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RECOMBINANT WHOLE CELL BACTERIAL BIOSENSORS IN THE SCREENING OF NATURAL EXTRACTS

Master of Science Thesis

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Luonnonaineuutteita on käytetty kauan kansanlääketieteessä muun muassa tulehdusten hoitoon. Modernin lääketieteen aikakaudella on kuitenkin siirrytty käyttämään myös synteettisiä lääkeaineita. Monet bakteerit ovat kehittäneet vastustuskyvyn jo käytössä oleville antibakteerisille aineille, kuten antibiooteille. Vastustuskykyiset bakteerit aiheuttavat terveysongelmia ja sairauksia erityisesti laitosympäristöissä.

Uusia antibakteerisia aineita etsitäänkin nyt kansanlääketieteessä käytetyistä luonnonaineuutteista. Eräs tällainen luonnonaineuute on kihokkiuute, jota on käytetty esimerkiksi yskänrohtona. Kihokkiuutteen sisältämillä yhdisteillä, kuten naftokinoneilla ja flavonoideilla on todettu terveydellisiä vaikutuksia. Pyöreälehtikihokin uutteesta on löydetty pieninä määrinä naftokinoneita, kuten 7-metyylijuglonia ja plumbagiinia, sekä flavonoidi kversetiiniä.

Luonnonaineuutteiden tutkimuksessa voidaan käyttää mittausmenetelmiä, joilla voidaan osoittaa sekä aineen toksisuus että sen biosaatavuus samaan aikaan. Koska luonnonaineuutteita seulotaan usein raakauutteina, vaikuttavat aineet ovat niissä pieninä pitoisuuksina, sekä erilaisia näytteitä on usein paljon. Menetelmien pitää siis lisäksi olla nopeita ja helppoja suorittaa, ja niiden tulee olla sensitiivisiä ja sovellettavissa monissa eri laboratoriossa.

Tämä työ tehtiin osana Tampereen teknillisen yliopiston ja Luonnonvarakeskuksen yhteistyökokonaisuutta, jossa tutkittiin luonnonaineuutteiden hyödyntämistä. Työssä tutkittiin sekä solullisesti lisätyn että kahdelta eri suolta kerätyn pyöreälehtikihokin (*D. rotundifolia*) etanoliuutteita geneettisesti muokatuilla bioluminoivilla bakteerisolukannoilla. Luonnonvarakeskuksen Parkanon koetoiminta-asemalta saatiin sekä pyöreälehtikihokkiuutteet että puhdasaineet, joita olivat kversetiini, plumbagiini, lawsoni ja jugloni. Rekombinantit biosensorisolut ovat bakteerisoluja, joihin on liitetty jostakin toisesta lajista geneettisiä reporttereja. Tässä työssä on käytetty neljää eri bakteerikantaa, joihin on lisätty valoa tuottava, bioluminesoiva ominaisuus. Bakteerikannat ovat *Staphylococcus aureus* RH4220, *Escherichia coli* K12 + pcGLS11, *Acinetobacter baylyi* ADP1 + pBAV1K-T5-LUX ja *P. putida*, joka muokattiin työn aikana bioluminesoivaksi. Muokkaus tapahtui transformoimalla plasmidi pBAV1K-T5-LUX elektroporaatiolla *P. putida* soluihin.

Kihokkiuutteet ja käytetyt puhdasainestandardit osoittivat antibakteerisuutta biosensoreilla. Vasteiden välillä oli kuitenkin eroja, minkä vuoksi jatkotutkimuksissa on tarpeen

ottaa huomioon eri kantojen erilainen sensitiivisyys. Esimerkiksi gram-positiiviset bakteerit voivat reagoida eri tavoin pyöreälehtikihokin sisältämille antibakteerisille yhdisteiden vaikutuksesta kuin gram-negatiiviset.

ABSTRACT

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Natural extracts have been used in traditional medicine as remedies to treat for example inflammations. During the era of modern medicine, there has been a shift towards usage of synthetic substances. However, many bacteria have already developed resistances against antibiotics or other antibacterial substances. These resistant bacteria cause serious health issues, especially in the hospital environment.

The natural extracts used in traditional medicine are new sources for antibacterial substances. One of these extracts is round-leaved sundew extract, which has been used for example as cough remedy. The compounds of sundew extract, such as naphthoquinones and flavonoids, have proven health effects. The extract of round leaved sundew includes naphthoquinones, such as 7-methyljuglone and plumbagin in small concentrations, as well as flavonoid quercetin.

New measurement methods, which can measure bioavailability and toxicity at the same time, can be useful in the screening of natural extracts. The natural extracts are usually screened as crude extracts, and therefore the concentrations of the active substances can be low, and there can be plenty of different samples to screen. Thus, the methods have to be sensitive, fast and easy to perform and easily adoptable into different laboratories.

This diploma thesis was done as a part of collaboration between Tampere University of Technology and the Natural Resources Institute of Finland (Luke). In this thesis, the antibacterial effects of ethanol extracted *Drosera rotundifolia* from pure tissue culture and plants gathered from two different peatlands were studied using genetically modified bioluminescent bacterial cells. Luke Parkano research station provided the round leaved sundew extracts as well as the standard substances, which were quercetin, plumbagin, lawsone and juglone. Recombinant biosensor cells are bacterial cells, which have been genetically modified to include reporter genes from another organism. In this work, four different biosensor strains were used, and they all included a bioluminescent genetic reporter. The strains were *Staphylococcus aureus* RH4220, *Escherichia coli* K12 + pcGLS11, *Acinetobacter baylyi* ADP1 + pBAV1K-T5-LUX and *Pseudomonas putida.* The last one was genetically modified during the work by transforming a bioluminescence plasmid pBAV1K-T5-LUX into the *P. putida* cells using electroporation.

The sundew extracts and the standards showed antibacterial effects on all of the strains. However, there were dissimilarities in the effects between the strains. Therefore, in further studies, it is important to consider the sensitivities of different bacteria. These variances might be caused for example by the gram positive bacteria having dissimilar reactions towards the compounds extracted from the round-leaved sundew than the gram negative.

PREFACE

This Master of Science thesis work was performed in Tampere University of Technology (TUT). Materials and support were also received from Natural Resources Institute of Finland (Luke).

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Emmi Poikulainen

SISÄLLYSLUETTELO

LIST OF FIGURES AND TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

ADP1	*Acinetobacter baylyi* ADP1
DDW	double-distilled water
CPU	counts per unit
d.w.	dry weight
G+	Gram positive
G-	Gram negative
LA	lysogeny agar
LB	lysogeny broth
MIC	minimal inhibition concentration
OD_{600}	optical disturbance at 600 nm
RCR	rolling circle replication
UV	ultraviolet
v/v-%	volume percentage
x g	times the gravitational force

1. INTRODUCTION

Natural extracts come from plants, and they can be prepared for example by boiling a part of the plant in hot water or soaking it in ethanol. For thousands of years, humans have used extracts from the nature to do different tasks. These tasks include seasoning, preserving, and healing. In traditional medicine the extracts have been used for example to treat headache, malaria, and different types of infections. (Ahn et al. 2004 & Rojas et al. 1992). After the rise of the modern medicine in the 1800–1900, many drugs are still derived from the nature (Keshavjee & Farmer 2012). In 1990s, synthetizing of new active compounds became easier, and the percentage of medicines of natural background dropped to approximately 50 % (Li & Vederas 2009).

In 1950s, drug resistance was first documented as a problem. For example in the treatment of tuberculosis, a study concluded that 1.3–2.6 % strains of the resistant to the antibiotics or other drugs used in the treatment. (Keshavjee & Farmer 2012). Since then, antibiotic and other drug resistances have become even more important factors (Keshavjee & Farmer 2012). There is a need to find out new medicines that would help combat the multi-resistant bacterial strains, and natural extracts are a promising source for the discovery of new antimicrobial agents (Li & Vederas 2009, Chemat et al. 2017). To be able to efficiently screen the small concentrations of the active compounds in natural extracts, new measurement systems also need to be discovered. The screening methods need to be sensitive, cost-effective and rapid. One potential solution is using recombinant whole cell bacterial sensors. (Nybond et al. 2015).

In traditional sense, biosensors are combining biological material to microelectronic systems, and thus allowing the detection of measured substance. There are biosensors that utilize for example enzymes or antibodies in their function. However, the whole cell bacterial biosensors utilize “a live, intact cell” as the biological material. This setup allows the evaluation of global parameters, like bioavailability and toxicity. (Belkin 2003). When the whole cell bacterial biosensors are recombinant, they combine a reporter gene derived from one organism to a host organism. There are different types of reporter systems available, but in this work, bioluminescence was chosen. Bioluminescence measurement devices are readily available in microbiology laboratories, and it is also a very sensitive reporter system. The genes encoding reporter properties can be introduced into the host organism using plasmid as a vector or then they can be recombined into the chromosomes. In this work, the plasmid vector method was used, and the DNA was not recombined into the chromosomes.

This thesis work has three aims. The first aim was to construct a new bacterial biosensor strain using an existing plasmid carrying bioluminescence property and *Pseudomonas putida*, a soil bacterium, which is susceptible to genetic manipulation. Furthermore, the aim was to confirm, that the constructed biosensor cell strain can be used a reliable measurement tool for toxicity and bioavailability. The second aim was to compare and contrast the effects of round-leaved sundew (*Drosera rotundifolia*) extract and pure substances similar to the substances found in the sundew extracts to four different strains of whole cell bacterial biosensor cells. In general, only one model organism is used to study the antimicrobial effects of an extract. In this study it was found out, that the model organisms should be carefully selected, as the same compounds can have varying effects on different bacteria, and thus the results might be dependent on the choice of the bacteria. Third aim was to compare the precision of the selected four biosensor strains by testing the same sundew extracts multiple times on the developed test method.

In chapter 2, the natural extracts, which were used as sample material in this thesis, are described in detail and the sundew plant is introduced. Chapter 2 also contains chemical information about the known active substances in the sundew extract. In chapter 3, the recombinant biosensor cells are introduced in detail, both about how they work and how they can be constructed. Chapter 4 describes the methods used in this thesis work. Chapters 5 and 6 are designated to the results and discussion respectively. Conclusions are presented in chapter 7.

2. ACTIVE COMPOUNDS IN NATURAL EXTRACTS

2.1 Natural extracts in general

Natural extracts have been used throughout the human history, for example Egyptians and Mayans have had their own extraction techniques. One of their most early usages has been for seasoning and preserving food, with extracts like rosemary, cloves or garlic. (Ahn et al. 2004; Chemat et al. 2017). Another historical usage of the natural extracts is their usages as traditional medicines. The natural extracts have been used as in wide variety of applications in the past. The uses include but are not limited to wound healing, headache medication, malaria medication, treatment of hyperpigmentation, and to relieve symptoms of lung congestion. In addition, they have been used as antimicrobial agents in a variety of infections, such as skin, wound, and stomach infections. (Rojas et al. 1992; Zhu & Gao 2008).

As multiresistant microbes, such as *Mycobacterium tuberculosis*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus* (MRSA) (Moskowitz et al. 2004; Bapela et al. 2006; Centers for Disease Control and Prevention 2014; De Bonis et al. 2016) are a current, and continuously worsening, problem, a new search for antimicrobial agents has emerged. Current trend in the pharmaceutical industry is trying to replace the synthetic substances with nature-derived alternatives. (Chemat et al. 2017). Traditional medicines, including the natural extracts, are now under the scrutiny to find new, promising antimicrobial substances. As the extracts have been traditionally used to treat infections, natural extracts represent an interesting source for new activities.

Currently, there are numerous ways to extract substances from nature. These techniques include maceration, solvent extraction, steam and hydro-distillation, cold pressing, and squeezing. Some of the most modern techniques involve ultrasound assistance to enhance the efficiency of the extraction process. These methods can be used to extract interesting substances including antioxidants, pigments, lipids and phytochemicals. (Chemat et al. 2017). The natural extracts are usually screened without any further handling, and thus the bioactive compounds might be present only in minute amounts (Nybond et al. 2015).

Different natural extracts have been studied for their effects on microbes. For example, Ahn et al. (2004) demonstrated that grape seed extract and pine bark extract have antimicrobial activities. These natural extracts inhibited the growth of *E. coli*, *Listeria monocytogenes* and *Salmonella typhimurium* in both growth medium and ground beef. Serra et al. (2008) further demonstrated the antibacterial effects of grape seed extract as well as

similar properties of olive extract. The olive and grape seed extracts also possessed antimicrobial activities for *Candida albicans* and *Saccharomyces cerevisiae*. In a study of African traditional medicinal plants of the *Euclea family*, their root extracts were found to be antimicrobial against *M. tuberculosis* (Joubert et al. 2006). The extracts of Finnish herbs (willow herb, *Chamaenerion angustifolium*; meadowsweet, *Filipendula ulmaria* and purple loosestrife, *Lythrum salicaria*) had bactericidal effects on *S. aureus* and *E. coli*. Also, the extracts of Finnish berries showed activities against bacteria, with crowberry (*Empetrum nigrum*) extract having the largest spectrum of target organisms. (Rauha et al. 2000). However, the effects of the new antimicrobial agents on humans have to be carefully assessed before using them, as the natural extracts can have some co-effects with the current medicines. For example, a Mexican study about natural extracts found out, that they can inhibit the enzymes of human liver, such as enzymes belonging to P450 family, which are related with the oxidative metabolism of various drugs. (Rodeiro et al. 2009).

The antimicrobial effects of the natural extracts can be caused by various mechanisms. Some of the speculated reasons include changes in the microbial cell membrane, which cause the cells to autolyze (Ahn et al. 2004). Some substances, especially organic solvents, are membrane active. This causes changes in the fatty acids of the cell membranes. These changes include isomerization of double-bonds in the fatty acids, which is a special reaction mechanism of bacteria for toxins in their environment. These changes can cause reduction in the fluidity of the cell membranes, but they let the bacteria to adapt better to their environment (Heipieper & De Bont 1994). Thus, as both the substances in the extracts and the solvents they are extracted with, can have effects on the bacteria, it is extremely important to control the amount of solvents in the samples to make sure that the reactions are only based on the active compounds.

The natural extracts contain a variety of chemical compounds. Many of the bioactive compounds are secondary metabolites of the plants they are extracted from. In contrast to primary metabolites, they are called secondary, because they are not directly related to the normal growth or reproduction of the plant. This means that the plant does not produce them, if it does not have enough energy or nutrients to do so, as they are not essential for its existence. The secondary metabolites are often related to the defense mechanisms of the plants, and many circumstances effect the production of the secondary metabolites. (Hohtola et al. 2005). Same secondary metabolites can be found from different plants, for example, grape seed extract contains polyphenolic and phenolic compounds, including quercetin (Ahn et al. 2006), which is one of the compounds found also in sundew extract, which is discussed in chapter 2.2.1.

2.2 Round-leaved or common sundew (*Drosera rotundifolia*)

First mentions of sundew plants are from 16th century, but they became more publicly known after Charles Darwin wrote a book about them in 1875 (Enroth 2009). Round-

leaved sundew (*Drosera rotundifolia*) is a carnivorous plant, whose main ranges are wet, oligotrophic habitats. It is widely distributed, and grows all over the northern hemisphere. A total of 110 sundew species grow all over the globe, with half of the species growing only in Australia. In Finland and Europe in general, two other species *D. anglica* and *D. intermedia* are also present. (Enroth 2009; Baranyai & Joosten 2016).

The habitats of *D. rotundifolia* include peatlands, that have acidic environment and microclimates, including high air humidity and lower temperatures. Round-leaved sundews usually grow on *Sphagnum* moss covered areas, and they survive over winter as buds covered in the moss. The soil is mostly poor in nutrients, such as nitrogen, phosphorus, potassium, calcium and magnesium, which the sundew compensates by being insectivorous. Calcium is even toxic for sundew in certain pH conditions. (Nordbakken et al. 2004; Egan & van der Kooy 2013; Baranyai & Joosten 2016). Nowadays, sundew is becoming endangered, as its traditional areas are taken for other purposes by draining the bogs, although in Finland, there is an abundance of peatlands at approximately 5.1 million hectares. The average density of *D. rotundifolia* plants in different collection areas in Finland was 45–56 plants per m^2, corresponding to 6.3–6.7 g of biomass. (Galambosi et al. 2000; Enroth 2009). Sundew is vulnerable to changes in its environment, including the increase in nitrogen levels, as the higher levels courage *Sphagnum* and other species to grow better and thus eliminating sundews by forcing them to grow higher to avoid being denied the sunlight (Svensson 1995).

Figure 1. *Whole sundew (Drosera rotundifolia) plants, which were collected from the peatlands of Western Finland for the extraction in this work. The tentacles of the leaves can be seen for example in the are marked with a red rectangle. Photo taken by Tytti Sarjala.*

Morphologically, the round-leaved sundew plant is small, with flowering stems ranging 4.1–14.7 cm, and its roots are usually poorly developed. Its leaves circle a basal rosette, and are approximately 20–50 mm long. The leaves have a light olive-green color on the lower sides, but also have reddish coloration on the upper sides due to anthocyanins, which are natural pigments that can also be found from the bilberries (*Vaccinium myrtillus*). The leaves also contain approximately 200 tentacles (see Figure 1), which contain highly viscous, sugary mucilage and which the sundew uses to catch and digest the insects. (Enroth 2009; Baranyai & Joosten 2016). The mucilage includes endochitinase to digest the chitinous parts of the insects it catches, but chitin can also be found in the roots of the sundew plant (Libantová et al. 2009; Baranyai et al. 2016; Jopcik et al. 2017). Each plant has 1–15 flowers, and each flower produces only a few hundred pollen grains, resulting in an average of 424 seeds produced per plant (Galambosi et al. 2000). In a fertilization experiment by Svensson (1995), 72 % of the sundew plants flowered. If the weather is not sunny, the flowers do not open at all and are self-pollinated. The sundew seeds are waterproof, and they can float on water for several months, which helps it to spread to new areas carried by flowing water. Sundew can also reproduce asexually through budding and root suckers. (Baranyai & Joosten 2016). The DNA and RNA content of *in vitro* grown sundew leaves has also been analyzed. The DNA content was 15 µg per 100 mg of plant tissue and RNA content 7 µg per 100 mg of plant tissue (Bekesiova et al. 1999).

Round-leaved sundew and its extracts have been used as a remedy for coughs and other respiratory tract illnesses for a long time (Baranyai et al. 2016). Other traditional uses include also use as potency medication and high blood pressure (Cederberg 2014). Traditionally, the sundew plant was collected in the beginning of the flowering period and then tried to be used in extracts or tinctures. Throughout the 1900s, the pharmacological properties of sundew began to be scientifically recognized. Currently, in Europe, there are 200–300 medicines or remedies that utilize sundew in its fresh or air-dried form. The sundew biomass required annually in Europe weighs 6–20 tons. It can be cultivated by *in vitro* propagation, indoor cultivation or outdoor cultivation, but currently the medicinal use is limited to wild grown populations of *D. madacasgariensis* from Madagascar and *D. rotundifolia* from Finland. (Baranyai & Joosten 2016; Baranyai et al. 2016). The limitation to the wild grown species might be due to the fact, that even when the chemical properties of the *in vitro* grown sundew biomass are qualitatively same as in the wild sundew plants, the quantity of the metabolites is usually much lower in the *in vitro* grown plants (Egan & van der Kooy 2013). The lower levels of the active secondary metabolites in the *in vitro* grown plants might be caused by the lack of stress factors in the laboratory compared to the natural habitats (Hohtola et al. 2005). However, the *in vitro* culturing might offer uniform quality of the plants, as they can be cloned (Baranyai et al. 2016).

In this thesis, both *in vitro* grown and Finnish wild *D. rotundifolia* were tested to find out, if the possible quantitative differences could be seen between the wild and *in vitro* grown samples (see 4.2.3).

2.2.1 Sundew extract

The active compounds in sundew extracts are mainly naphthoquinones and flavonoids, which are natural pigments (López López et al. 2014). Naphthoquinones are bicyclic polyphenolic compounds that contain normally one or multiple ketone groups and are structurally related to naphthalene with carbonyl groups located in the positions 1 and 4 (Figures 2B, 2C and 2D). They are organic compounds and their reactivity is high. (López López et al. 2014). Naphthoquinones are biosynthesized through polyketide precursors in shikimic acid pathway (7-methyl juglone) or acetate-polymalonate pathway (plumbagin). In addition to being found in plants, they are also produced in the metabolism of some fungi, algae and bacteria (Egan & van der Kooy 2013; López López et al. 2014).

