The possible transgenerational effects on *Daphnia magna* exposed to crude oil-derived hydrocarbons

Master's thesis

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Abstract

Understanding the effects of crude oil spills is especially meaningful regarding the Baltic Sea. This marine area is considered to be a high-risk zone for oil spills due to large amounts of crude oil trafficking. Crude oil consists mainly of diverse hydrocarbons of which the polycyclic aromatic hydrocarbons (PAHs) are especially toxic for living organisms. Exposure to crude oil has been documented to cause physical damages to body tissues, intoxication, and acute death. Direct metabolic changes reflected by altered oxygen consumption rates of model organisms, such as Daphnia magna, have also been observed in prior research investigating the effects of exposure to crude oil-derived hydrocarbons. The long-term impacts of this type of hydrocarbon exposure have not been investigated extensively and lack of knowledge might cause a deficiency in risk assessments evaluating the impacts of oil spills on the marine ecosystem of the Baltic Sea.
The purpose of this master's thesis was, therefore, to examine possible long-term effects of crude oil-derived hydrocarbon exposure on Daphnia magna. In the experiment, 48-hour-aged Daphnia magna in generation F0, were exposed to 30%-V, 10%-V and 0%-V concentrations of the water-soluble fraction of crude oil for a 48-hour period. Oxygen consumption rate and reproduction success of the exposed individuals were measured following the exposure and the same parameters were monitored in two following generations (F1 & F2) to see if the exposure-caused alternations that were carried over generations. The filial generations were only monitored, not exposed to any hydrocarbons. The most important result of the present thesis was that the individual oxygen consumption rate in the heavily exposed population (30%-V) stayed homogenous and stable throughout generations while the two additional populations (10%-V and 0%-V) showed unequal variances and significant differences in the rates of individual oxygen consumption between generations. These findings suggest that heavy hydrocarbon exposure derived from crude oil could cause a reduction of variation that is carried over generations and indicate that events such as oil spills could affect exposed populations throughout generations.

Keywords: Crude oil hydrocarbons, transgenerational effects, *Daphnia magna*, The Baltic Sea, oil spill

Svenskspråkig sammanfattning

Råolja är en icke-förnybar naturresurs som används i produktionen av många vardagliga förnödenheter som bränsle och plast (Harayama et al. 1999). Råolja är en brännbar vätska som består huvudsakligen av molekyler som byggs upp av kol och väte, dvs. kolväten (Rudzinski & Aminabhavi 2000). Vissa kolväten, som bensen och andra aromatiska kolväten, är giftiga för levande organismer, och således betraktas råolja som ett miljöskadligt ämne (Almeda et al. 2014).

Råoljeutsläpp i miljön förekommer regelbundet till följd av olyckor eller likgiltighet (HELCOM 2016). Risken för marina oljeutsläpp är speciellt stor i Östersjön eftersom trafiken i dessa områden är livlig och försvåras av krävande farleder med grynnor och smala partier (Suzdalev et al. 2014). År 2013 uppskattades det att 250 miljoner ton råolja samt av den raffinerade produkter transporterades årligen över Östersjön (BRISK 2013). På grund av den höga risken för oljeutsläpp är det viktigt att känna till möjliga effekter av oljeexponering i länder med kust längs Östersjön.

Tidigare oljeutsläpp i Östersjön har ökat förståelsen av akuta effekter hos marina organismer orsakade av råoljeexponering, men långtidseffekter har inte studerats systematiskt (Elmgren et al. 1983). På grund av att långtidseffekterna av råoljeexponering inte är så utförligt studerade, är betydelsen av oljeutsläpp för Östersjöns marina miljö svåra att bedöma. Målet med detta examensarbete har varit att belysa möjliga långtidseffekter av exponering för kolväten som härstammar från råolja och således bidra till kunskapen om möjliga långtidseffekter av den här exponeringen.

Speciellt oljeutsläpp utgör ett hot för akvatiska miljöer eftersom det i form av ett hydrofobt ämne sprider sig effektivt längs vattenytan (Harayama et al. 1999). Förutom detta, kan naturkrafter som vågor bidra till att giftiga kolväten upplöses i vattnet (Albers 1995). Den effektiva spridningen leder även till att ett lokalt spill påverkar ett omfattande område (EPA 1999). Den delen av olja som upplöses i en kolumn av vatten kallas för *Water-solube Fraction* (WSF) och den består för det mesta av polycykliska aromatiska kolväten (PAH), heterocykliska föreningar, resiner och asfaltener (Akaishi et al. 2004). I denna studie tillverkades en syntetisk WSF enligt Martínez-Jerónimo et al. (2003) anvisningar för att kunna efterlikna en naturlig kolväteexponering i samband med oljeutsläpp.

Metabolisk respons hos försöksorganismer har dokumenterats i samband med tidigare experiment där organismer har blivit exponerade för kolväten som härstammat från råolja (Christiansen et al. 2010). Eftersom största delen av alla metaboliska processer är syreberoende, är förändringar i organismers individuella syreförbrukning en faktor som speglar deras metaboliska respons (Kleiven 1992). Förändringar i reproduktionsframgång har även blivit dokumenterade till följd av tidigare experimentella kolväteexponeringar (Geiger & Buikema 1982). På grund av att både individuell syreförbrukning och reproduktionsframgång verkade vara lovande parametrar för att mäta effekter av kolväteexponering, utnyttjades dessa i denna studie.

