen (essential for amino acids) through disrupted nitrogen metabolism in *F. antipyretica* (100 and 500 µM Pb). However, according to Rau et al. (2007) Pb of 25-100 µM have little impact on Fv/Fm. Hence, the concentrations detected in Skut 1 and 2 probably does not impact *F. antipyretica*. Concentration of TOC and DOC was higher at Ref.

loc., possibly originating from forest debris. Concentrations of pesticides and elements were lower at the end of the exposure period for all streams.

HOBO-logger light measurements are expected to be less accurate and with higher variability than ideal, due to shading by accumulated debris above loggers attached to poles, and due to loss of one logger at Skut 2 (data from only one logger was used). This result in lower accuracy, however is not considered to have significant impact on the results. The fish-eye pictures were taken approx. two weeks after the field study was finished, when there were less leaves on deciduous trees. Hence, it was assumably more shading during the study in the two Skuterud locations, containing deciduous trees. However, as indicated by sun movement in pictures, there was significant shading by trees and little direct sun at all locations.



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