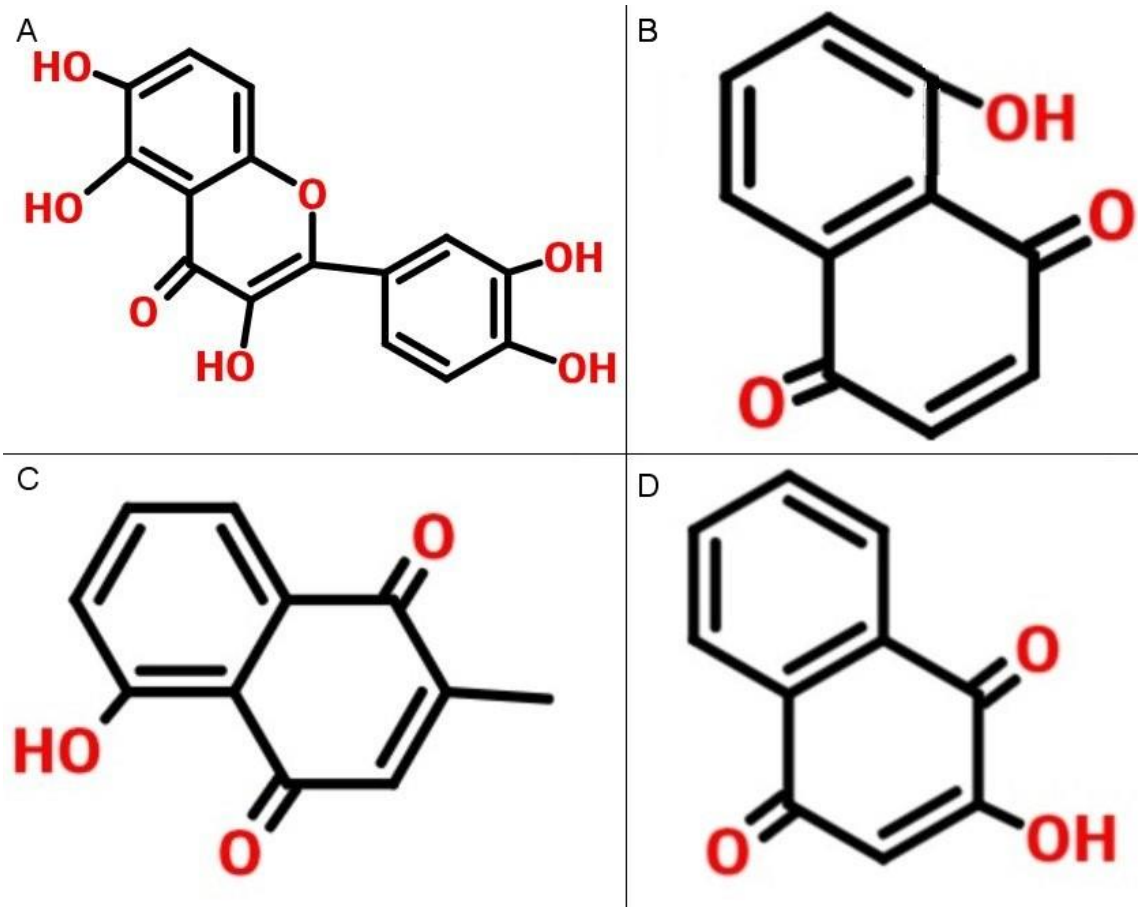

Figure 2. *Naphthoquinones and flavonoids present in sundew extracts and used in this thesis as control substances.* ***A.*** *Quercetin.* ***B.*** *Julone.* ***C.*** *Plumbagin.* ***D.*** *Lawsone. All figures are drawn with Chemical Structure Editor (Metamolecular).*

The naphthoquinone content of Finnish sundews is higher than that of the sundews from South of Europe (Hohtola et al. 2005), which makes it an interesting sample material for antimicrobial testing. The trend is most likely caused by the strong variation of temperature and light of the Nordic seasons. The naphthoquinones have been extensively studied for their antimicrobial effects. They also have antispasmodic, analgesic and antipruritic effects. (Bapela et al. 2006; Hakakian et al. 2017).

The concentration of different naphthoquinones varies between the sundew species, but in *D. rotundifolia*, 7-methyljuglone (5-hydroxy-7-methyl-1,4-naphthoquinone, an optical isomer in structure compared to plumbagin in Figure 2C) is the most common one, and for example, plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone, in Figure 2C) is found only in minute concentrations. In one study, *D. rotundifolia* was found to have 0.129–0.184 % dry-weight (d.w.) of 7-methyljuglone. *D. intermedia* had 0.752–0.946 % d.w. of plumbagin (Baranyai et al. 2016). In another study, the concentration of 7-methyljuglone was found to be 1.0–2.3 % d.w. in *D. rotundifolia* (Kämäräinen et al. 2003). As an example of the varying plumbagin concentrations, they were for *D. adeleae* and *D. aliciae* 0.006–0.039 % and 0.001–0.040 % from the fresh weight respectively

(Marczak et al. 2005). The concentration of the 7-methyl juglone in *D. rotundifolia* depends also on the plant part, with highest concentration in the flowers (1.73–2.46 % d.w.). The leaves and stems had lower concentrations, 0.52–0.95 % d.w. and 0.24–0.35 % d.w respectively. (Galambosi et al. 2000). In *in vitro* propagation, the concentration of 7-methyljuglone can be enhanced by addition of salisylic or jasmonic acid. The concenctration of 7-methyljuglone in salisylic acid treated shoots of *D. capensis* was 0.013 mg/kg d.w., which was 2.1 times higher than the control. (Ziaratnia et al. 2009).

The weather conditions during the growth of the sundew plant can also affect the naphthoquinone content. In a Finnish study, a negative correlation was found between rain amount and the measured 7-methyljuglone content. *Vice versa*, a positive correlation was found between the 7-methyljuglone content and the average temperature measured in June of the picking year. Thus, the conditions that are suitable for photosynthesis seem to increase the production of secondary metabolites, including naphthoquinones. However, no correlation was found between the shading of the growth place and usage of fertilizers and the concentration. (Kämäräinen et al. 2003; Hohtola et al. 2005).

In other studies, possible reasons for varying naphthoquinone content have also been discussed. The reasons include infection of the plant tissues, nutrient availability, the habitat and genetic variation of the plants. In a Finnish study, the best habitat producing the highest naphthoquinone concentration was a lake shore bog. (Kämäräinen et al. 2003; Joubert et al. 2006). Both plumbagin and 7-methyljuglone can also be present in *D. rotundifolia* or *D. intermedia* plants in bound, glucosidal forms, called hydroplumbagin glucoside and rossoliside respectively. The glucosidal versions are converted to the compounds during methanol extraction process through hydrolysis. (Budzianowski 1996; Budzianowski 1997; Egan & van der Kooy 2013).

Other active compounds in the sundew extracts include flavonoids, such as quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one, in Figure 2A) and ellagic acid (2,3,7,8-Tetrahydroxy-chromeno[5,4,3-cde]chromene-5,10-dione). Depending on the study, *D. rotundifolia* has 0.077–0.141 % or 0.187 % dry-weight of quercetin, but also higher concentrations (4 %) have been reported after hydrolysis of the leaves, stems or roots (Budzianowski 1996; Budzianowski 1997; Zehl et al. 2011; Baranyai et al. 2016). In another study, the ethanol extract of *D. rotundifolia* had 0.49 % of quercetin in it (Paper et al. 2005). Ellagic acid is a known antioxidant, and it comprises 0.17–2.36 % of the dry weight. The main effects of flavonoids are anti-inflammatory and spasmolytic activity. All currently known flavonoids that have been extracted from *Drosera* species include a cathecol or pyrogallol moiety in the B-ring of their structure. A- and B- are aromatic rings form the skeleton of the flavonoid. Other important flavonoids found in *D. rotundifolia* are isoquercetin and hyperoside, which have proven to inhibit human neutrophil elastase, and can, thus, be used for treating inflammatory lung diseases. (Marin et al. 2005; Fukushima et al. 2009; Egan & van der Kooy 2013).

One study (Fukushima 2009) found out that *D. rotundifolia* ethanol extract fractions suppressed changes caused by inflammatory reaction in human mast cells, which are an important part of the inflammatory response in human body. The inflammatory reaction was induced with activated T-cell membranes. The extracts prevented adhesion and extension of the mast cells as well as returned the gene expression related to inflammation back to the base level of the control cells. The gene suppression was dose dependent for the *D. rotundifolia* extracts. However, it was not clear, which of the components in the extracts caused the suppressive effects. In agreeance, Paper et al. (2005) found out that the ethanol extract of *D. rotundifolia* also possessed anti-inflammatory properties. They performed HET-CAM (horioallantoic membrane) assays for fertilized chicken eggs and 500 µg plumbagin per 10 µl pellet was enough to inhibit 98 % of the adverse reactions caused by sodium dodecylsulfate.

Another example of the antimicrobial effects of *Drosera* extracts was found against the oral streptococci microbes. In the study, the aerial parts of *Drosera peltata* were extracted in organic solvents. Ether extracts had MIC of from 100 µg/ml to over 500 µg/ml, chloroform extracts 31.25–250 µg/ml and ethyl acetate extracts 250–500 µg/ml. The chloroform extract was also fractioned, and one of the fractions was identified to be plumbagin. For the plumbagin fraction, the MIC was as low as 1–2 µg/ml. (Didry et al. 1998).

2.2.2 Antimicrobial effects of naphthoquinones and flavonoids

The antimicrobial effects of 7-methyljuglone from an African tree (*Euclea natalensis*) was studied together with classical tuberculosis drugs. The minimal inhibitory concentration (MIC) of 7-methyljuglone against *M. tuberculosis* was 0.5 µg/ml, and when it was applied together with isoniazid, their MICs were decreased six-fold, which indicated synergistic effects on the *M. tuberculosis*. (Bapela et al. 2006). Also synthetic derivatives of 7-methyljuglone showed antimicrobial activity against *M. tuberculosis* (Mahapatra et al. 2007).

Plumbagin, extracted from a tropical shrub *Plumbago zeylanica*, has also demonstrated antibacterial activity against *E. coli*, *S. aureus* and *P. aeruginosa* (Jeyachandran et al. 2009). In addition, plumbagin extracted from *Plumbago rosea* showed antimicrobial activity against *S. aureus* in concentration of 5 µg/ml. Nair et al. (2016) proved, that plumbagin's antimicrobial activity was better against gram positive (G+) than gram negative (G-) bacteria. The group furthermore proved that the antimicrobial activity of plumbagin could also be effective in skin infections caused by *S. aureus*.

The antimicrobial effects of the naphthoquinones are likely caused by several different mechanisms, and they are structurally connected to several commercially used antibiotics. These antibiotics include daunomycin (see Figure 3A) and myxopyronin (Figure 3B). The shared structure is the hydroxyquinone structure, which is related to their capability to form adducts with cell's macromolecules. (Rahmoun et al. 2012). Plumbagin's biological

activity can be based on its ability to create reactive oxygen species and thus inducing apoptosis. Plumbagin induced 2.5-fold increase in creation of reactive oxygen species in *S. aureus* (Nair et al. 2016). It also activates cell signaling routes, inducing expression of Nrf2 genes (Cayman Chemical 2015). Nrf2 is a regulator for antioxidant response (Jaiswal 2004), thus plumbagin induces the expression of antioxidant enzymes. Furthermore, plumbagin increases the concentration of intracellular superoxides. Atavaquone, another naphthoquinone, inhibits oxygen consumption of the bacterial cell. (Bapela al. 2006).

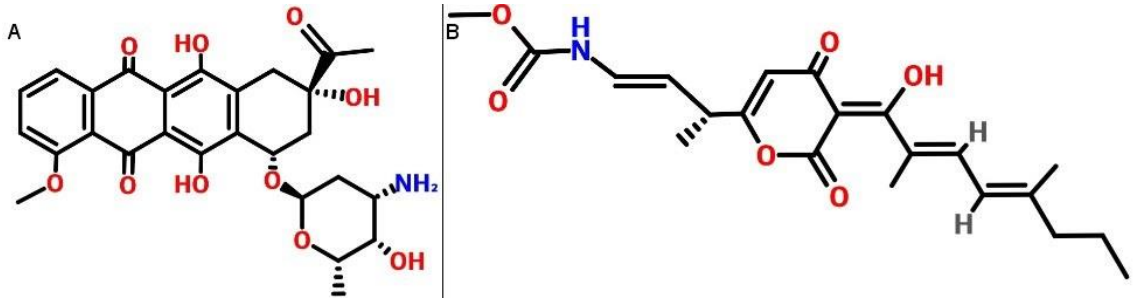

Figure 3. ***A.*** *Chemical structure of daunomycin antibiotic.* ***B****. Chemical structure of myxopyronin antibiotic. Both figures are drawn using Chemical Structure Editor (Metamolecular).*

Furthermore, with other naphthoquinones, such as lawsone, bioactivities might be based on the generation of reactive oxygen species. They can interact with biological targets and for example prevent the electron transportation. The naphthoquinones can accept one or two electrons, causing them to change into highly reactive anion radicals. The naphthoquinones can also generate superoxide, which leads to the formation of hydrogen peroxide (H_2O_2). Hydrogen peroxide can permeate through cell membranes and, thus, damage proteins and lipids, causing oxidative stress for the cell. It can even lead to apoptosis. (López López et al. 2014).

Plumbagin also has inhibitory effects on NADPH oxidase 4, and other naphthoquinones can inhibit topoisomerases, which regulate the winding of DNA during its replication. The enzyme-based effects of the naphthoquinones happen also because they can act as subversive substrates to many enzymes, but it is not the only reason. Many of the effects are complicated and they carry non-specific reactivity against many biological substances in the cells. Furthermore, plumbagin can protect cells from lack of glucose or oxygen via its stimulation of Nrf2, which was discussed above. (Mahapatra et al. 2007; López López et al. 2014; Klotz et al. 2014; Cayman Chemical 2015)

Contradictory information on the effects of naphthoquinones on human cells have been published. For example, naphthoquinones are cytotoxic for human MRC-5 fibroblast cells (Bapela et al. 2006). In another study, plumbagin did not show any toxicity towards *D. melanogaster* (fruit fly), a common toxicity model organism (Nair et al. 2016). Thus, there is no clear consensus on the usability of the naphthoquinones in human subjects.

Quercetin is known to prohibit allergic and inflammatory reactions, and it can even relieve depression symptoms (Cederberg 2014). Quercetin has shown effects of anti-carcinogenicity and neuroprotection, and it has also been studied for its antibacterial properties (Jaisinghani 2017). Quercetin's antimicrobial properties were demonstrated in a Finnish study (Rauha et al. 2000), where quercetin and its structural isomer, morin, were found to be toxic to *S. aureus*, *E. coli* and *P. aeruginosa*.

Lawsone and juglone are natural naphthoquinones that can be found in the plants. Juglone is isolated from different species of walnut trees, including their roots. Lawsone is originated from the henna plant, which has also been used in traditional medicine as well as a cosmetic dye. Lawsone has been found to carry antifungal activity against *Candida albicans* and its derivatives are antiparasitic against *Toxoplasma gondii* (López López et al. 2014). The antibacterial function of lawsone depends on the ketone function in its structure (Rahmoun et al. 2012). It has been used as antibacterial dye for technical textile development (Yusuf et al. 2012). Juglone has been used in traditional medicine to alleviate the symptoms of acne, parasites, cancer and fungal infections. It has been proven to induce stress response in *S. aureus* by creating reactive oxygen species and inhibiting protein synthesis and cell division. (Wang et al. 2016).

3. BIOSENSOR CELLS

3.1 Recombinant biosensor cells

By definition, biosensors combine biological elements to microelectronic systems, and thus enable the detection of measured substance (Belkin 2003). There are different types of biosensors, including cell-free and living biosensors. The cell-free biosensors, cellular components are isolated from cells and used outside them. The measurement conditions are defined and can be modified closely by the user. In this thesis, however, living biosensor cells were used. Biosensor cells are, for example, bacterial or yeast cells that are used to perform the measurements. The bacterial biosensors can also be genetically modified to enhance the usability of the living biosensor cells. The recombinant biosensor cells contain DNA elements from evolutionarily distant organisms. (Galluzzi & Karp 2006). This paragraph has defined the usage of the term recombinant biosensor cell for the purposes of this thesis work.

The living, recombinant biosensor cells combine gene promoters, which are responsive to the substance being measured and a reporter gene. (Ko & Kong 2017). There are various reporter genes, which can produce fluorescent, bioluminescent and colorimetric signals (Yagur-Kroll et al. 2015). In this study, the reporter system is based on the *luxABCDE* operon, which causes bioluminescence. Bioluminescence is an useful detection method for the measurements, as it is sensitive, and the signal can be monitored in a simple and rapid manner. In addition, the background signal associated with the bioluminescence is low, which improves the noise to signal ratios in the measurements. (Santala et al. 2016). Bioluminescence genes from *Photorabdus luminescens* are also stable in higher temperatures (up to 45 °C) (Vesterlund et al. 2004), which increases their usability in various applications.

The biosensor cells can be divided into two categories: constitutive and inductive sensors. In inductive sensors, the catabolism of the biosensor bacterial cells is affected (Ko & Kong 2017). The more bioluminescent light the bacterial cells produce, the more their catabolism is affected by the measured substance or circumstance, and thus they are sometimes called “lights on” biosensor cells (Xu et al. 2013). For example, inductive biosensor made from *E. coli* was used to demonstrate the harmful effects of ultraviolet radiation (UV) on the cells and establish a test to find out the UV-protective properties of natural extracts. The UV light cause damage to the cell’s DNA, which then induces the bioluminescent reaction. When the cells are protected from the UV light, such as covering with aluminum foil or by the natural extracts, their luminescence signal is lower. (Tienaho et al. 2018).

In constitutive bacterial biosensors, the metabolism of the cells is affected (Ko & Kong 2017). The measurements are based on the fact, that the metabolic well-being of the bioluminescent bacterial cell is directly affecting the intensity of the measured bioluminescence (George et al. 2017). In contrast to the inducive sensors, they are called "lights off" sensors (Xu et al. 2013). Their function for cytotoxicity is based on the necessary enzymes of the cell being inhibited. Under normal conditions, the biosensor cells express *luxABCDE* and produce bioluminescence following the reaction equation, which the luciferase enzyme catalyzes (Vesterlund et al. 2004):

$$RCHO + O_2 + FMNH_2 \rightarrow RCOOH + FMN + H_2O + \text{light (at 490 nm)}. \quad (1)$$

In the reaction equation (1), RCHO corresponds to long chain fatty acid that is oxidized in the reaction to fatty acid RCOOH. $FMNH_2$ represents the reducing power of the flavin mononucleotide (Stewart & Williams 1992) and FMN is the flavin mononucleotide in reduced form. The light produced is blue-green, and its wavelength is 490 nm. (Vesterlund et al. 2004).

When cytotoxic compounds are present, the gene transcription and protein translation are not as active as without the cytotoxic compounds. This happens because of the inhibition of the enzymes. The reaction (1) includes the reducing power ($FMNH_2$), which links it to the metabolic activity of the bacteria (Vesterlund et al. 2004). Thus, also luciferase becomes less active, as its coding genes, *luxAB*, are less expressed. *LuxA* codes the α subunit of the enzyme and *luxB* the β subunit (Stewart & Williams 1992). Expression of *luxC* (acyl-CoA reductase) and *luxD* (acyl-ACP thioesterase) are also inhibited. In addition, the enzyme that participates in production of substrate (RCHO in reaction equation (1) to luciferase, acyl-protein synthase and its gene *luxE* is inhibited. As the toxicity increases, the inhibition escalates as well, which lowers the intensity of the bioluminescent signal. (Cui et al. 2018), Thus, the more cytotoxic the compound studied is, the lower the bioluminescent signal.

Biosensor cells reply to the growing need for highly sensitive, rapid, inexpensive and high-throughput detection methods. The protocols are generally simple to perform, and the cost-effectivity is due to the lack of conventional chemical analyses, which require specialized equipment and take time. The whole-cell biosensor methods also allow the simultaneous evaluation of antimicrobial nature and bioavailability of the measured substance. Compared to other cell models, such as models including mammalian cells, the bacterial cells are also effortless to grow, manipulate and sustain, which further explains their usability in screening or measuring various substances. (Elad et al. 2015; Ko & Kong 2017; Cui et al. 2018). Previously, they have been extensively studied in their properties for environmental samples, such as heavy metal detection (Xu et al. 2013) . However, they are also extremely well suited for screening of the natural extracts, as they have

enough sensitivity to detect small amounts of the substances as well as possibility to convert the bacterial biosensor cells into high-throughput screening methods. (Nybond et al. 2015).

3.2 Genetic engineering of biosensor cell

In genetic engineering, the non-native genetic material can be integrated into the chromosomal host DNA or it can be introduced as a plasmid into the host cell. Plasmids are small circular DNA molecules, which often carry genes for virulence or antibiotic resistance. (Smillie et al. 2010). Plasmid DNA is extrachromosomal in the bacterial cells, and its replication is not directly depended on the growth of the bacterial cells. The plasmid still uses the machinery of the host cell for replication (Scott 1984).

The plasmids can be transformed into the host bacteria using electroporation. The efficiency of the transformation depends on for example the concentration of plasmid DNA used. (Dower et al. 1988). In the electroporation, the cells are subjected to an electric field briefly. The electric field interacts with the lipids in the cell wall or cell membrane. This creates pores to the cell surface and the DNA plasmids can pass through these pores into the inside of the cell. The electric field also acts as a driving force, which pushes the charged molecules, including negatively charged DNA, into the cell (Weaver & Chizmadzhev 1996). Afterwards the electroporated cells are cultivated in selective media to confirm the success of the transformation (Dower et al. 1988).

The bacterial strains that include the *lux* in plasmid form are typically temperature sensitive, and grow and express the plasmid best at temperatures, which are 7–10 °C lower than the wild type bacteria would do (Stewart & Williams 1992). The transformed cells also usually need to be cultivated in a medium containing their proper selection marker. If this is not done, there is no selection pressure, which leads to plasmid instability. When the plasmid is instable, the cell division might result in one of daughter cells not carrying the plasmid anymore. (de Taxis du Poët et al. 1986).

3.3 Relevant biosensor cells

Gram positive (G+) bacteria may be more sensitive to changes in the growth conditions than the gram negative (G-) bacteria. This is due to the differences in their structure. G+ cells lack an outer membrane and have, thus, been found to respond more readily to temperature and pH changes (Ahn et al. 2004). The cell wall structure of the G+ bacteria is not as complex as in the G- bacteria, which makes them more susceptible to the permeation of antimicrobial compounds (Serra et al. 2008).

The selected bacteria were from two different sources. *E. coli* and *S. aureus* are bacteria that live in the human body. *E. coli* is found in the digestive tract, and *S. aureus* in the respiratory tract, including nose and the skin. *A. baylyi* and *P. putida*, on the other hand,

are soil bacteria. Some of the secondary metabolites, such as naphthoquinones discussed in chapter 2.2.1, are the plant's defense mechanism against pathogenic soil bacteria and fungi. For example, the antimicrobial activity of 7-methyljuglone in the sundew plant might decrease the competition of nitrogen between the plant and harmful soil bacteria. (Kämäräinen 16 et al. 2003). In addition to pathogenic soil bacteria, also useful soil bacteria can be found. The useful soil bacteria can promote the growth of a plant, by for example fixing nitrogen into the root nodules of legume plants. This fixation is associated with an increased flavonoid production in the legume plant. (Vessey 2003). As the both pathogenic and useful soil bacteria can induce increased levels of naphthoquinones and flavonoids, it is interesting to compare the antimicrobial effects of the sundew extract between the bacteria of human and soil origin, which might be the original targets of these secondary metabolites.

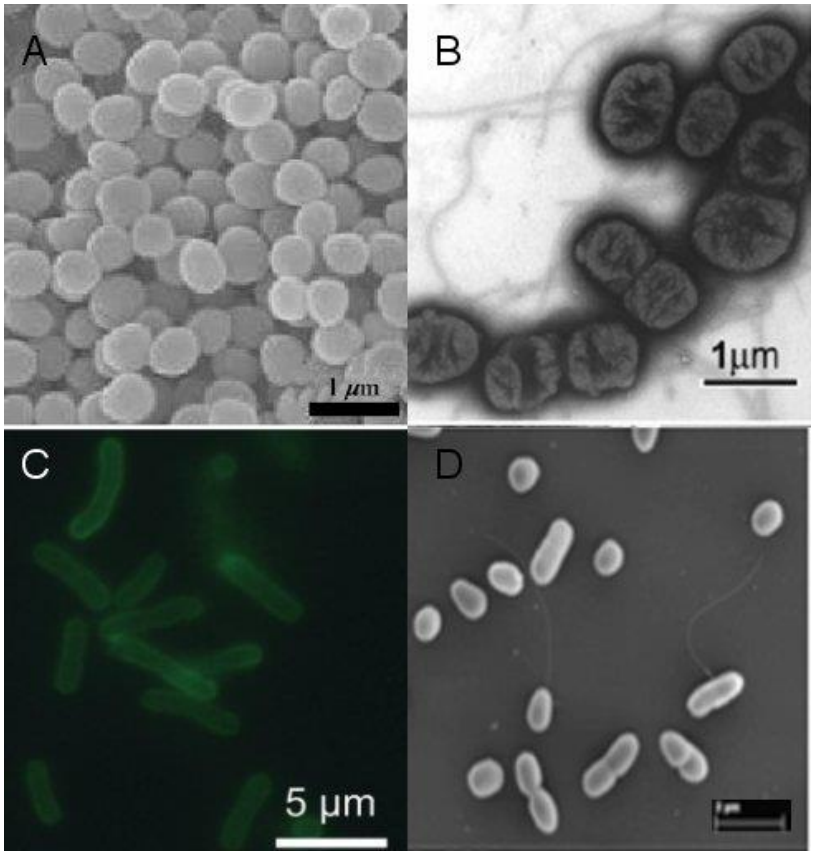

Figure 4. *Microscopic images of the bacteria used in this thesis. The images are not necessarily from the same strain as used in this work, but more used as reference to see the general shape of each of the bacteria. Images are picked from literature and are cropped to suitable size.* ***A.*** *S. aureus (Cho et al. 2005).* ***B.*** *E. coli K12 (Pal et al. 2007).* ***C.*** *Fluorescent A. baylyi ADP1 (Zhang et al. 2011).* ***D.*** *P. putida (Poblete-Castro et al. 2014)*

In the following sub-chapters, each of the bacteria are introduced along with some of the current biosensor cell applications hat have already been carried out in each of the bacterial species.

3.3.1 *Staphylococcus aureus* RH4220

Staphylococcus aureus is a round shaped (see Figure 4A), G+ bacterium that can be isolated from mucosal surfaces of humans. It mainly resides in the anterior nares, but when the individual has immune disruption, it can become pathogenic and colonize skin. On the skin *S. aureus* can cause pyoderma, impetigo, ecthyma (different types of skin infections) and cellulitis (infection of fat tissue). In heart muscle, it can cause endocarditis and in the bones osteomyelitis. (Nair et al. 2016).

S. aureus can also cause biofilms, which are especially harmful in hospital environments. It becomes highly resistant to common antibiotics once in the biofilm, because of the polysaccharide matrix of the biofilm. These biofilms can accumulate in harmful places, such as implantable medical devices or urine catheters. (Nair et al. 2016). *S. aureus* is a normal cause for healthcare related infections, and 15–45 % of *S. aureus* strains are methicillin resistant (MRSA). MRSA caused over 72 000 infections in USA in 2014, with majority of the cases being health care related (Centers for Disease Control and Prevention 2014). Thus, it is important to find new antimicrobial agents, which could help the treatment of MRSA. The screening of natural extracts using *S. aureus* biosensor cells might prove beneficial for the discovery of these new substances.

S. aureus luminescent biosensors have been previously used in measurement of tetracycline antibiotic and nisin. The study demonstrated their use as a screening method for detection of antimicrobial substances. The bioluminescence levels were 20 times higher than that of the background, and the signal could be detected early during their incubation, which made the strain suitable for screening applications. (Vesterlund et al. 2004). However, lux operon is not expressed in G+ bacteria as well as in G- bacteria, which might cause lower light signals. (Mesak et al. 2009). Bioluminescent *S. aureus* cells have also been used in in vitro imaging of *S. aureus* infections in mice models. Plaut et al. (2013) were able to demonstrate that the bioluminescence could be measured through the skin of the mouse and were, thus, able to monitor the infection, and its healing.

3.3.2 *Escherichia coli* K12 + pcGLS11

Escherichia coli is G- bacterium with rod-like shape (Spratt 1975) and flagella (see Figure 4B). It mainly resides in the gastrointestinal tract of humans and other vertebrates. It is the most common organism in the guts, which can use both aerobic and anaerobic respiration. Some of the strains are pathogenic, while other are harmless or even beneficial for the host organism. (Tenaillon et al. 2010). The main diseases caused by pathogenic *E. coli* are diarrhea and other enteric diseases. They can also cause infections outside the gastrointestinal tract such as in the urinary tract and in extreme cases, they have been shown to cause meningitis. The pathogenic *E. coli* bacteria carry some virulence factor, which can be for example a plasmid, but also deletions of the DNA compared to non-pathogenic strains can happen. (Kaper et al. 2004).

The whole genome of *E. coli* K12 strain is known, and it was first isolated from a patient with diphtheria in 1922. It is one of the most studied organisms, and the molecular processes have been most widely studied in *E. coli*. It was used in the early studies of genetics and phages in 1940s and 1950s, which caused it to become a common model organism in biochemistry and molecular biology. (Hayashi et al. 2006).

Due to the fact, that it is such a commonly known and well-researched microbe, *E. coli* has also been proven to be a favorable host in biosensor cell applications. The signal level in *E. coli* pcGLS11 biosensor cells was 10 000 times as high as the background, which demonstrates the good ability of the sensor to emit light (Vesterlund et al. 2004). As gut bacteria have been shown to have a linkage to the rise of antibiotic resistance (Tenaillon et al. 2010) and the extra-intestinal cases of *E. coli* are mainly affecting people with compromised immune systems and already are antibiotic resistant (Biran & Ron 2018), it is also an interesting model organism for finding new antimicrobial substances.

E. coli has already been employed in many different applications. For example, Jha et al. (2018) developed a fluorescent *E. coli* based biosensor for the detection of protochatuate. It was able to detect protochatuate and cathecol and showed minor cross-reactivity with other similar compounds. Willardson et al. (1998) demonstrated the usage of *E. coli* microbial sensor in the detection of xylene, toluene and benzene in water and soil samples. They cloned the *E. coli* with a plasmid containing *xyl*R regulator from *P. putida* mt-2 and bioluminescence in the form of *luc* firefly luciferase gene. Their biosensor cell was able to detect concentrations of toluene that were in the µM range. The *E. coli* biosensor cells have also been used as a panel to screen drugs for their effects. It was demonstrated that the different reporter and promoter genes applied to the different *E. coli* strains together could provide a comprehensive picture of the drugs screened (Elad et al. 2015). A luminescent *E. coli* K12 sensor has also been used in the detection of tetracyclines in poultry meat. The biosensor was constructed to include *luxABCDE* and its expression was controlled with tetracycline induced promoter *tetA*. Concentrations as low as 5 ng/g could be measured in the system. (Virolainen et al. 2008).

3.3.3 *Acinetobacter baylyi* ADP1

Acinetobacter baylyi is an aerobic, G- bacterium that can be found in soils. It is rod-shaped (see Figure 4C). It is nonpathogenic, and its genome has been fully sequenced. It is also relatively effortless to genetically manipulate. It is also metabolically versatile and a robust bacterium, able to tolerate different environments. (Peleg et al. 2008; Cui et al. 2018). These facts make it an interesting option for model organism.

In addition, it is related to a pathogen, *Acinetobacter baumannii*, which is known to develop resistance to antibiotics. *Acinetobacter baumannii* is most often isolated in hospital environments, and it causes various serious diseases. Such diseases include meningitis after neurosurgery and neonatal necrotizing fasciitis, which is also known as flesh eating

infection. (De Bonis et al. 2016; Dhawan et al. 2017). Understandably, there is a need for new antimicrobial treatments to prevent this rising health issue from getting worse. Namely, as the populations of developed countries get older, also the healthcare related infections might be on the rise, as the older people are more vulnerable to these nosomical infections (Strausbaugh 2001). As *A. baumannii* and *A. baylyi* belong to the same genus, it is likely that their responses to same antimicrobial substances would be similar. Thus, *A. baylyi* is a useful model in the search of new antibiotics from natural extracts.

Acinetobacter baylyi strain ADP1 has demonstrated its usefulness in screening of antimicrobial substances. In a study about photoactivated, antimicrobial dyes, ADP1 with pBAV1C-T-lux plasmid was used as one of the model organisms (George et al. 2017). Previously, ADP1 has also been used as a whole cell biosensor to detect cytotoxicity of heavy metal pollutants in seawater. In the study, it was validated against a fish model for cytotoxicity, and it was found to correlate well. It also proved to be sensitive, as concentrations as low as 0.02 mg/l of mercury were detected. (Cui et al. 2018).

Other topics studied with ADP1 include determination of salicylic acid from plant leaves. Huang et al. (2006) constructed a fusion of salicylic acid hydrolase operon with *luxABCDE*. The resulting biosensor cell induces bioluminescence in the presence of salicylic acid, in even minute concentrations of 5 nM. They injected the biosensor cells into the leaves of a tobacco plant and were able to detect the bioluminescent light reaction *in situ* with an imaging technique. ADP1 has also been used as a biosensor for simultaneous detection of alkanes and their synthesis intermediates (Lehtinen et al. 2017). In the study, both bioluminescent and fluorescent markers were used, and highest signals were received from dodecane. The application is useful in the metabolic engineering, as the production pathways can be more easily optimized. The variety of *A. baylyi* ADP1 applications proves that it is practical in the biosensor cell studies.

3.3.4 Pseudomonas putida

Pseudomonas putida is an aerobic, G-, soil bacterium, which is a root colonizer of plants and has bioremediation potential. These two properties make it a profitable bacterium for the environment. (Holtwick et al. 2001; Gajjar et al. 2009). *P. putida* has been completely sequenced. Nelson et al. (2002) sequenced the strain KT4220. Holtwick et al. (2001) managed to extract three different plasmids, that replicate in rolling circle manner.

Usually, *P. putida* is rod-shaped (Figure 4D), but when starved of the carbon-source, the shape shifts to small, round or coccoid form, and 3–24 hours from the beginning of carbon starvation they tend to clump and stick to surfaces. When the medium lacks sulfate, nitrogen or phosphate, the *P. putida* cells are highly motile under microscopic observation. (Givskov et al. 1994). MIC of ethanol (EtOH) for *P. putida* is 7 v/v-% (percentage by volume), however EtOH increases the fluidity of the cell membrane of *P. putida* (Heipieper & de Bont 1994). *P. putida* can also degrade phenols, and it increases in size when

faced with organic solvents. It can degrade up to 600 mg/ml when freely residing in the medium, but if immobilized, the tolerance is even higher at 1000 mg/L. However, in higher concentrations, the degradation rate is slower. As discussed in chapter 2.2.1, naphthoquinones are polyphenolic compounds, and therefore it is interesting to test their effects on a biosensor cell that can potentially degrade phenolic compounds. (Holtwick et al. 2001; Chung et al. 2003; Neumann et al. 2005).

In the *Pseudomonas* genus, there is an opportunistically pathogenic species, that can resist single-antibiotic treatments, namely, *Pseudomonas aeruginosa*. It and *P. putida* share 85 % of their DNA, but *P. putida* lacks the main virulence factors. (Nelson et al. 2002). *P. aeruginosa* is mainly harmless for healthy people but it is one of the leading reasons of infections caught in healthcare. Examples of these healthcare related infections caused by *P. aeruginosa* include blood stream, ear, urinary tract and surgery wound infections. It is also hard to treat because of its intrinsic resistance to antibiotics, especially if used without another antibiotic. New treatments are needed to help the treatment of *P. aeruginosa*. (Moskowitz et al. 2004; Pires et al. 2015; George et al. 2017). Thus, similarly to *A. baylyi* and *A. baumannii* (see sub-chapter 3.3.3), *P. putida* could be used as a model organism to study new antibacterial substances for their effects on *P. aeruginosa* in a more secure manner.

P. putida is also a relatively susceptible organism to be genetically modified, and it has been used especially in the monitoring of environmental samples. For example, Gajjar et al. (2009) constructed a biosensor cell from *P. putida* KT2440 strain, which had a *luxAB* fusion with copper (Cu) responsive promoter. They demonstrated that 5 mg/L of nanoparticle-sized Cu was enough to cause a drop in the bioluminescent signal, and that the results behaved in a dose dependent manner. A loss of signals at 0.2 mg/L of silver (Ag) and 7 mg/L of zinc (Zn), both in nanoparticle size, was also measured. Furthermore, it was reported that mixtures of these nanoparticle heavy metals were more toxic to the biosensor cells than the Ag, Zn and Cu alone. Another example of *P. putida*'s usage as an environmental biosensor is the biomonitoring of groundwater samples near gasoline leakage sites (Ko & Kong 2017). It was able to detect pollutants such as toluene and xylenes from the samples. In addition, Phoenix et al. (2003) demonstrated that *P. putida* biosensor can measure naphthalene, benzene, toluene and crude oil in addition to gasoline. Naphthalene can also be detected in gaseous phase samples using luminescent *P. putida* biosensor cells. The usage of gaseous phase measurand instead of the aqueous phase increases the sensitivity of the sensor, by decreasing the detection limit from 0.5 µM of naphthalene to 50 nM. (Werlen et al. 2004).

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals

Ethanol was obtained from Altia, Finland. Labema, Finland supplied tryptone and yeast extract. NaCl, KH_2PO_4 were from Merck, USA. Kanamycin was from Janssen, USA; erythromycin from TCI, Japan and ampicillin from Sigma, USA. K_2HPO_4 was from VWR International, USA. Glycerol was obtained from Sigma, USA.

For the *in vitro* propagation of sundew, Luke Parkano Research Station purchased Murashige and Skoog Basal Medium, $FeSO_4 \cdot 7\ H_2O$, sucrose, *myo*-inositol, benzylaminopurine and 1-naphthaleneacetic acid from SigmaAldrich, USA. Also, the pure control substances (juglone, lawsone, plumbagin and quercetin) were purchased from SigmaAldrich, USA by Luke.

4.1.2 Bacterial biosensor strains

All of the bacterial strains used in this thesis are constitutive biosensor cells. The strains used include *Staphylococcus aureus* RH4220, *Escherichia coli* K12 pcGLS11, *Acinetobacter baylyi* ADP1 (DSM 24193), and *Pseudomonas putida* (DSM 291). Both G- (*E. coli*, *P. putida* and *A. baylyi*) and G+ bacteria (*S. aureus*) were selected for this study. All of them were imaged using IVIS imaging system (Xenogen Caliper, USA) to detect their bioluminescence with a cooled camera and precision filters. The detected bioluminescence is measured in calibrated units of photons per second. The values are automatically color coded and placed over photographic images in the figures by the Living Image Software that is used to operate the IVIS system. (Yang 2010). The bioluminescence illustrations (Figure 5) show that all of the strains are producing bioluminescence.

The *S. aureus* and *E. coli* K12 were kind gifts from professor emeritus Matti Karp, and *A. baylyi* carrying pBAV1k-T5-LUX plasmid and *P. putida* were generously supplied by assistant professor Ville Santala. *E. coli* K12 + pcGLS11 was developed by Kurittu et al. (1998) and the plasmid described by Frackman et al. (1990). The usage of *S. aureus* biosensor strain was first described by Vesterlund et al. (2004). ADP1 sensor strain was developed by in previous research (unpublished). The strains were stored at - 80 °C in 40 % glycerol.

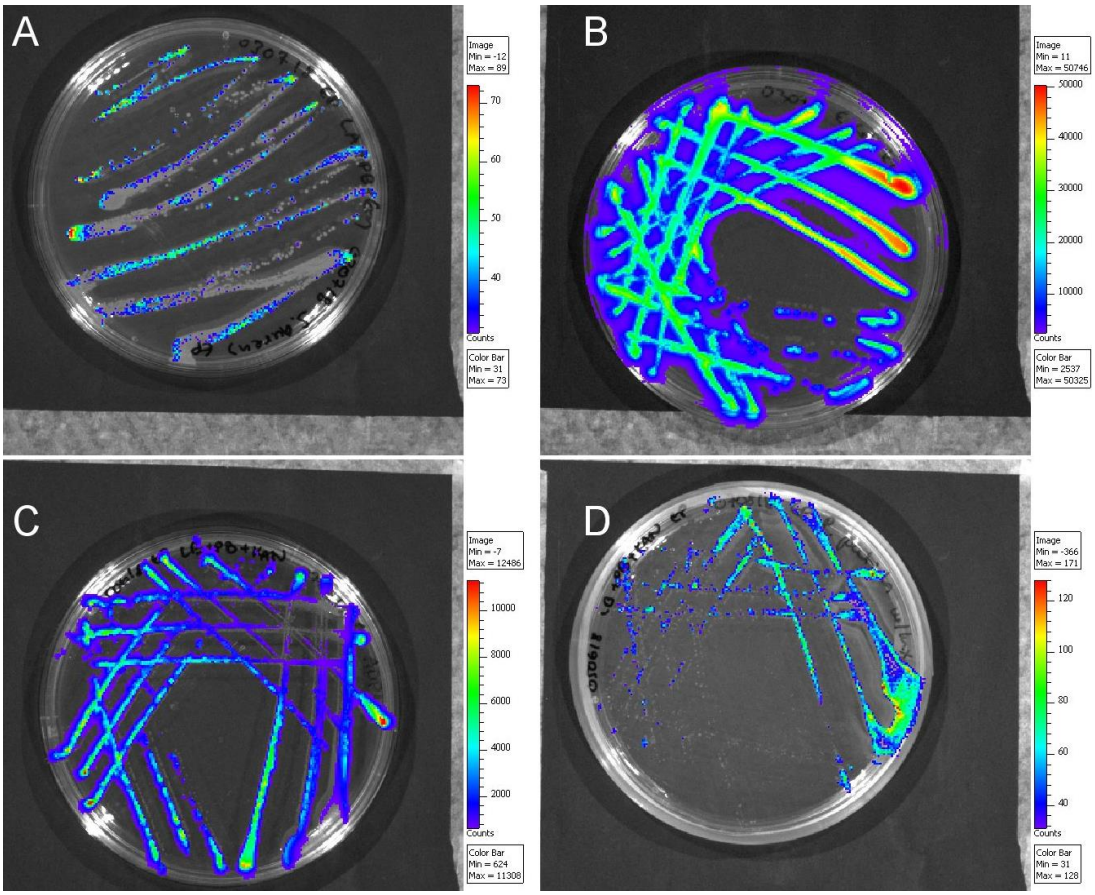

Figure 5. *IVIS pictures of all of the biosensor cells used in this work. IVIS images reveal the bioluminescence of the bacteria. For the imaging parameters, exposure time was set to 1 s, binning to medium and F/Stop to 1. The highest luminescence is detected in the areas with red coloring, and the lowest at dark blue coloring.* ***A.*** *S. aureus RH4220.* ***B.*** *E. coli K12 + pcGLS11.* ***C.*** *A. baylyi ADP1 + pBAV1k-LUX.* ***D.*** *P. putida + pBAV1k-LUX.*

4.1.3 The pBAV1k-T5-LUX plasmid

The plasmid pBAV1k-T5-LUX was a kind gift from Ichiro Matsumura (Addgene plasmid # 55800). The design of pBAV1k-T5-LUX was reported by Bryksin and Matsumura (2010). It was designed to be an efficient replicant in both G+ and G- bacteria. The previous plasmid designs, such as pWV01, suffered from instability in non-selective conditions, as their rolling circle replication was less efficient than their copy number control mechanisms, while they were in non-native host cells. Figure 6A represents one of the *E. coli* strains (XL1-Blue) including a pBAV1K-T5-LUX plasmid cultivated on kanamycin containing plate. For this study, the plasmid was isolated from this *E. coli* strain. The IVIS image (see 4.1.2) portrays the bioluminescent light produced by the strain. The replication of the plasmid has been efficient, as all of the colonies on the plate exhibit bioluminescence.

The different areas of the plasmid can be seen in an illustrated plasmid map in Figure 6B. The new minimal plasmid includes only copy number elements that are necessary for the conversion from single-strand DNA to double-strand. The plasmid includes the *lux* genes of *Photorabdus luminescens* (*luxABCDE*) as well as the selection marker from *Enterococcus* (KanR) and T5 promoter. The usage of the whole lux operon eliminates the need for an external substrate for the bioluminescence reaction (Huang et al. 2006).

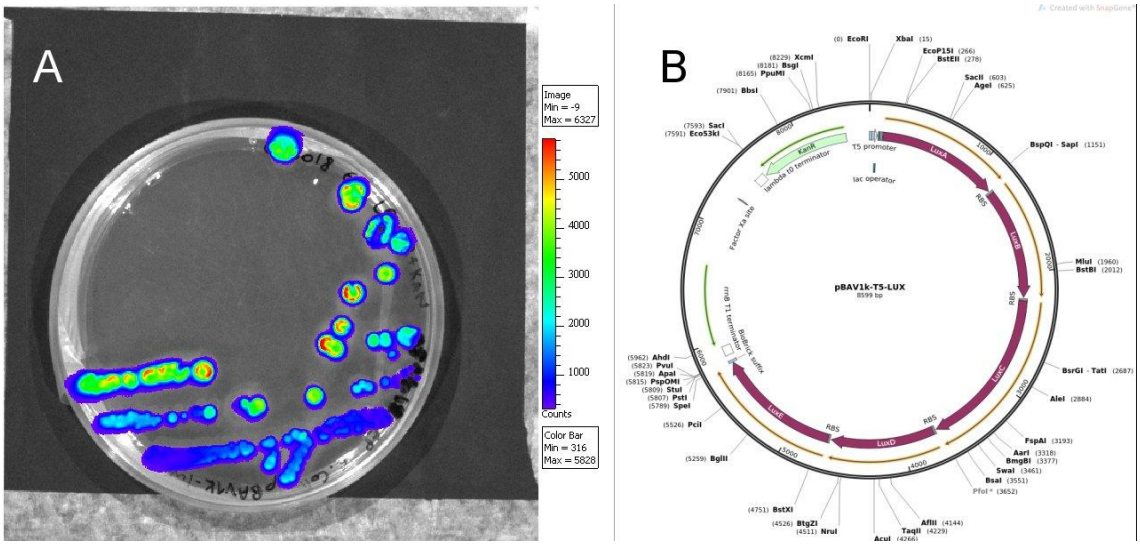

Figure 6. ***A.*** *An IVIS image of E. coli XL1-Blue, containing the plasmid pBAV1k-T5-LUX. For the imaging parameters, exposure time was set to 1 s, binning to medium and F/Stop to 1. The highest luminescence is detected in the areas with red coloring, and the lowest at dark blue coloring.* ***B.*** *Illustrated plasmid map of plasmid pBAV1k-T5-LUX. (addgene.org 2018).*

Bryksin and Matsumura (2010) also demonstrated that the plasmid was a high copy number using the green fluorescent protein version of the it (pBAV1K-T5-*gfp*). In two different strains of *E. coli*, MDS42 recA-Blue and INV Alpha F', the copy number was 357 and 251 respectively, compared to 60 copies of another plasmid (pGK12) in *E. coli* They also showed that it was more straightforward to clone genes into the plasmid than previously. The plasmid also proved to be more resistant to non-selective surroundings, with 18 % of *E. coli* colonies still being kanamycin resistant after 80 generations, whereas 0 % of the pgK12 colonies were chloramphenicol resistant after the same amount of generations.

4.2 Methods

4.2.1 Plasmid isolation

The *E. coli* XL1-Blue (Stratagene, USA) containing the plasmid pBAV1k-T5-LUX was cultivated on lysogeny agar (LA) plates containing tryptone 10 g/L; yeast extract 5 g/L; NaCl 10 g/L and agar 15 g/L. The plates were supplemented with 10 % of sterile filtered

1 M phosphate buffer (PB) with pH 7.0 and 50 µg/ml kanamycin. The plates were incubated at 30 °C for 2 days. On the day before the isolation, a working stock was created by inoculating one colony of *E. coli* XL1-Blue to into 5 ml of autoclaved, sterile lysogeny broth (LB), which contained tryptone 10 g/L; yeast extract 5 g/L and NaCl 10 g/L. The LB was supplemented with 10 % of sterile filtered PB and 50 mg/ml kanamycin. The stock was incubated at 30 °C and 300 rpm for 17 hours. In total, three of similar tubes were inoculated.

On the day of the isolation, the bioluminescence of the stock was measured using Chameleon Multilabel (Hidex Oy, Finland) microplate reader to determine which of the strains produces most light, and thus has the biggest plasmid concentration. An aliquot of 1.5 ml of the stock was centrifuged in a sterile Eppendorf tube at 10 000 x g for one minute and the supernatant was discarded. Another 1.5 ml of the stock was added to the same tube, and the centrifugation was repeated, and the supernatant was discarded again. Thus, the cells used for the isolation were obtained from a total volume of 3 ml of the stock.

The isolation was performed immediately after the harvesting of the cells. The isolation was completed with GeneJET Plasmid Miniprep Kit (ThermoScientific, USA), using the kit's own instructions. Briefly, the cells were resuspended into resuspension solution of the kit, the cells were lysed and neutralized with the kit's own reagents. The DNA was bound to the spin column by centrifugation, and it was washed twice with the washing solution of the kit. The centrifugations for the isolation were performed at 12 000 x g, and the washing steps lasted for 1 minute. Then, the DNA was eluted with double distilled water (DDW). After the isolation, the DNA content was measured with NanoDrop (ThermoFisher Scientific, USA).

4.2.2 Transformation of *Pseudomonas putida*

Prior to the transformation, a fresh overnight culture was prepared by inoculating one colony of the *P. putida* into 5 ml of sterile lysogeny broth (LB). The overnight culture was cultivated in a shaker at 30 °C and 300 rpm for approximately 16 h.

The transformation protocol was developed by Meinhardt (Meinhardt 2002). In short, the growth medium is inoculated with fresh overnight culture of *P. putida* and grown to the optical density of 0.8 at 600 nm (OD_{600}). The cells are harvested by centrifugation, washed twice with ice-cold glycerol, and finally resuspended in the glycerol. These electrocompetent cells are then electroporated with the target plasmid. The protocol was completed with the following changes. LB was used instead of the standard 1-medium. The volume of the cultivations was scaled down from 50 ml to 5 ml, with similar changes of the other volumes. The OD_{600} was 0.698 after the growth period before the harvesting of the cells. The harvest centrifugation was performed for 2 minutes at 5000 rpm producing 11 200 x g for the first and second centrifugations and at 12 000 x g for 1 minute during

the last centrifugation. The *P. putida* cells were electroporated with BIO-RAD Micropulser™ (Bio-Rad Laboratories Inc., USA).

The final electroporation parameters were 2.39 kV and 5.70 ms for the control (no plasmid) and 2.39 kV and 5.10 ms for the *P. putida* with plasmid. The selective plates were similar to the LA plates described in chapter 4.2.1. The LB added right after the electroporation was warmed to 30 °C prior to the addition to ensure the best survival of the electroporated cells. After plating the cells to the LA-kanamycin plate, they were cultivated in 30 °C for 18 h 30 min and in room temperature for 4 days before the colonies were observed. The plates were imaged with IVIS imaging system (see chapter 4.1.2) to confirm the success of the transformation visually.

After the transformation was confirmed, seven uniformly shaped and bioluminescent colonies were chosen for further testing. The purpose was to find a colony that produces highest bioluminescence signals and simultaneously small errors for triplicate samples. Colonies 1–5 were inoculated into sterile LB supplemented with 10 % of PB and 50 µg/ml kanamycin. Colony P was inoculated in LB supplemented with 50 µg/ml kanamycin, but no PB. The purpose was to test whether PB would enhance the growth and luminescent signal of the transformed *P. putida*. Colony K was inoculated in LB supplemented with PB, but no kanamycin to demonstrate the effect of the lack of selection pressure, which should decrease the luminescent signal given.

The strains were imaged using IVIS imaging system and their bioluminescent signals were monitored in Chameleon Multilabel plate reader for 90 minutes. The best strains were plated again on the LA and kanamycin plates and imaged again using IVIS to make the final selection of the used strain. After the first round of tests was done, all of the strains, besides K, were made into glycerol preparations by adding 4.5 ml of 80 % glycerol into 4.5 ml of the cell cultivation media and mixing them gently but thoroughly. The glycerol preparations were divided into 750 µl Eppendorf tubes and stored in -80 °C for long term storage.

4.2.3 Sundew collection, *in vitro* propagation and extraction

The wild samples of round-leaved sundew (*Drosera rotundifolia*) were collected in June of 2018 from two different peatlands located in Parkano, Western Finland. In Appendix A, a location map of Parkano can be seen. Three samples were collected from Lehtolamminneva and three from Kivineva. Three samples of laboratory grown *D. rotundifolia* were also used.

The laboratory tissue cultivation of *D. rotundifolia* has been described in Pelkonen (2017). The development of the method was part of Leila Korpela's, Natural Resources Institute of Finland (Luke), project focusing on cultivation of *D. rotundifolia* ("Kihokkia lääkekasviksi Pohjois-Satakunnan heikkotuottoisilla turvemailla"). The cultivation and

extraction were performed by Anneli Käenmäki and Eeva Pihlajaviita in Luke Parkano Research Station as a part of Tytti Sarjala's project "Bioactive compounds in forestry-based bioeconomy". Additionally, the collection of the round-leved sundews from the peatlands was performed with the help of staff in Parkano Research Station. I wish to thank all of the people involved in this part of the work.

To describe the methods briefly, sterile *D. rotundifolia* seeds were inoculated on ½ Murashige-Skoog (½ MS) plates. The plates contained 2.2 g/l of Murashige and Skoog Basal Medium. Its contents are specified in their product information data sheet (Murashige and Skoog Basal Medium). The modifications to the ½ MS media were previously described by Pelkonen (2017). The changes included changing the $FeSO_4 \cdot 7\ H_2O$ concentration to 0.039 mg/l and *myo*-inositol to 100 mg/l; supplementing the media with benzylaminopurine (0.1 mg/l), 1-naphthaleneacetic acid (0.05 mg/l), sucrose (30 g/l); and adding 6.5 g/l of agar. The pH was adjusted to 5.8.

The inoculated plates were sealed with parafilm and incubated in room temperature (approximately 25 °C). The light cycle was 16 hours of light and 8 hours of darkness. After 7 weeks of incubation, the plant cell mass was divided in sterile conditions and the divided masses were placed on new ½ MS plates. A new division was done every 3-4 weeks. After third division, the cells were plated on ½ MS plates that did not contain benzylaminopurine or 1-naphthaleneacetic acid. This modification was done to enhance the root formation as presence of cytokines might hinder it. (Kim & Jang 2004; Pelkonen 2017).

Two batches of the sundew extracts were prepared in Natural Resources Institute Finland, Parkano research unit. The sundew samples were weighed fresh. From the laboratory grown samples, the green parts (the leaves) were used. The sundew sample was weighed, and 4 grams of it was ground in mortar with a pestle and moved to a pre-weighed sterile Falcon tube. Next, 28 ml of EtOH (ethanol) was added to the tube. The sample extracted into EtOH for approximately one hour and the tubes were vortexed periodically throughout the extraction. It was then centrifuged for 15 minutes at 10 g and the supernatant was collected. Then the EtOH was evaporated. For the first extract batch, the evaporation happened in a vacuum centrifuge with a cooling unit (Rotational-Vacuum-Concentrator RVC 2-18, Cold Trap CT 02-50SR, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). For the second batch, the evaporation was performed in the TUT premises by me. It was performed in a fume hood at room temperature by opening the caps of the vials and letting them evaporate for 6 days. After the 6 days of evaporation, the rest of the ethanol was evaporated using nitrogen evaporation. The nitrogen evaporation took approximately 4 hours to complete.

The dried samples were stored in -20 °C before the dilutions. The dried samples were dissolved into sterile DDW to achieve stock concentrations of 10 mg/ml. The dissolving was enhanced with strong vortexing and mixing by pipetting the DDW back and forth in sterile conditions. Further dilutions of 0.625, 1.25, 2.50 and 5.00 mg/ml were prepared in

DDW. The testing further dilutes the samples by factor 1/3; final concentrations in the test wells were, thus, 0.208, 0.417, 0.833, 1.67 and 3.33 mg/ml. All of the dilutions were stored in -20 °C between the test runs and new dilutions were prepared weekly.

4.2.4 Naphthoquinone and flavonoid standards and ethanol controls

All of naphthoquinone standards were kindly offered by Jenni Tienaho and Tytti Sarjala from the Natural Resources Institute Finland and prepared by dissolving the naphthoquinone in 99.9 % ethanol. As 7-methyljuglone, which is the most common naphthoquinone in *D. rotundifolia*, is not commercially available, it could not be included as one of the standard compounds to be studied. Plumbagin and quercetin were studied along with two other naphthoquinones lawsone (structure seen in Figure 2D) and juglone (Figure 2B). The concentrations of the stock volumes were 10 mg/ml for juglone, 5 mg/ml for lawsone and plumbagin and 2 mg/ml for quercetin. The stock solutions were stored in brown glass vials to prevent the ambient light from interacting with the naphthoquinones. The storage temperature was + 4 °C for the stock solutions.

The tested concentrations varied for each of the standards. Dilution series were planned for each of the naphthoquinones around the literature values of the minimal inhibition concentrations (MICs). The MIC was 37.5 µg/ml for juglone against *S. aureus* (Wang et al. 2016). The MICs for lawsone against *E. coli*, *P. aeruginosa* and *A. baumannii* were ≥ 512 µg/ml (Rahmoun et al. 2012). For plumbagin the MIC against *S. aureus* was 1.56 µg/ml (Paiva et al. 2003). For quercetin, the MIC was 20 µg/ml against *P. aeruginosa* and *S. aureus* and 300 µg/ml against *E. coli* (Jaisinghani 2017). This was done in order to ensure that a dose response could be detected while testing.

For juglone, the tested concentrations per well were 3.13, 6.25, 12.5 and 25.0 µg/ml (EtOH contents from the original dissolving in EtOH 0.03, 0.06, 0.13 and 0.25 volume percentage, v/v-%). For lawsone, the concentrations per well were 42.7, 85.3, 171, 341 µg/ml (EtOH contents 0.85, 1.7, 3.4 and 6.8 v/v-%). For plumbagin, 0.130, 0.260, 0.520 and 1.04 µg/ml (EtOH contents 0.00, 0.01, 0.01 and 0.02 v/v-%); and for quercetin 5.00, 10.0, 20.0 and 100 µg/ml (EtOH contents 0.25, 0.49, 1.0 and 5.0 v/v-%). The further dilutions were done in sterile DDW and stored in dark and in + 4 °C between the test runs. Fresh dilutions were prepared after 48 hours of storing to prevent errors caused by degradation.

The ethanol controls were prepared by diluting 99.9 % EtOH in DDW. For initial testing of the strains, concentrations of 0.33 v/v-%, 1.67 v/v-% and 16.7 v/v-% per well were used. These concentrations were chosen based on previous unpublished studies, as 0.33 % represents no damage, 1.67 % minimal damage and 16.7 % deadly damage to the cells. The used concentrations for other tests were 6.8 % per well and 16.7 % per well for naphthoquinone tests, as maximum EtOH-% in the naphthoquinone dilutions was 6.8 %. In

sundew extract tests, 0.33 % per well was used, as the used juglone controls contained maximum of 0.06 % of EtOH per well. The ethanol controls were stored in + 4 °C between the test runs.

4.2.5 Testing

For the testing, the bacterial strains were cultivated on LA plates as described in chapter 4.2.1. Each of the bacteria, however, had their own antibiotic marker and the plates were thus supplemented with the suitable antibiotic. For ADP1 and *P. putida* with the pBAV1k-T5-LUX plasmid, the supplement was kanamycin (50 µg/ml) as with the plasmid isolation and transformation. For *E. coli* K12 pcGLS11, the antibiotic was ampicillin (100 µg/ml) and for *S. aureus* RH4220 erythromycin (5 µg/ml). The other strains were incubated in 30 °C, except *S. aureus* in 37 °C. Incubation time for *P. putida* was at least 40 h, whereas for all of the others it was approximately 16 h.

After the incubation periods, the produced luminescence was imaged on the IVIS platform. Only colonies, which were emitting luminescence and were small and regular in shape were selected for the further steps. This ensures the homogeneity of the selected colony as well as the luminescent light emission. The selected colonies were marked on the bottom of the plate using a marker, so that same plates could be used for several days. However, new plates were prepared once a week, so that the bacteria stayed fresh and metabolically functioning. All of the other strains were stored at + 4 °C, but ADP1 in room temperature.

Fresh working stocks were prepared on the day prior to the testing. *E. coli*, ADP1 and *S. aureus* were cultivated in media containing LB, PB and antibiotic. The PB proportion was 10 %. The antibiotic concentrations were 100 µg/ml ampicillin (*E. coli*), 50 µg/ml kanamycin (*ADP1*) and 5 µg/ml erythromycin (*S. aureus*). For *P. putida*, the medium contained only LB, and the supplementing antibiotic was kanamycin (50 µg/ml). One colony was inoculated into each of the sterile tubes containing the described media, with at least two tubes for each of the biosensor cell strains. The stocks were incubated in 30 °C and 300 rpm shaking for approximately 18 hours.

The working stocks were screened in duplicate with Chameleon Multilabel (Hidex Oy, Finland) or Fluoroskan Ascent FL (ThermoScientific, USA) microplate readers before running the tests. Chameleon Multilabel was used for EtOH, naphthoquinone and first part of sundew extract tests and Fluoroskan Ascent FL was used for the last part of the sundew extract tests. When using the Fluoroskan Ascent FL, proper light shields were used according to the manual of the equipment. The stock that produced the highest signal was selected for the test. However, if two stocks had similar signals, the one with smaller variation was selected for the test.

An aliquot of 50 µl of the samples were pipetted in triplicate into the wells of a 96-well, opaque white plate (Corning, USA). To ensure that the surviving cells will have enough nutrition, 50 µl of LB was added during the incubation time. Bacterial stock (50 µl) was added to the wells. DDW was used as a negative control sample, whereas EtOH was used as a positive control sample in concentration of 16.7 % for naphthoquinone tests. Another EtOH control was added, with concentration of 6.8 %, as this was the highest EtOH concentration of the selected naphthoquinone standards. For the extract tests, 0.33 % EtOH was used to confirm that for the juglone control, the reaction was caused solely by juglone, and not by EtOH, which was present in the juglone controls. The 96-well plate was incubated for 90 minutes and the bioluminescence measured with 5-minute intervals with the Chameleon or the Fluoroskan Ascenet FL microplate readers.

4.2.6 Data analysis and image handling

All of the data was analyzed using Microsoft Excel 2016 (Microsoft, USA). The figures with data plots were drawn using Origin8 (OriginLab, USA). Images were cropped using Corel Paintshop Pro X8 (Corel Corporation, Canada) and paneled with ImageJ (Schneider et al. 2012). Chemical structures were drawn using Chemical Structure Editor (Metamolecular LLC, USA), accessed online (Metamolecular).

Inhibition percentages were calculated using the following equations:

$$Inhibition - \% = 100 \times \frac{Average(Blank_t) - Average(Sample_t)}{Average(Blank_t)} \quad (2)$$

$$CV - \% = 100 \times \frac{Standard\ deviation(Sample_t)}{Average(Blank_t)} \quad (3).$$

In equations (2) and (3), *t* is the incubation time passed before the measurement. Inhibition-% is the inhibition percentage and CV-% denotes the coefficient of variation. Blank are the wells including only DDW and biosensor cells. The signals of sample wells were compared to the same time point, as also the cells in sample wells grow during the incubation, meaning that the signal from the blank wells will also change over time.

Table 1. *Samples and number of parallels in testing and data analysis.*

Origin of sample	Parallel samples from origin	Parallel samples in one test run	Wells per test run per one parallel sample
Vacuum centrifuge dried			
Lehtolamminneva	3	1	3
Kivineva	3	1	3
Laboratory	3	1	3
Air/nitrogen evaporated			
Lehtolamminneva	3	3	3
Kivineva	3	3	3
Laboratory	3	3	3
Controls			
EtOH	1	1	3
Naphthoquinone/Flavonoid	1	1	3

Table 1 summarizes the sample origins and number of parallel tests performed for each of the samples. For the EtOH and naphthoquinone control compounds, the calculations of inhibition percentages and CV-% were done from the triplicate wells in one test run, as presented in Table 1. Three parallel samples were picked from two different bogs and three parallel samples were from laboratory grown sundews. For the first, vacuum centrifuge dried, batch of the sundew extracts, the calculations were based on three parallel samples in one test run. This totals to 9 parallels for each of the origins (see Table 1). For the second, nitrogen evaporated, batch of sundew extracts, the test was performed on three occasions, that is to say three different test runs were completed. The calculations were thus based on either 27 parallels for the second batch (see Table 1). Obvious outliers, in other words signals that were unusually large or small compared to the other signals from the same sample, were omitted in the calculations.

5. RESULTS

5.1 Plasmid isolation and transformation

The plasmid pBAV1K-T5-LUX was successfully isolated from E. coli XL-1 Blue. The DNA content measured after Miniprep isolation with Nanodrop was 99.1 ng/µl. The plasmid isolate (10 µl) was added to *P. putida* P8 strain prior to the electroporation. Thus, the plasmid content used was 991 ng, and the concentration during the electroporation was 19.82 ng/µl. The plasmid was transformed with the electroporation into the *P. putida* P8 strain.

As can be seen from Figure 7A and 7C, neither the control plate (A), containing *P. putida* and no plasmid, nor the plate containing *P. putida* (C) and the plasmid presented any colonies after 18.5 hours of incubation in +30 °C. The plates were further incubated in room temperature (approximately 21–27 °C) for 4 days, after which the colonies were observed on the plate containing the plasmid and photographed, as can be seen from Figure 7D. The lack of colonies in the control plate is seen in Figure 7B. The light production and growth in the transformed *P. putida* started slowly.

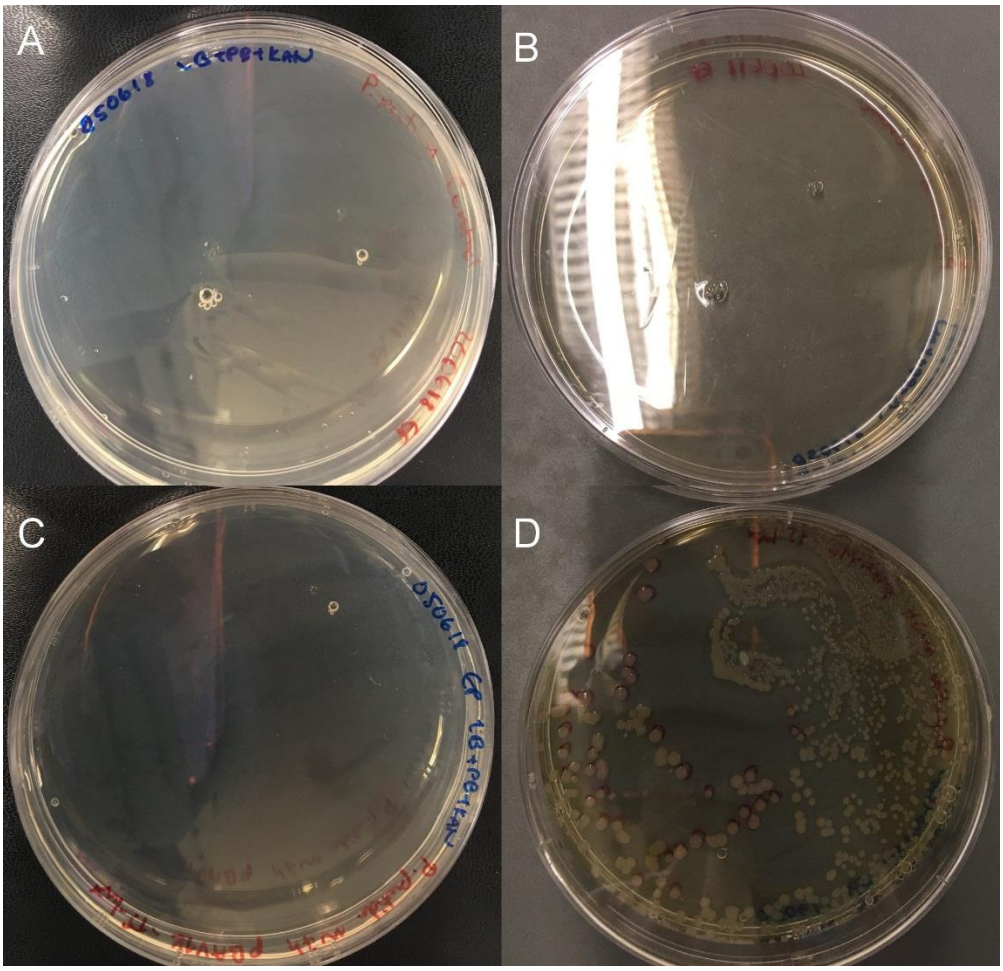

Figure 7. *Photographs of the agar plates plated with transformed P. putida.* ***A.*** *Control of electroporated P. putida without plasmid in electroporation step. After 18.5 hours of incubation in +30 °C no colonies can be seen.* ***B.*** *Control of electroporated P. putida without plasmid in electroporation step. After 18.5 hours of incubation in +30 °C and 4 days incubation in ambient room temperature. No colonies can be seen.* ***C.*** *Transformed P. putida with plasmid after 18.5 hours of incubation in +30 °C. No colonies formed.* ***D.*** *Transformed P. putida with plasmid after 18.5 h incubation in +30 °C and 4 days incubation in ambient room temperature. Colonies have only grown on this plate. Photos taken by Emmi Poikulainen.*

The bioluminescence of the transformed *P. putida* plate was also imaged twice using IVIS imaging platform (see chapter 4.1.2). No bioluminescence was detected after 18.5 h of incubation in +30 °C (Figure 8A). After the colonies were detected, another image was taken to confirm the successful transformation. A bioluminescence reaction was detected, and the image is represented in Figure 8B. As bioluminescence was detected on the selective kanamycin plate, the transformation was deemed successful.

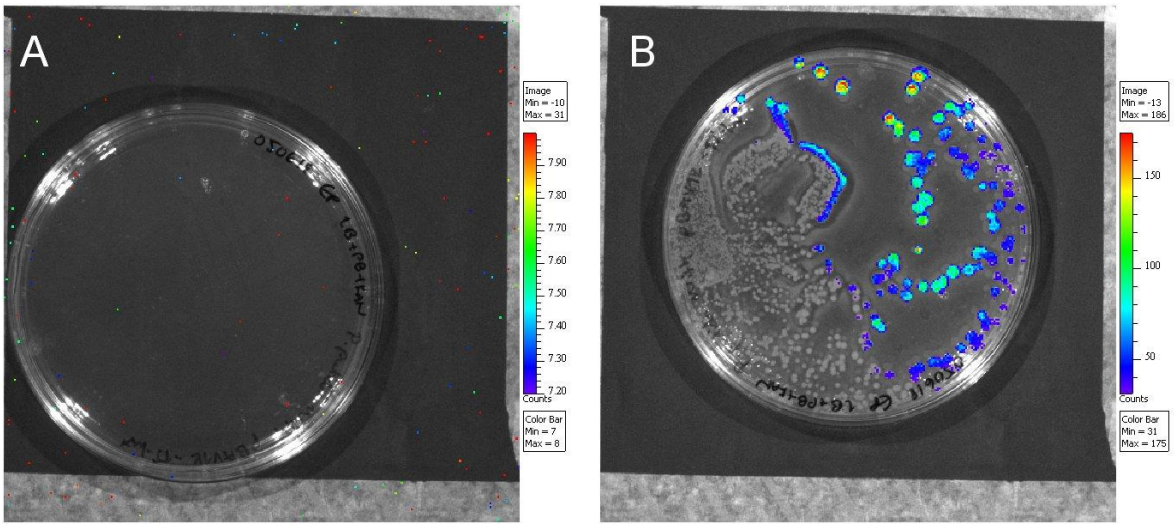

Figure 8. *IVIS luminescence pictures of the transformed P. putida. For the imaging parameters, exposure time was set to 1 s, binning to medium and F/Stop to 1. The highest luminescence is detected in the areas with red coloring, and the lowest at dark blue coloring.* ***A.*** *After 18.5 hours of incubation at + 30 °C. No colonies or bioluminescence detected at this time point. Corresponds to the photograph in Figure 7C.* ***B.*** *After 18.5 hours of incubation at + 30 °C and 4 days of incubation at ambient room temperature. There are colonies, which are bioluminescent. Corresponds to the photograph in Figure 7D.*

Not all the colonies on the kanamycin agar plate were bioluminescent, as can be seen on the left side of the plate in Figure 8B, and only bioluminescent colonies were selected for further testing, as well as making the glycerol preparations.

5.2 *P. putida* with pBAV1k-T5-LUX further testing

After the selected colonies from the bioluminescent *P. putida* were cultivated firstly in liquid medium described in 4.2.2. The tubes containing the medium and the bacteria were imaged using IVIS imaging system after 22.5 h of incubation. From the Figure 9A, it can be seen that no significant luminescence is detected in the control tube (on the left) or the tube containing no kanamycin (on the right). However, a significant luminescence signal can be seen in the middle, which contain the strain P. The highest luminescence detected in the tube P was 449 CPU (counts per unit).

In Figure 9B, the other strains and their bioluminescence are represented in an IVIS image, with number order of the strains from left to right being 1, 2, 3, 4 and 5. All of the strains exhibit bioluminescence, with the highest value in the image being 386 CPU. Strains 3 and 4 have the highest bioluminescent signals in 8B, as both have red areas. The strains 1, 2 and 5 have lower luminescence compared to 3 and 4. The colors of the IVIS image are only comparable inside the image, but the highest CPU values can be compared from image to image. Thus, the strain P showed highest bioluminescence.

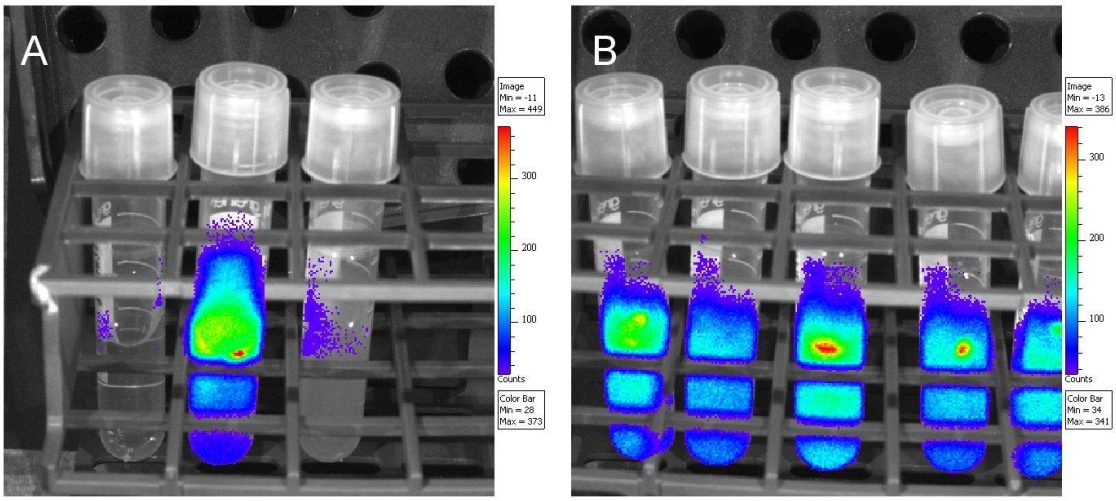

Figure 9. *P. putida colonies picked for further testing in an IVIS imaging picture. For the imaging parameters, exposure time was set to 1 s, binning to medium and F/Stop to 1. The highest luminescence is detected in the areas with red coloring, and the lowest at dark blue coloring.* ***A.*** *From left to right: Control; colony P incubated without PB; colony K incubated without kanamycin. Only small background luminescence can be seen in control tube, high luminescence in the P tube and low luminescence in K tube.* ***B.*** *From left to right: colonies 1, 2, 3, 4 and 5. All cultivated as described in the methods. Colonies 3 and 4 have slightly higher luminescence than 1, 2 and 5. Highest luminescence of all tubes is seen in tube P.*

All of the strains were tested for their ability to produce bioluminescent light signal consistently. In the test, the strains' signal from Chameleon Multilabel microplate reader measurements were compared to a background signal measured from three wells containing only LB and DDW. The results can be seen in the Figure 10. Consistently with the results obtained from IVIS imaging (Figure 9) the highest signal was produced in the strain P. Furthermore, strain K shows signal almost comparable to the background as in the IVIS image. Also, for the strains 2, 5 and 3, the two methods seem to agree, with 3 being a bit higher in bioluminescence and 2 and 5 a bit lower. However, for strains 1 and 4, the relative order of the signals is different in the IVIS and Chameleon Multilabel measurements. The order of microplate reader measurement was deemed to be correct, as the results were numeric, where as in IVIS, the interpretation is mostly done visually.

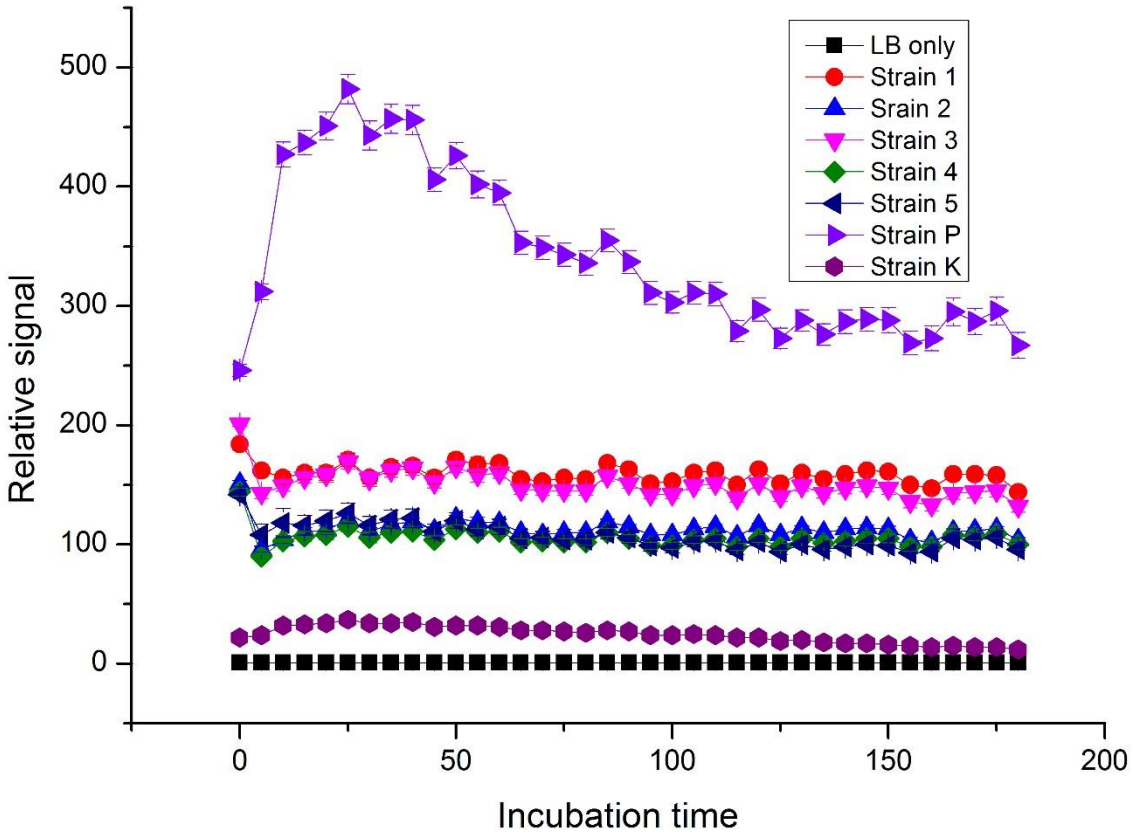

Figure 10. *Bioluminescence signal measured from the tested strains with a microplate reader. Strains 1–5 originated were cultivated in medium containing LB, PB and kanamycin. Strain P was cultivated without phosphate buffer, and strain K without kanamycin. Relative signals were calculated by dividing the signal with the signal of LB only control. Error bars represent CV-percentage. Highest luminescence is observed in strain P. Strains 1 and 3 show medium luminescence; strains 2, 4 and 5 produce a slightly lower luminescence. Almost no luminescence is observed from the Strain K.*

Based on the luminescence imaging and microplate reader measurements, strains P and 1 were selected to further cultivations. They were plated on the LB agar plates containing kanamycin and PB. After 40 hours of incubation in + 30 °C, they were imaged using IVIS, and strain P was found to have higher luminescence (data not shown). Thus, strain P was selected to be used in all of the tests, including EtOH, naphthoquinone and extract tests. In Figure 11, a photograph of both of the strains are shown, and the colonies have grown in both of the plates. However, the colonies have also formed a halo effect around them during the incubation.

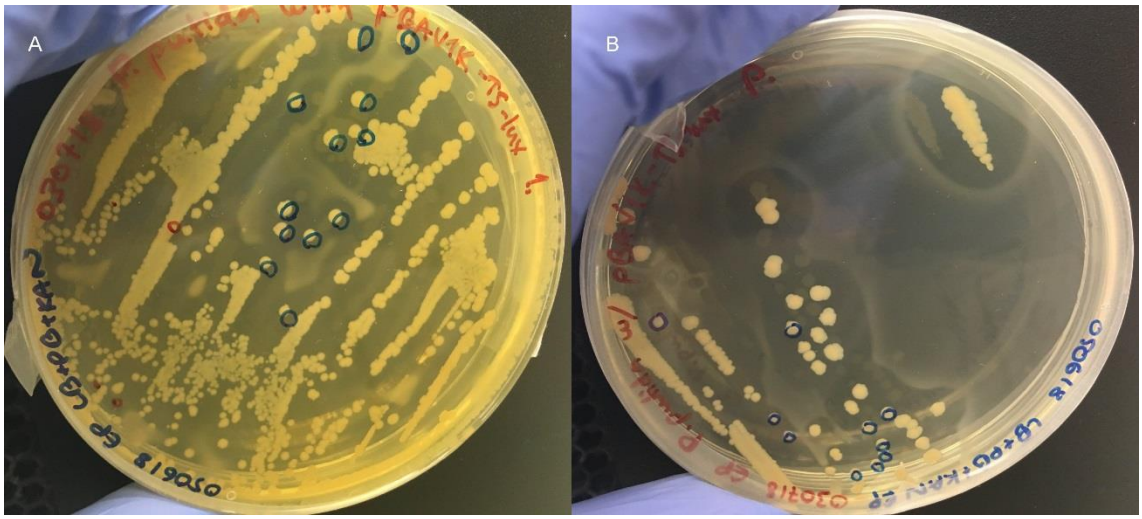

Figure 11. *Two of the transformed P. putida strains tested and the halo zone effect formed around the colonies. Agars were pictured after 40 hours of incubation at + 30 °C.* ***A.*** *Strain 1.* ***B.*** *Strain P. It was chosen for the testing of the samples, because of highest luminescence signals. Photos taken by Emmi Poikulainen.*

The similar halo effect (Figure 11) was also seen from time to time in other plate cultivations of the transformed *P. putida*.

5.3 Naphthoquinone and flavonoid standards and EtOH controls

EtOH controls were tested on each of the biosensor cell strains. As can be seen from Figure 12, the strains showed different sensitivities to the tested EtOH concentrations. While *E. coli* K12 (Figure 12B) and *A. baylyi* ADP1 (Figure 12C) were not greatly affected by the smallest concentration of 0.33 volume per cents (v/v-%), *S. aureus* RH4220 (Figure 12A) and the transformed *P. putida* (Figure 12D) showed inhibition percentages of approximately 20 %.

Similarly, no big effect was seen in *E. coli* or ADP1 when the EtOH v/v-% was raised to 1.67 % per well. However, for *S. aureus* the inhibition-% was now approximately 40 % at the highest time point and for *P. putida* almost 70 %. All of the strains showed clear inhibition when the EtOH v/v-% was 16.7 %. *S. aureus* showed a decreasing inhibition percentage as the incubation time progressed when incubated with the highest ethanol concentration.

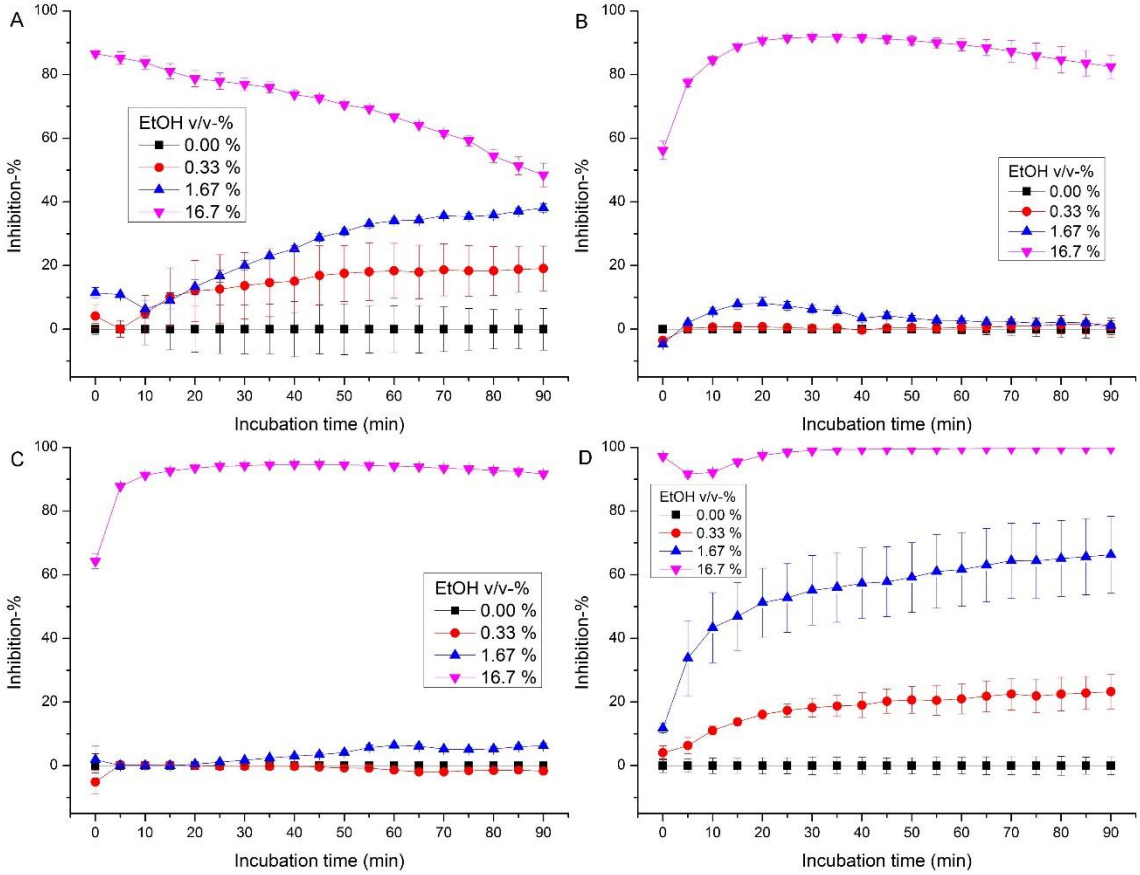

Figure 12. *Bioluminescent signal inhibition-% measured from each of the cell strains when incubated with various EtOH concentrations. Error bars represent CV-%.* ***A.*** *S. aureus RH4220.* ***B.*** *E. coli K12 + pCGLs11.* ***C.*** *A. baylyi ADP1 + pBAV1k-LUX.* ***D.*** *P. putida + pBAV1k-LUX.*

The concentrations selected for the naphthoquinone and flavonoid standards tested were based on previous studies on minimal inhibition concentrations (MIC) of each of the naphthoquinones. The MIC was 37.5 µg/ml for juglone against *S. aureus* (Wang et al. 2016). The MICs for lawsone against *E. coli*, *P. aeruginosa* and *A. baumannii* were ≥ 512 µg/ml (Rahmoun et al. 2012). For plumbagin the MIC against *S. aureus* was 1.56 µg/ml (Paiva et al. 2003) . For quercetin, the MIC was 20 µg/ml against *P. aeruginosa* and *S. aureus* and 300 µg/ml against *E. coli* (Jaisinghani 2017). The tested concentrations ranged from colorless to vibrantly yellow. These colors can be seen in Appendix B.

Table 2 includes the inhibition-% values measured in this work for all different naphthoquinones. The values are measured after 50 minutes of incubation. For juglone, at 50 minutes, the MIC for *E. coli* and *A. baylyi* was 12.5 µg/ml. *S. aureus* and *P. putida* were not fully inhibited at 50 minutes of incubation even by the highest concentration of 25.0 µg/ml. In contrast, *S. aureus* seemed to be extremely sensitive to lawsone, as even the smallest concentration (42.7 µg/ml, 0.85 % EtOH) inhibited over 90 %, whereas the EtOH control (6.8 %) inhibited 65.0 ± 8.0 %. *E. coli* and *P. putida* were over 90 % inhibited by 85.3 and 171 µg/ml respectively. However, *A. baylyi* was not inhibited over 90 % even by the highest concentration of 341 µg/ml.

Table 2. *Inhibition percentages (Inhib-%) of different naphthoquinones, measured with different biosensor cells. CV-% is the percentage of the signal CV-% of the three sample wells divided by the signal of the blank sample. Measured after 50 minutes of incubation.*

Substance, c (µg/ml)	*S. aureus* Inhib.-%	CV-%	*E. coli* Inhib.-%	CV-%	*A. baylyi* Inhib.-%	CV-%	*P. putida* Inhib.-%	CV-%
Juglone								
3.13	19.8	9.3	42.8	1.2	36.1	13.4	9.6	4.4
6.25	44.7	1.6	69.7	0.6	70.8	4.8	25.6	0.3
12.5	67.2	0.7	97.3	1.3	97.3	1.5	43.2	1.0
25.0	86.3	0.7	100.0	0.0	95.8	4.8	72.3	0.3
Lawsone								
42.7	91.1	1.3	80.3	1.5	70.6	3.3	64.5	1.7
85.3	96.9	0.4	90.8	0.7	75.6	5.1	79.8	1.3
171	99.1	0.1	97.3	0.4	79.3	8.2	90.6	1.6
341	99.7	0.0	99.1	0.1	89.1	6.1	97.8	0.2
Plumbagin								
0.130	8.5	5.9	1.9	2.4	3.7	3.1	8.7	1.1
0.260	16.6	5.3	6.8	3.3	13.4	2.4	20.9	1.2
0.520	27.3	2.1	13.5	3.9	21.8	4.5	37.7	1.6
1.04	45.5	1.1	28.6	1.4	37.3	2.7	52.6	0.6
Quercetin								
5.00	23.4	3.9	21.2	4.1	10.2	2.6	26.7	1.1
10.0	37.0	3.8	29.5	3.3	22.1	2.1	42.6	1.1
20.0	54.5	0.5	42.1	2.5	42.8	1.9	55.9	0.5
100	68.7	20.0	63.6	2.0	51.4	8.7	75.4	1.0
Controls								
Blank	0.0	4.7	0.0	1.5	0.0	2.0	0.0	5.1
EtOH 6.8 %	65.0	8.0	58.8	5.3	66.7	5.7	77.8	4.3

The most sensitive organism to plumbagin in the highest concentration was *P. putida* (Table 2), followed by *S. aureus,* which were inhibited by 52.6 ± 0.6 % and 45.5 ± 1.1 %. Even the highest concentration of plumbagin (1.04 µg/ml) did not inhibit either *E. coli* or *A. baylyi* by more than approximately third. Similarly, quercetin caused highest inhibition in *P. putida* (75.4 % ± 1.0 % in highest concentration of 100 µg/ml per well). Second highest inhibition was observed for *S. aureus*, followed by *E. coli* (both inhibited over 60 % by the highest quercetin concentration). The least effect was seen on *A. baylyi*. However, as the CV-% was higher (20.0 %) for *S. aureus* than for the other species, its order is not as certain as for the other three species.

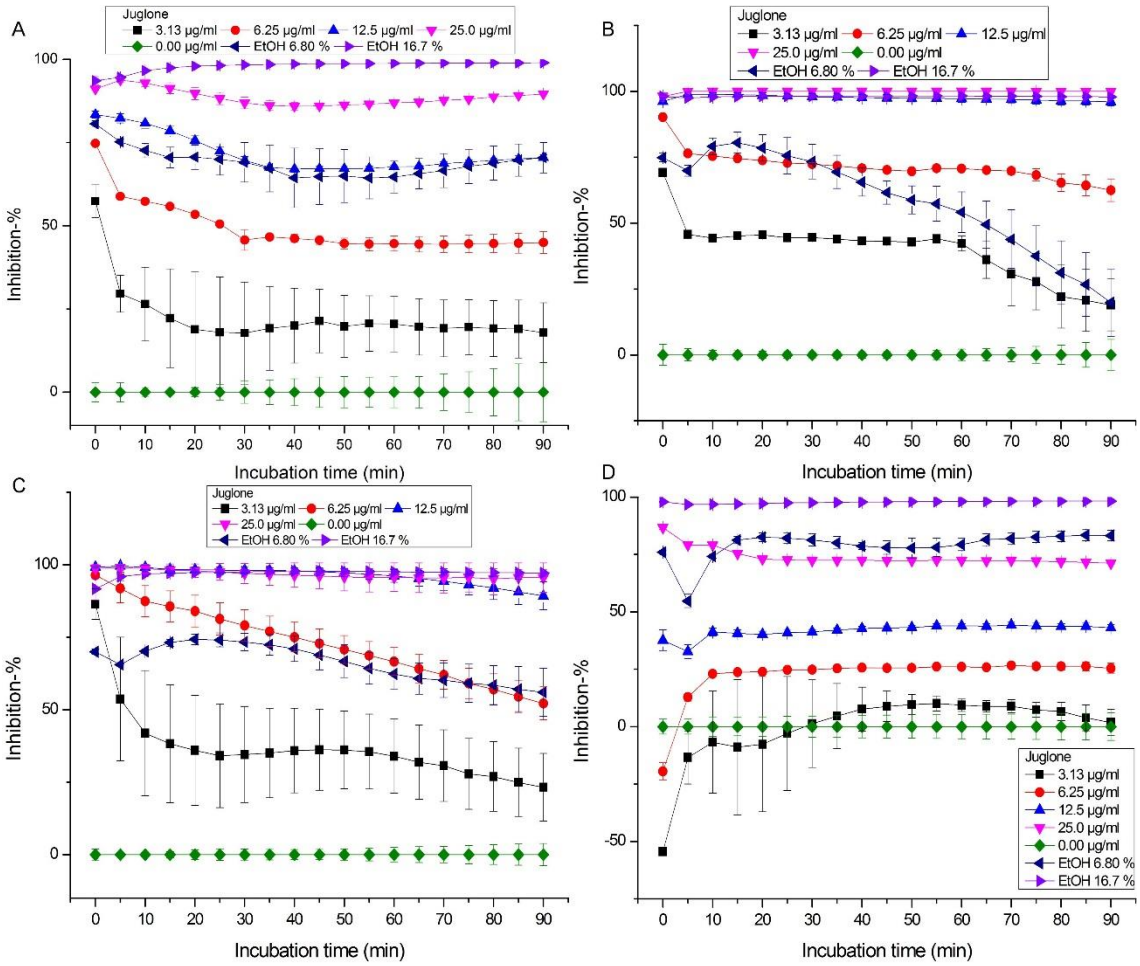

Figure 13. *Inhibition percentages (-%) for all of the biosensor strains when incubated with juglone control concentrations. Error bars represent CV-%.* ***A.*** *S. aureus RH4220.* ***B.*** *E. coli K12 + pCGLs11.* ***C.*** *A. baylyi ADP1.* ***D.*** *P. putida + pBAV1k-LUX.*

In Figure 13, a closer look is taken at the results of incubating the bacterial biosensor cells together with different concentrations of juglone. For all of the biosensor cells, the response to juglone was dose-depended, so that the lowest concentration produced lowest inhibition and the rest were in order as well, at the latest after 35 minutes of incubation. At some incubation points, however, the CV-% were higher, and thus overlapping, which hinders the evaluation of the results. *E. coli* K12 (Figure 13B) and *A. baylyi* ADP1 (Figure 13C) were the most sensitive strains for juglone, as the 12.5 and 25.0 µg/ml concentrations inhibited their growth almost 100 %. For *S. aureus* (Figure 13A) and *P. putida* (Figure 13D) only 25.0 µg/ml of juglone (0.25 % v/v-% EtOH) showed a inhibition-% nearing 100 % after the full incubation time.

5.4 Sundew extracts

Sundew extracts were obtained from Natural Resources Institute Finland (Luke), Parkano research unit. The extracts of round-leaved sundew collected from the peatlands had a

dark, reddish purple color (Figure 14A). Whereas, the laboratory grown sundews produced a vibrant green extract (Figure 14B). In Appendix B, a figure of one test plate is presented, where the colors of tested concentrations can be seen.

Figure 14. *The color differences of D. rotundifolia extracts.* ***A.*** *The round-leaved sundew extracts from plants collected from the peatlands. In front row, the extracts are from Kivineva and in the back row, from Lehtolamminneva.* ***B.*** *The extracts made from laboratory-grown round-leaved sundew. Photos taken by Tytti Sarjala.*

The results, measured with Chameleon Multilabel microplate reader, for the first batch (vacuum centrifuge dried) sundew extracts are represented in Figure 15A–D and Table 3. Lehtolamminneva (Figure 15A) and Kivineva (Figure 15B) extracts inhibited *P. putida* over 60 % even in the smallest concentration of 0.206 mg/ml. The inhibition was higher than that of 9.38 µg/ml juglone control. For both of extracts made from nature grown sundews, the inhibition followed a dose dependent pattern. On the contrary, the extracts of laboratory grown sundew only showed over 60 % inhibition in the two highest concentrations (Figure 15C), and the three lower concentrations did not behave dose dependently. Figure 15D shows a comparison between the three extract sources after 50 minutes of incubation. At that time point, all extracts inhibited the *P. putida* more than the juglone control, and the inhibition effect of EtOH 0.33 % can be neglected, as it is relatively close to 0 %. Lehtolamminneva and Kivineva extracts show a remarkably similar inhibition dose response, and the laboratory grown extract exhibits a lower inhibition-%.

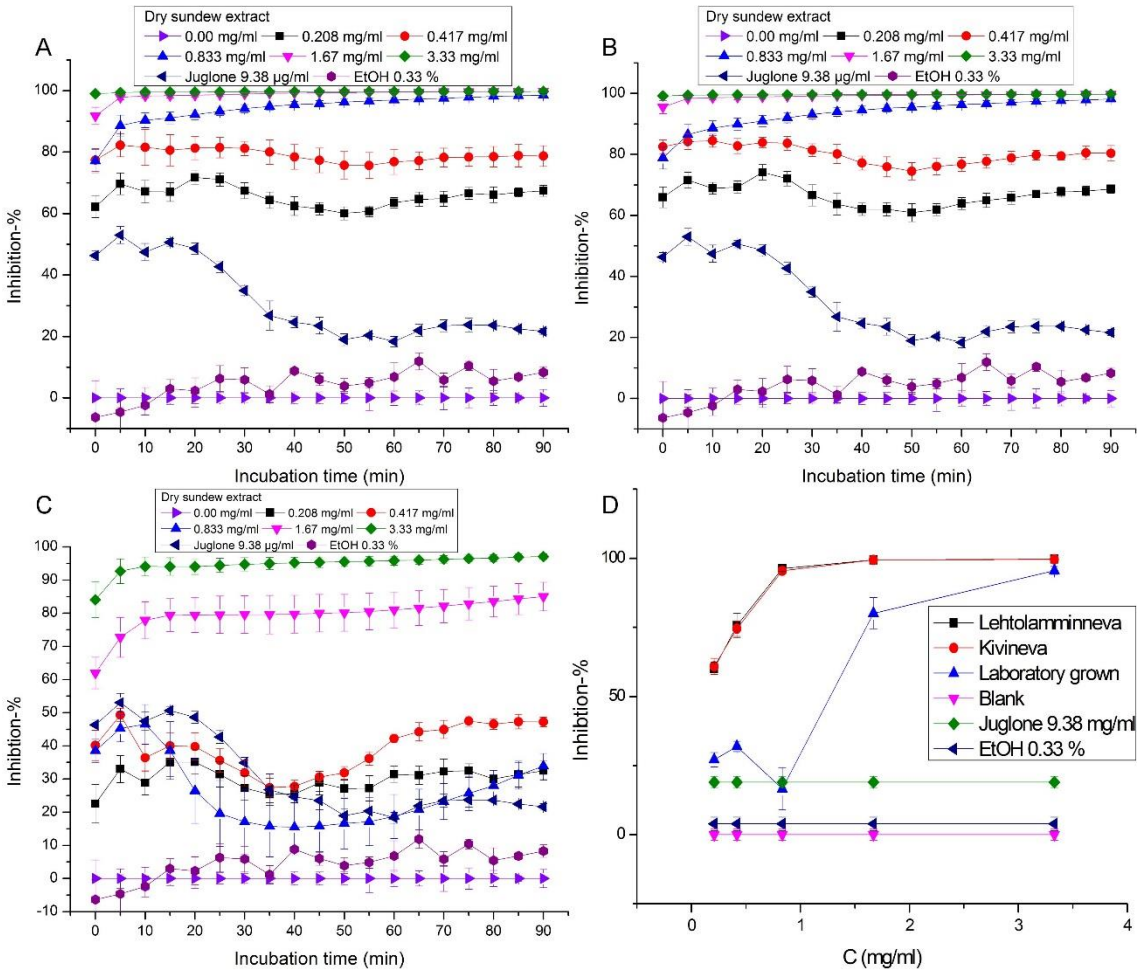

Figure 15. *The inhibition percentages (-%) caused when P. putida + pBAV1K-T5-LUX was incubated with the first batch of sundew extracts, dried with the vacuum centrifuge. The error bars represent CV-%. Values are collected from two different measurement occasions, the controls, 0.208 mg/ml and 0.417 mg/ml were measured in the same test and the rest of the concentrations in another. Dry sundew extract means the dry weight of the dried extract that was re-dissolved per milliliter into the sterile double distilled water to achieve the concentrations tested.* ***A.*** *Extracts from Lehtolamminneva peatland.* ***B.*** *Extracts from Kivineva peatland.* ***C.*** *Extracts from laboratory grown sundew.* ***D****. Comparison of the three extracts after incubation of 50 minutes. The concentration of dried sundew extract is denoted with c.*

In a comparison with Figure 15D, Table 3 represents the response of all of the biosensor cells after 50 minutes of incubation to all three sundew extracts. For *S. aureus*, the Lehtolamminneva and Kivineva extracts cause a dose dependent inhibition reaction. One concentration of the laboratory grown extracts even causes “negative” inhibition, which can be interpreted as the extract promoting the growth of *S. aureus*. For *A. baylyi* and *E. coli*, the dose dependency can be detected also for laboratory grown sundew extracts. However, the overall trend is that inhibition percentages are lower for extracts of the laboratory grown than for the extracts of the round-leaved sundews collected from the peatlands at similar concentrations, besides the highest concentration. *A. baylyi* is the most

sensitive to the first extract batch, as the inhibition percentages are generally higher than for the other sensor cells, and the CV-% are low, and do not explain the differences seen from 0.833 mg d.w./ml.

Table 3. *The average inhibition percentages (Inhib.-%). CV-% is the coefficient of variation. Jug. is the juglone control. The samples were from the first batch of the sundew extracts, which were dried with a vacuum centrifuge. Values are collected into one table from two different test runs. Controls and two concentrations (0.208 mg/ml and 0.417 mg/ml) were measured in the one test run and the rest of the concentrations in another. A lack of space on the 96-well plate prevented the measurement of all concentrations in the same test run. All values are measured after 50 minutes of incubation.*

Extract (mg d.w./ml)	*S. aureus* Inhib.-%	CV-%	*E. coli* Inhib.-%	CV-%	*A. baylyi* Inhib.-%	CV-%	*P. putida* Inhib.-%	CV-%
Lehtolammin-neva								
0.208	68.1	6.0	54.8	2.5	68.6	2.9	60.1	2.2
0.417	81.4	3.3	68.6	3.1	84.1	8.7	75.7	4.5
0.833	89.5	5.0	70.8	4.4	99.6	0.2	96.3	1.0
1.67	98.4	1.2	87.4	3.1	99.9	0.0	99.4	0.2
3.33	99.8	0.1	98.1	1.6	99.9	0.0	99.7	0.0
Kivineva								
0.208	61.4	2.6	55.6	2.9	61.5	2.3	60.9	2.9
0.417	76.1	2.3	70.6	1.5	82.7	5.4	74.5	2.8
0.833	80.5	9.4	71.9	3.5	99.3	0.5	95.5	1.2
1.67	97.8	1.9	89.6	3.4	99.9	0.1	99.4	0.2
3.33	99.7	0.2	98.9	2.2	99.9	0.0	99.6	0.1
Laboratory grown								
0.208	8.1	31.1	14.6	1.0	24.7	4.4	27.1	2.6
0.417	-32.4	23.8	25.5	1.8	39.0	5.0	31.9	1.9
0.833	41.8	10.0	44.1	4.6	63.3	6.7	16.6	7.7
1.67	72.8	11.9	62.6	8.2	92.8	8.0	80.1	5.6
3.33	88.2	3.1	88.8	8.6	99.2	0.9	95.6	1.4
Controls								
Jug. 9.38 µg/ml	24.0	0.9	8.9	0.8	40.7	2.2	19.0	1.9
Jug.18.8 µg/ml	34.7	5.1	19.9	3.6	96.3	0.8	37.9	2.2
Blank	0.0	3.7	0.0	1.2	0.0	2.8	0.0	2.0
EtOH 0.33 %	2.2	0.4	-6.6	0.5	-0.2	2.5	3.9	2.4

The Figures 16–19 and Table 4 represent the results from the second, air and nitrogen evaporated extract batch. The results were measured using Fluoroskan Ascent FL microplate reader, and thus are not directly comparable with the results from Figure 15A–D and Table 3, which had been measured using the Chameleon Multilabel reader. The two highest concentrations 1.67 mg/ml and 3.33 mg/ml of the extracts were also discarded, as they almost fully inhibited all of the strains in the first batch.

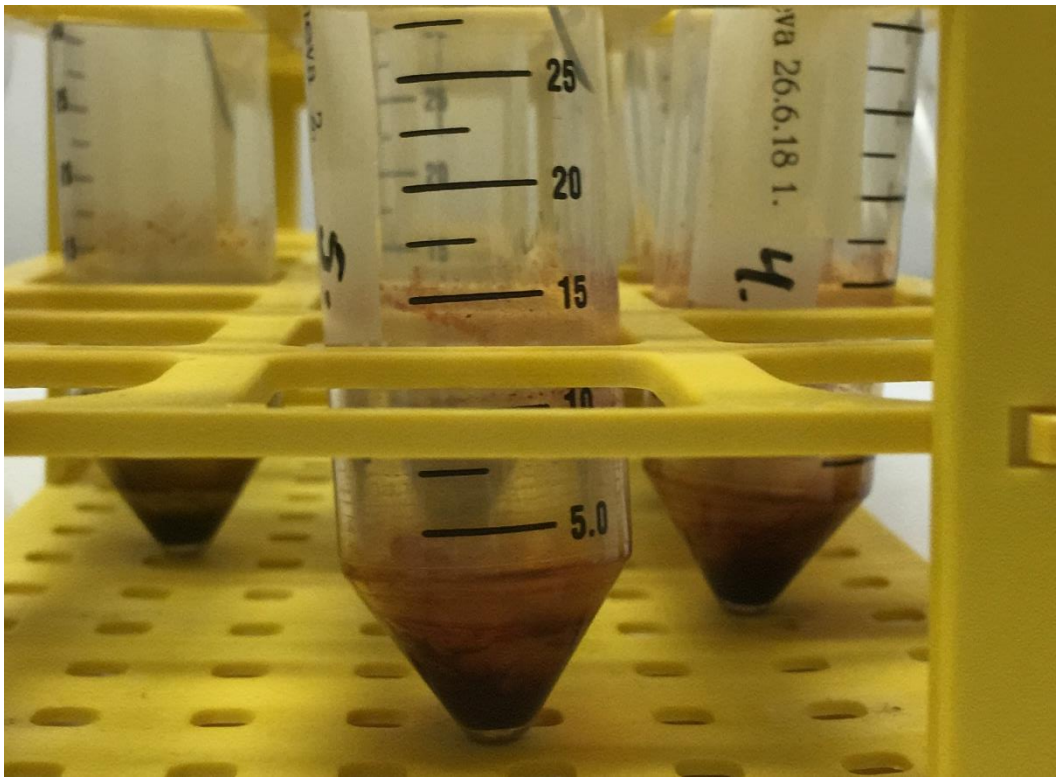

Figure 16. *The second batch of sundew extracts being dried with nitrogen evaporation. The evaporation has lasted approximately 20 hours, but the extracts are not completely dry yet. The sundews for the extracts were picked from the two peatlands. The tubes 4 and 5 (on the right-hand side and in the middle) are from Kivineva peatland and the tube on the left is from Lehtolamminneva. The nitrogen is flowing from the needle tips, which do not touch the extracts. The color of extracts is somewhat darker than in the first batch, and the red-brown hues are slightly more pronounced. Photo taken by Emmi Poikulainen.*

In Figure 16, the color of the second batch of extracts can be seen. The extracts were in the process of nitrogen evaporation when the photo was taken, and it has lasted approximately 20 hours. The nitrogen is flowing from the needles on the top of the figure, and the needles did not touch the extracts during the drying process. The extract in the left tube was originated from Lehtolamminneva collection, and the extracts in the two tubes on the middle and right from Kivineva collection. The color is not as vibrant as in the first batch (see Appendix B and Figure 14), but it is darker. The reddish brown hues are also slightly more pronounced in the second batch.

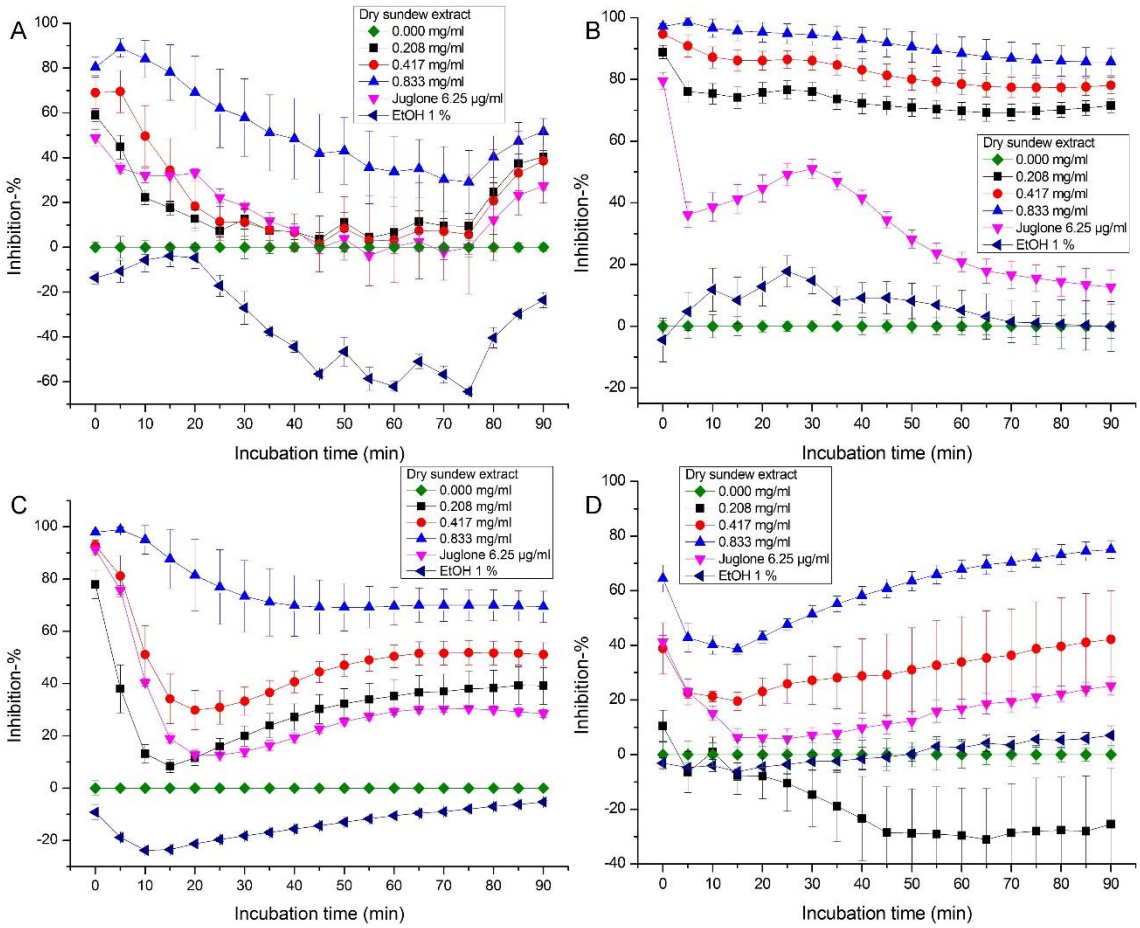

Figure 17. *Inhibition percentages (Inhibition-%) caused by the air and nitrogen evaporated sundew extracts from Lehtolamminneva peatland. Error bars represent CV-%.* ***A.*** *S. aureus RH4220.* ***B.*** *E. coli K12 + pCGLs11.* ***C.*** *A. baylyi ADP1.* ***D.*** *P. putida + pBAV1k-LUX.*

Figure 17 demonstrates the effects of Lehtolamminneva sundew extracts on all of the bacterial strains in one test run. The results were mainly dose dependent, only exception are the 0.206 mg/ml and 0.417 mg/ml with *S. aureus* (Figure 17A), which gave very similar inhibition percentages. *S. aureus* and *A. baylyi* (Figure 17C) were also stimulated to grow by the 0.33 % EtOH control. The most sensitive was *E. coli* (Figure 17B), as even the smallest concentration inhibited the growth approximately by 80 %. *P. putida* (Figure 15D) was not inhibited but stimulated by the smallest concentration, and was also overall the most tolerant to the extracts from Lehtolamminneva peatland. However, all of the extracts were producing less light, and thus probably more toxic, at least in some concentration, compared to the juglone standard. The CV-% were also overall higher in these results compared to the results of the measurements executed with Chameleon Multilabel microplate reader.

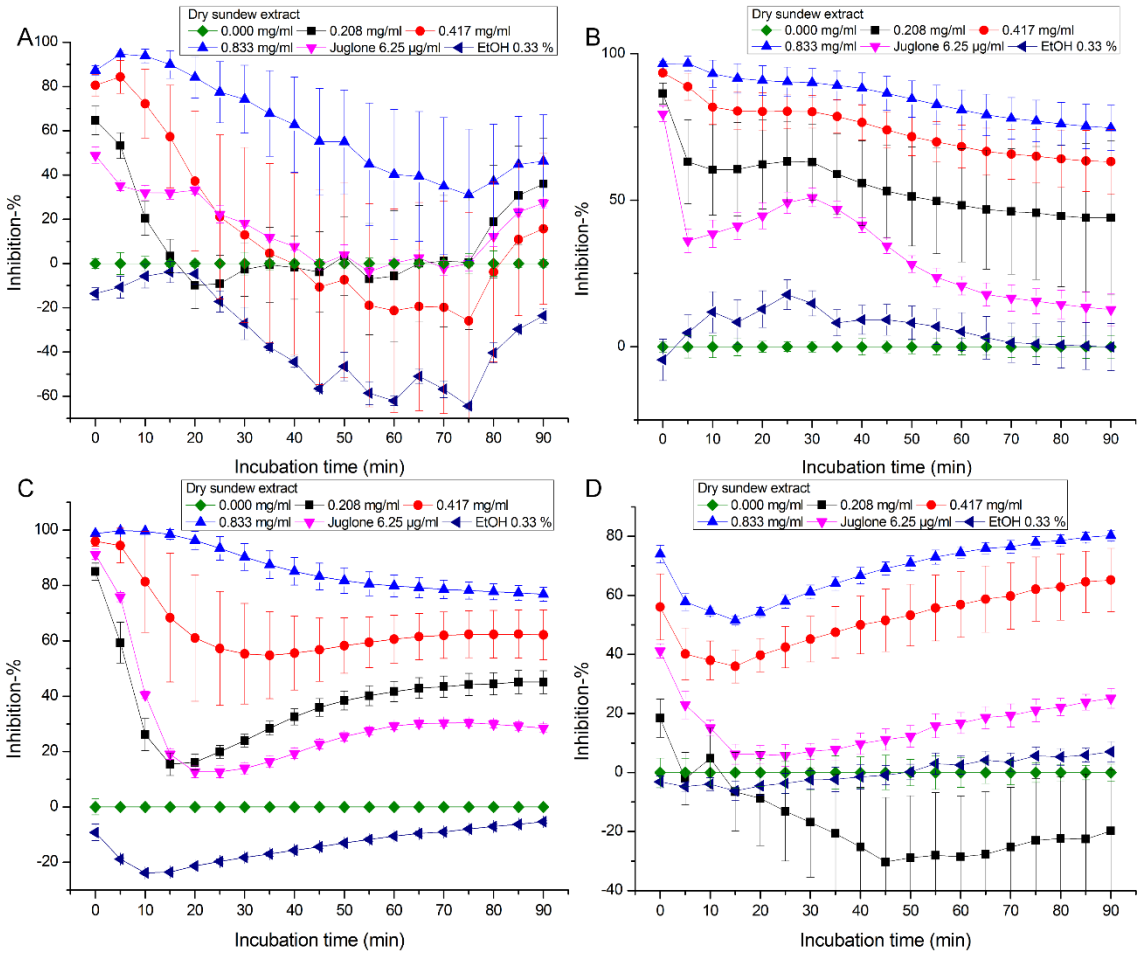

Figure 18. *Inhibition percentages (Inhibition-%) caused by the air and nitrogen evaporated from Kivineva peatland. Error bars represent CV-%.* ***A.*** *S. aureus RH4220.* ***B.*** *E. coli K12 + pCGLs11.* ***C.*** *A. baylyi ADP1.* ***D.*** *P. putida + pBAV1k-LUX.*

Figure 18 describes the results obtained with the extracts of Kivineva sundews. The results, especially for *A. baylyi* (Figure 18C), but also for other strains, are similar compared to the results from Lehtolamminneva sundew extracts, presented in the previous figure. The results were similarly dose depended besides for *S. aureus* (Figure 18A). The CV-% were even higher in these tests, especially with *S. aureus* and *P. putida* (Figure 18D), but also in *E. coli* (Figure 18B).

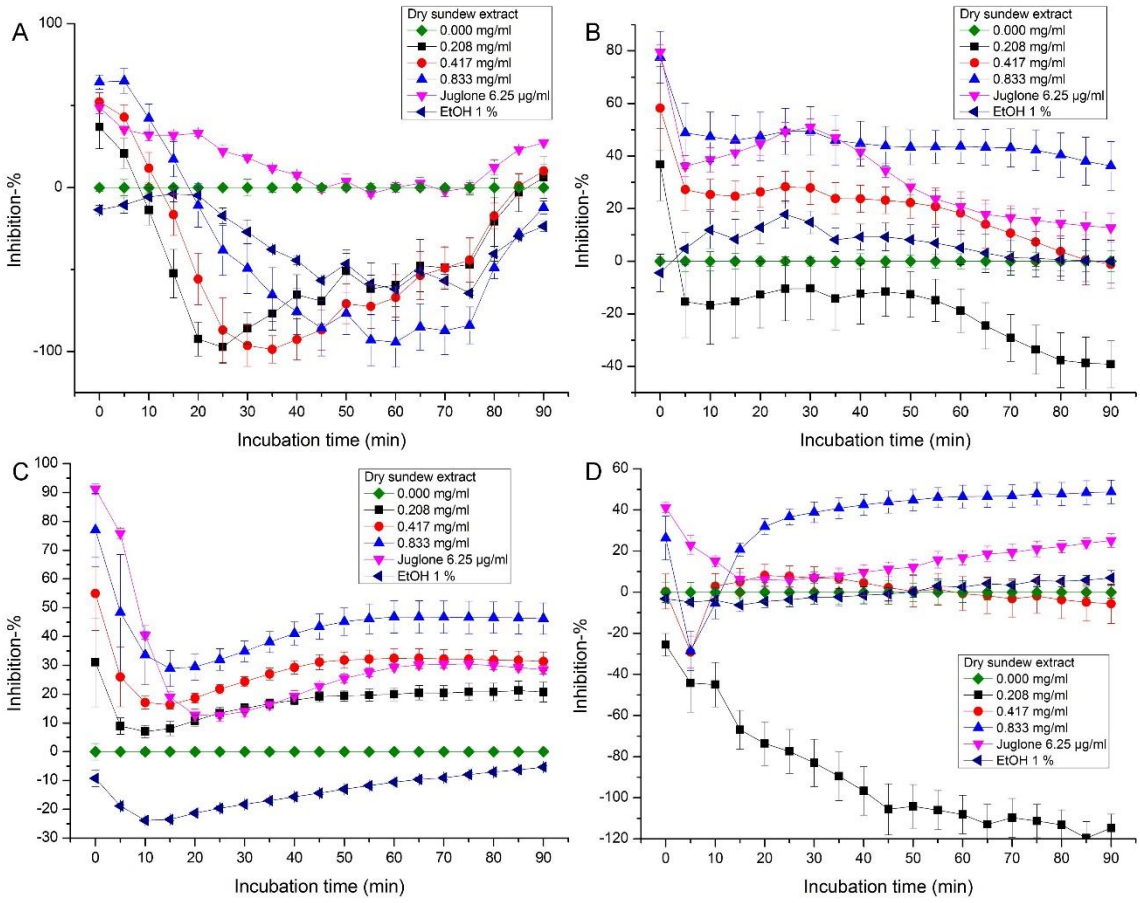

Figure 19. *Inhibition percentages (Inhibition-%) caused by the air and nitrogen evaporated sundew extracts from laboratory grown sundews. Error bars represent CV-%.* ***A.*** *S. aureus RH4220.* ***B.*** *E. coli K12 + pCGLs11.* ***C.*** *A. baylyi ADP1.* ***D.*** *P. putida + pBAV1k-LUX.*

The results of air and nitrogen evaporated laboratory grown sundew extracts (Figure 19) are quite different compared to the other results obtained. *S. aureus* (Figure 19A) was stimulated to grow by all of the extracts. In addition, *E. coli* showed stimulation with the lowest concentration (Figure 19B), and the inhibition was lower for the higher concentrations than with the wild grown sundew samples in Figures 15–16. *A. baylyi* (Figure 19C) behaved most similarly to the other extracts, and showed dose dependent inhibition, although the inhibition-% were lower. The highest concentration caused approximately as much inhibition as the lowest concentration in the other tests (40 %).

P. putida (Figure 19D) was stimulated to produce more light by the lowest concentration (0.208 mg/ml) of the laboratory grown sundew extracts. Similar effect could also be seen in *S. aureus* and *E. coli*, but their responses were not as pronounced as with *P. putida*. For *S. aureus* the highest stimiluation was slightly less than -100 in inhibition-% and for *E. coli* -40 inhibition-%, whereas for *P. putida* it was over -120 inhibition-%. Thus, the luminescent light signal of those *P. putida* wells was over 2-fold compared to that of the

blank sample (0.000 mg/ml). The medium concentration showed no large effect compared to the blank, and the highest inhibited the *P. putida* moderately. The CV-% of these tests were relatively low.

The air and nitrogen evaporated extracts were tested 3 times in total. Each of the test runs was run independently – using a new working stock of the bacterial samples as well as storing the samples as described in the materials and methods. The averages calculated from the three test runs, with three extracts from each bog or laboratory and each extract pipetted in triplicate, are shown below in Table 4. The CV-% were high, when calculated between all the three of the test runs, for *S. aureus* 31.6–92,8 %, for *E. coli* 6.0–29.3 %, for *A. baylyi* 14.1–46.7 % and for *P. putida* 18.4–57.6 %. However, the reactions caused by the extracts were mainly dose dependent even when taking all the data into account. Only for the extracts originating from Kivineva two lowest concentrations of the samples (0.208 mg/ml and 0.417 mg/ml) were not behaving dose dependently with *S. aureus*. However, the high CV-%s between the tests hinder the interpretation of the results.

Table 4. *The average inhibition percentages (Inhib-%) of three test runs, three parallel samples from each of the origin and in triplicate wells (total of 9 tests or 27 wells per extract). CV-% is the coefficient of variation. Jug. is juglone control. The samples were from the second, nitrogen evaporated, batch of extracts. Measured after 50 minutes of incubation.*

Extract (mg d.w./ml)	*S. aureus* Inhib.-%	CV-%	*E. coli* Inhib.-%	CV-%	*A. baylyi* Inhib.-%	CV-%	*P. putida* Inhib.-%	CV-%
Lehtolam-minneva								
0.208	26.7	49.0	46.8	13.9	26.8	30.3	-6.4	53.7
0.417	42.5	49.7	62.7	9.6	38.8	26.4	29.6	38.2
0.833	66.2	37.2	80.4	7.4	61.3	19.8	59.7	22.1
Kivineva								
0.208	36.1	49.2	46.3	15.0	29.8	28.7	-0.7	47.2
0.417	36.0	69.7	64.9	8.3	51.0	23.8	46.5	30.9
0.833	70.4	31.6	81.5	6.0	74.2	14.1	67.0	18.4
Laboratory grown								
0.208	-23.5	66.3	16.4	34.3	16.4	33.0	-14.9	57.6
0.417	-21.7	79.9	39.6	22.6	28.1	28.6	20.2	40.1
0.833	-11.4	92.8	54.2	16.0	39.5	25.1	45.6	27.3
Controls								
Jug. 9.38 µg/ml	33.9	68.4	34.1	22.4	37.1	15.6	17.6	35.5
Jug. 18.8 µg/ml	47.0	55.8	49.2	29.3	40.7	17.3	20.4	35.3
Blank	0.0	49.3	0.0	20.8	0.0	37.7	0.0	41.7
EtOH 1%	-28.8	67.7	-1.6	12.5	-18.6	46.7	1.3	43.3

6. DISCUSSION

The first attempts to transform the plasmid pBAV1K-T5-LUX into *P. putida* P8 (data not shown) were unsuccessful all together. This was attributed to too low isolated DNA content, and the elution buffer was changed to DDW, which helped to achieve the higher concentration described in the results. The first incubation after the successful transformation of the pBAV1K-T5-LUX plasmid into the *P. putida* took almost 5 days. For further studies, a slightly lower concentration of kanamycin, 30 µg/ml instead of 50 µg/ml, could be used to promote the growth after the transformation.

P. putida strain K showed poor bioluminescence in the preliminary tests. This is in accordance with the idea that the plasmid is not stable in the host unless there is selection pressure that favors the genes brought in by the plasmid (Dower et al. 1988). The halo zone effect, detected when cultivating strains 1 and P, did not have any effect on the measurements. However, no comprehensive explanation was found for the effect during the study. Cho et al. (2007) have used a different halo zone method to determine enzyme activities of endophytic bacteria, including *Pseudomonas* sp. Thus, it is possible that *P. putida* also excretes some enzymes, which causes the effect. Further studies would be needed to confirm this hypothesis.

Gajjar et al. (2009) speculated that the complex formation of bacterial growth media, with its inorganic and organic materials, might influence the bioavailability of heavy metals. They avoided the problem by harvesting the biosensor cells from the culture media by centrifugation and then resuspending the cells into sterile DDW. If that is also the case with the active substances in the natural extracts, it might be interesting in further studies to examine the impact of changing the medium from LB to for example minimal media, or to minimize the effect by not adding LB for the test incubation.

P. putida can achieve a high-degree of resistance to ethanol, if it is suddenly starved from its carbon source (Givskov et al. 1994). They can also be preconditioned to tolerate more ethanol, raising their MIC from 7 v/v-% to 9 v/v-% (Heipieper & de Bont 1994. The results of the ethanol tests in this thesis indicated lower tolerance for ethanol. Compared to the other bacteria used in this study, *P. putida* was the most sensitive to ethanol. This was demonstrated with the highest inhibition percentage of all of them at 1.67 v/v-% of ethanol, which is significantly lower than that reported by Heipieper and de Bont (1994). However, the previous studies were done using *P. putida S12* (Heipieper & de Bont 1994) and KT2442 (Givskov et al. 1994), which are different strains than used in this study. Also, no carbon source starvation or preconditioning were done for the cells, which could explain the differences between these results and the previous results. However, the 0.33 % of EtOH control in the extract tests increased the light signal emitted of *P. putida* compared no EtOH in the second batches of Lehtolamminneva and Kivineva extract tests,

which could indicate that small EtOH concentrations could be used with *P. putida* biosensor strain.

S. aureus RH4220 showed interesting behavior when incubated together with ethanol. In Figure 12A, the highest EtOH v/v-% caused initially a high inhibition percentage. However, as the incubation continued, the inhibition-% started to decrease, unlike in other cases where the continued incubation usually increased the inhibition-%. A reason for this behavior is thought to be that the added EtOH inhibited the light production initially in most of the *S. aureus* bacteria, but some of the bacteria were left intact and able to recover and continue the light production. Thus, when the time progressed, recovery of the remaining *S. aureus* caused a growth in the light production in the sample. As the samples were supplemented with the 50 µl additional LB during the incubation, the growing bacteria had an ample supply of nutrients. The bacteria in the control well, however, were not initially inhibited by EtOH or any other agent. Thus, the bacteria in the control wells were consuming the nutrients of LB already earlier in the incubation. As the inhibition-% were calculated against the signal from the control wells, this difference in nutrient availability might have boosted the metabolic activity of EtOH incubated *S. aureus* compared to the control well's.

Furthermore, especially in in the measurements done with Fluoroskan Ascent FL microplate reader, the CV-% of *S. aureus* tests were high and the inhibition-% lower compared to the tests conducted with Chameleon Multiplate and the tests made with other biosensor cells. The raw data signals received from Fluoroskan Ascent FL reader were low; for example, in the measurement of Kivineva extracts, represented in Figure 18A, the highest absolute signal received from any sample or control during the whole incubation time was 0.2706 CPU (counts per unit). *S. aureus* might not be as efficient in expressing the *luxABCDE* operon as the other bacteria used in this work, as it is G+ (Mesak et al. 2009). Thus, the smaller absolute signal levels might be explained with this. When the absolute signal levels are low, even minute absolute variation (such as the highest absolute variation in the described measurement: 0.0352 CPU) in the measured values leads to large CV-%s.

The problem of high CV-% was not as pronounced in the measurements conducted with Chameleon Multilabel due to the overall sensitivity of that reader being higher than that of Fluoroskan Ascent FL. This is highlighted by the signal to noise (S/N) ratios of each of the measuring devices. The noise signal was measured in previous studies in one time point from two wells of an empty 96-well plate (data not shown). Signal for Chameleon Multilabel was picked as the highest absolute signal in *S. aureus* test plate during the whole incubation of the first batch of the sundew extracts (results presented Table 3). The highest signal in that test was 1 124 818 CPU. The S/N ratio was calculated with a simple division of the highest CPU value received in the described tests by the noise signal from

the empty wells. For Fluoroskan Ascent FL, the S/N ratio was 366, whereas for the Chameleon Multilabel it was 7 352. In conclusion, Chameleon Multilabel had approximately 20 times lower S/N ratio than the Fluoroskan Ascent FL.

Another reason for higher CV-%s in the second batch might be the drying method. As the first batch was dried with vacuum centrifuge, the process was exactly same for all of the samples. However, as the second batch was first evaporated in the air in room temperature and then with the nitrogen gas in room temperature, there might have been slight differences in the evaporation conditions between the different samples. The conditions that could have changed include the temperature, the flow of the nitrogen to each of the tubes and the time of evaporation. Each of the samples was also only visually inspected to confirm that it was dry, thus variations in the completeness of the drying could also have happened. All of these factors might contribute to the differences noticed in the measurements between different samples.

A. baylyi ADP1 is versatile in its usage of carbon sources (Huang et al. 2006). Therefore it is slightly unexpected that it as sensitive as measured in this study towards juglone. In addition, lawsone showed relatively constant inhibition-% of 70.6–89.1 % despite the almost 8-fold increase in the concentration. This is in accordance with the fact, that ADP1 cannot use naphthalene as its carbon source (Wei et al. 2005) and naphthoquinones are structurally related to naphthalene as discussed in sub-chapter 2.2.1. However, plumbagin did not show that high inhibition-% for ADP1. The reason for this could be that it was added in minute concentrations (highest being 1.04 µg/ml per well), thus, did not much inhibit ADP1. Quercetin is not a naphthalene related molecule, but a flavonoid, and is relatively non-antimicrobial to ADP1.

Similarly to *S. aureus*, *A. baylyi*'s light production was also stimulated by 0.33 % of EtOH in the extract tests. This suggests, that the non-toxic concentration of ethanol can be beneficial for ADP1. Nwugo et al. (2012) found out that up to 2 % of ethanol is well tolerated in *A. baumannii*. The ethanol stimulated a stress response in *A. baumannii*, which caused production of indole acetic acid in the organism. The indole acetic acid could be, in turn, used by *A. baumannii* as a carbon source. Thus, small percentage of ethanol could enhance the conditions for the bacteria and even increase its virulence. A similar mechanism could cause the finding in this study for 0.33 % EtOH and *A. baylyi*.

In the naphthoquinone and flavonoid control tests, the ethanol concentration of 6.8 v/v-% was used to control the effect of EtOH compared to the effect of naphthoquinones and flavonoids. Lawsone was the naphthoquinone control with the highest EtOH content, which was the same 6.8 % in highest concentration of lawsone. As seen from Table 2, lawsone, in all of the concentrations, inhibited *S. aureus* and *E. coli* more than the EtOH control. For *P. putida* the 171 µg/ml (3.4 v/v-% EtOH) showed more inhibition compared to the EtOH control, and for *A. baylyi* the concentrations was the highest one 341 µg/ml.

This indicates, that the lawsone had an inhibitory effect on all of the strains, and not all of the inhibition was caused by the EtOH.

For juglone, *S. aureus* was inhibited more by 25 µg/ml than by the EtOH control, and for *E. coli* and *A. baylyi*, similar effect could be seen at 6.25 µg/ml and at 12.5 µg/ml, respectively. *P. putida* was not inhibited more by any of the concentrations of juglone than by the EtOH control. However, the EtOH contents of juglone were drastically less than the EtOH control (0.25 v/v-% maximum vs. 6.8 v/v-%). Thus, further tests with other ethanol concentrations are needed to determine the extent of juglone's inhibitory effect on *P. putida*. For all of the other strains, juglone showed more inhibitory effects than the EtOH control, and thus it can be concluded that the inhibition is mainly caused by the naphthoquinone for those strains.

For all of the strains, plumbagin caused inhibition, which was lower than that of the EtOH control. However, similarly to juglone's effect on *P. putida*, the ethanol content of plumbagin concentrations was noticeably smaller, topping at 0.02 %. Thus, more tests are required to prove the extent of its inhibitory effects. *S. aureus* and *P. putida* had similar inhibition when incubated with the highest quercetin content (100 µg/ml, 5.0 v/v-% EtOH) than with the ethanol control. *E. coli* was inhibited more by quercetin than by the EtOH control. Only *A. baylyi* showed less inhibition, and would require more tests to confirm the inhibitory effect. To conclude on the effects of ethanol included in the standard substance dilutions, it would have been beneficial to include other ethanol contents to the tests from the beginning to confirm the results better for all of the substances and strains.

The color differences observed in the sundew extracts grown in the laboratory versus those collected on the bogs might be due to the fact that *in vitro* grown sundews typically have less or different secondary metabolites compared to the wild grown sundews as discussed in chapter 2.2. This might also be the reason, why the second batch of extracts made from laboratory grown sundews showed favorable effects to all strains except ADP1 at least in one measurement and one concentration. Both *E. coli* and *P. putida* showed increased luminescence when they were subjected to 0.208 mg/ml of the second batch extracts from the laboratory grown sundews. *P. putida* also exhibited this with the same concentration of the extracts made from the sundews picked from the two peatlands. Thus, it is likely that the extracts include nutrients that the bacteria can use, but the nutrients play no role in the higher concentrations as the inhibitory substances, such as naphthoquinones, are still present at a concentration higher than their MICs. Only when the extract is diluted, and the concentration of the inhibitory substances decreases below the MIC, can the bacteria use the beneficial components available in the extract. *S. aureus* showed this behavior with all of the laboratory originated extracts, which suggests that the extracts have a lower concentration of inhibitory substances.

In addition, the colorfulness of the extract and pure substance samples might have affected the results received in this study. The bioluminescent light produced by the lux operon peaks at 490 nm (blue), although different species of bacteria might emit at different peak wavelengths (Hastings 1996; Vesterlund et al. 2004). If there are colorful substances present in the samples, which absorb light at the same wavelengths, the intensity of the bioluminescent signal might be decreased. This effect is probably higher in the extract samples than in the pure substance samples, as the extract samples contain more blue hues (green and purplish colors seen in Figure 14 and Figure 16 as well as in the Appendix B) than the pure substances, which were more yellowish or orange colored (Appendix B). Further studies should be done to assess the effects of the color on the data received.

These further studies could be started by testing substances which are colorful, but do not have known bactericidal effects, such as food dyes. For example, brilliant blue food dye demonstrated only very little antimicrobial activity against human pathogens (Baab et al. 1983) and could be thus used as a starting point for testing the effect of color intensity on these bacterial biosensor strains. The tests could be similar to the ethanol tests performed in this study, with varying concentrations of the colorful dyes. After these tests, a color control could be added to the tests of natural extracts or pure substances, in addition to the ethanol control used in this study. Another option for further research could be development of new bacterial biosensor strains. For example, by developing inducible (lights on) biosensors, which detect naphthoquinones or flavonoids, higher levels of bioluminescence could be achieved. Thus, even if some of the bioluminescent light were absorbed by the colorful samples, there would be more light produced to detect overall. Therefore, the low sensitivity of the measurement devices would not limit the measurements as much as in this study.

Overall, the results obtained in the screening part of the study suggest, that different bacterial biosensors behave differently, when faced with same natural extracts. In previous studies, it has been typical to screen the extract using only one model organism, such as the *E. coli*. The results on the effects of the sundew extracts on each of the strains is inconclusive, as the two different batches could not be directly compared. The batches were dried differently, because it was tested (data not shown) that the first drying method (the vacuum centrifuge) might also destroy some of the naphthoquinones from the sundew extracts. Therefore, it would have been interesting to compare, if the second batch actually was more toxic than the first batch. The change of measurement device in the middle, however, prevents this sort of comparison. The equipment was changed due to a malfunction in the original equipment, and thus could not have been avoided. For further studies, it would be important to test the four bacterial biosensor strains using the same natural extracts and the same measurement system to confirm the results.

What can be said, however, is that within the first experiments, the Lehtolamminneva and Kivineva samples were in good accordance, and that they showed higher inhibition than

the laboratory grown samples, which is in accordance to the second batch samples. The inhibition, however was different for each of the strains. The differences could be seen especially between *S. aureus* and the other three strains. *S. aureus* is G+ bacterium and the other three are G-. This is a likely explanation for the response differences.

The comparison of the results is also difficult due to the high CV-%, especially in between the test runs. This highlights the fact, that even though the used bacterial biosensor strains were sensitive enough to detect even minute amounts of standard substances (down to µg/ml concentrations), their results are not currently consistent enough to be used as the only research method.

However, because the test protocol is fast and suitable for high-throughput screening, and the results within one test run are quite consistent, they can be used to screen libraries of natural extracts. The usefulness of the bacterial biosensor cell tests is, that the extracts, which do not show any activity, can be discarded. When a substance or an extract shows antimicrobial properties in these tests, it should be selected for further testing. Methods for the further tests of the selected extracts or substances include for example diffusion methods, thin-layer chromatography-bioautography methods, dilution methods and flow cytometry (Balouiri et al. 2016).

7. CONCLUSIONS

The first aim of this thesis was to construct a new bacterial sensor strain using an existing plasmid containing the lux operon, pBAV1K-T5-LUX, and *Pseudomonas putida*. After a few minor problems, such as low concentration of the plasmid and long incubation times after the electroporation, the transformation was performed successfully. The aim was also to confirm, that the constructed *P. putida* can be used a biosensor cell. This was done in two steps. Firstly, different colonies were compared against each other to find the most luminescent strain with as small as possible errors. Such a strain was obtained, and it was found to grow best in a medium without phosphate buffer. Also, it was confirmed, that selection pressure affects the stability of the plasmid in the strain. Its capability to detect toxicity was confirmed using ethanol controls.

The second aim was to compare and contrast the effects of round-leaved sundew (*D. rotundifolia*) extract and pure compounds that can be found in either the round-leaved sundew extract or other natural extracts. In addition to the constructed *P. putida*, three pre-established cell strains were used. The strains were *E. coli* K12 + pcGLS11, *S. aureus* RH4220 and *A. baylyi* ADP1 + pBAV1K-T5-LUX. The four tested compounds were juglone, lawsone, plumbagin and quercetin. Out of these, quercetin is a flavonoid and others are naphthoquinones. All of the standard compounds caused inhibitory effects on all of the strains. However, the effect's magnitude depended on both the strain and the substance. These differences show, that it is important to select the suitable biosensor strains carefully for each applications.

Third aim of this thesis was to compare the accuracy of the selected four biosensor strains by testing the same extracts multiple times on the developed test method. The variance within the same test run was quite low; the CV-% was 0.0–23.8 %. However, when taking multiple test runs into account, the CV-% were much higher. Despite the higher CV-%, dose-dependency could be established in almost all of the samples even when taking three test runs into consideration. This suggests that the bacterial biosensor cells are suitable to be used for screening purposes of natural extracts, but that the extracts that exhibit inhibitory effects in the biosensor cell tests, should be further assessed using other methods to confirm their effects on both bacteria and human subjects alike.

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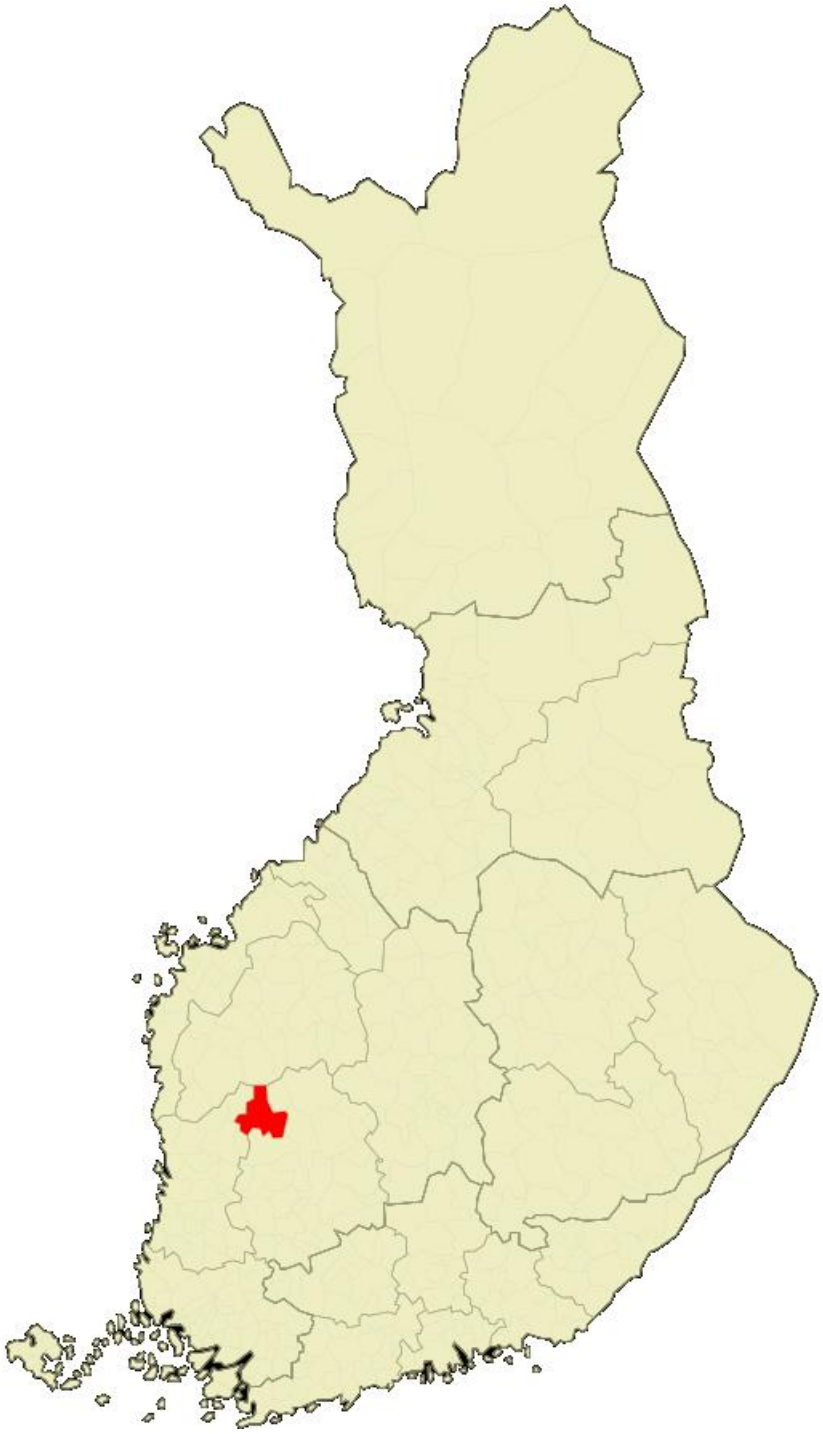
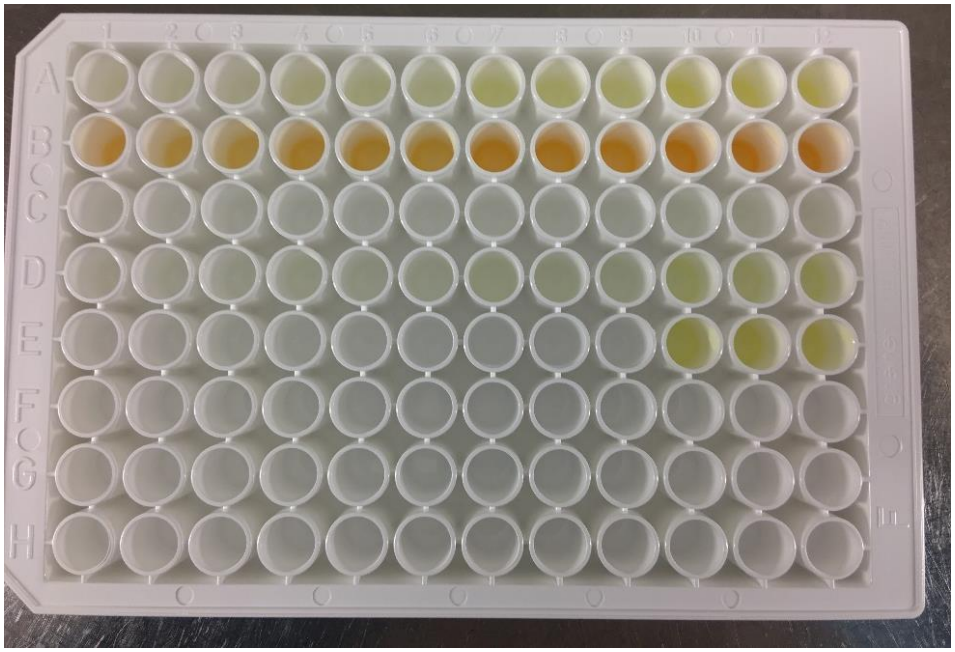
APPENDIX A: Location of the peatlands

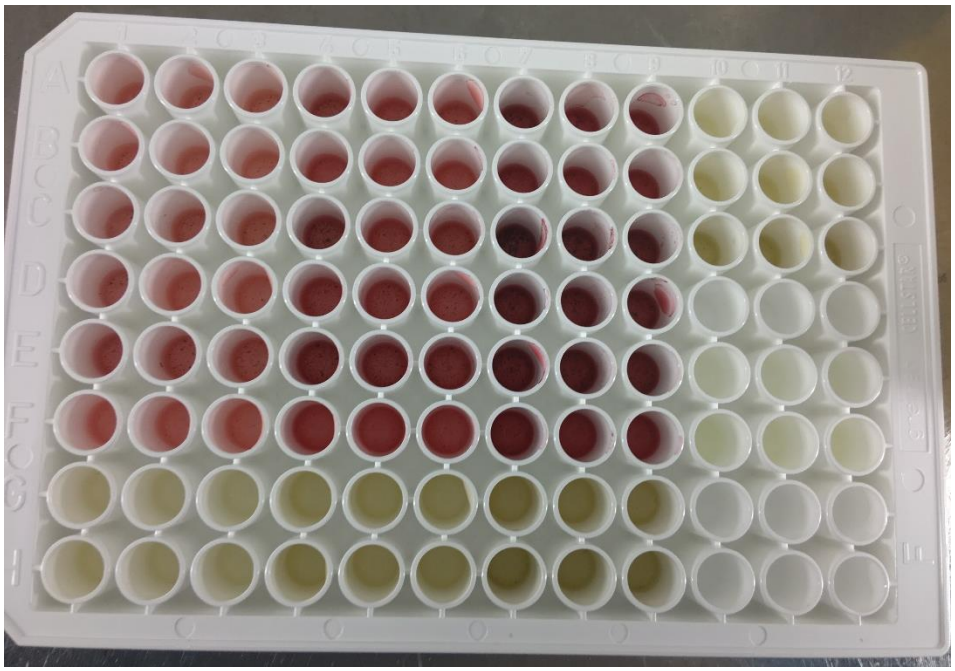
The peatlands, Lehtolamminneva and Kivineva, were located in Parkano, Western Finland. In this map, the municipality of Parkano is highlighted with red color. The map is reproduced from Wikimedia Commons public domain, user: BishkekRocks/ Wikimedia Commons / Public Domain.



APPENDIX B: Colors of the tested concentrations



Naphthoquinone standards on a test plate prior to addition of the cells or LB. Each row has triplicates of each of the tested concentrations, beginning with the lowest on the left. Row A: Juglone. Row B: Lawsone. Row C: Plumbagin. Row D: Quercetin. Row E: EtOH and 300 mg/ml Quercetin.



Sundew extracts on a test plate prior to addition of the cells or LB. Each row has triplicates of each of the tested concentrations, beginning with the lowest on the left. Rows A–C, columns 1–9: Lehtolamminneva. Rows D–F, columns 1–9: Kivineva. Rows G–H, columns 1–9 and rows A–C, columns 10–12: Laboratory grown sundew.