I denna studie exponerades två populationer (F0) av *Daphnia magna* för två olika koncentrationer av WSF (10 volymprocent WSF och 30 volymprocent WSF) medan en tredje population förblev som en icke-exponerad kontrollgrupp. Exponeringsperioden av F0-generationen började då individerna var 48-timmar gamla och räckte 48 timmar. Efter exponeringsperioden mättes effekterna av exponeringen genom att följa med individuell syreförbrukning ($\dot{M}O_2$) och reproduktionsframgång. Avkomman av de olika behandlade populationerna vid F0-generationen studerades sedan likadant under två generationer (F1 & F2) då de inte längre exponerades för kolväten som härstammade från råolja. Följande generationer (F1 & F2) undersöktes för att se ifall exponeringen av föräldrarna i F0-generationen hade orsakat förändringar som överfördes från föräldrar till följande generation (transgenerationella effekter).

Daphnia magna är ett lämpligt försöksdjur då man vill forska i möjliga transgenerationella effekter, eftersom kräftdjurarten ifråga förökar sig asexuellt i gynnsamma omständigheter (t ex. laboratorium) (Kleiven et al. 1992). Asexuell reproduktion leder till att avkomman är genetiska kopior av föräldrarna och till att eventuella individuella skillnader därmed snarare borde bero på epigenetisk variation orsakad av miljöfaktorer än på variation som följd av genetisk rekombination (Dukić et al. 2019). Alla individer i föräldragenerationen (F0) härstammade från en enskild slumpmässigt vald individ av arten *Daphnia magna* och representerade således samma genotyp. Förhållandena för de skilda populationerna i alla generationer kontrollerades för att motsvara varandra i fråga om temperatur, ljus, pH och näring. Endast exponeringsgraden till WSF varierade under exponeringsperioden mellan populationerna i F0-generationen (kontroll, 10 volymprocent WSF och 30 volymprocent WSF).

Resultaten i denna studie anger att varken den individuella syreförbrukningen eller reproduktionsframgången i F0-generationen påverkades signifikant av varierande WSF-exponeringsgrader, eftersom inga signifikanta skillnader observerades mellan individer med olika exponeringsgrad (Kruskal-Wallis Test $p > 0,05$). Däremot hittades skillnader i individuell syreförbrukning mellan populationer bland de följande generationerna, F1 och F2. Individerna som härstammade från populationen som blivit utsatt för den kraftigaste kolväte-exponeringen (30 volymprocent WSF) representerade betydligt högre syreförbrukning än individerna som härstammade från den icke-exponerade kontrollpopulationen eller de individer som härstammade från den mindre exponerade populationen (10 volymprocent WSF) (pairwise comparison Kruskal-Wallis, $p = 0.000$). Inga signifikanta skillnader i reproduktionsframgång hittades mellan de olika populationerna inom de följande generationerna.

Intressanta resultat erhölls då individuell syreförbrukning hos olika generationer inom varje population jämfördes med varandra. Det framkom att alla generationer (F0, F1, F2) av den kraftigast kolväteexponerade populationen representerade homogen individuell syreförbrukning (Levene's Test for Homogeneity of variances, $F = 0,656$, $p = 0,523$). Den individuella syreförbrukningen förblev oförändrad mellan de olika generationerna (independent samples Kruskal-Wallis Test, $H = 3,537$, $p = 0,171$). Alla dokumenterade värden för syreförbrukning mellan olika generationer av den kraftigast exponerade

populationen förblev även inom normaldistributionen (Shapiro-Wilks p >0.05). Dessa resultat tyder på att den kraftiga kolväteexponeringen förorsakade en sorts metabolisk stabilitet som överfördes från den exponerade föräldragenerationen (F0) till följande icke-exponerade generationer (F1 & F2).

Till skillnad från populationen som härstammade från de kraftigast exponerade individerna visade både den mildare kolväteexponerade såväl som kontrollpopulationen heterogen individuell syreförbrukning mellan de olika generationerna (Levene's Test for Homogeneity of variances, p = <0.001). Även signifikanta skillnader i individuell syreförbrukning kunde observeras mellan olika generationer inom båda populationerna (Kruskal-Wallis p <0.05). Vissa generationer representerade även värden av individuell syreförbrukning som stod utanför normaldistribution (Shapiro-Wilks p <0.05). Växlande syreförbrukning tyder på att en mildare exponering inte förorsakar likadan rigiditet som den kraftigare exponeringen.

En del av den använda syntetiska 30 volymprocent WSF-lösningen konserverades direkt efter tillverkningen och en annan del av lösningen fick avdunsta i dragskåp under exponeringsperioden i 48 timmar. Dessa sampel analyserades kemiskt i samband med experimentet för att få en uppfattning om de kemiska komponenterna som försöksdjuren exponerades för. Den totala kolvätehalten av lösningen nådde 3.6 mg/l i det direkt konserverade samplet. Bara en bråkdel av kolvätena anses vara direkt giftiga för levande organismer. En stor del av dessa giftiga molekyler hör till PAH-föreningar. Det visade sig att PAH-halten i lösningen var 27 µg/l men minskade drastiskt till 1.9µg/l under avdunstningen. I den syntetiska WSF lösningen var majoriteten av PAH-föreningarna naftalen men även små mängder av fenantren, fluoren och acenaftylen hittades. Dessa giftiga molekyler är lättflyktiga och avdunstar snabbt även i samband med oljeutsläpp som sker i naturen (Albers 1995).

Kolvätehalten i vattenkolumnen i samband med riktiga oljeutsläpp är svår att jämföra direkt med experimentella kolvätehalter vid användning av syntetisk WSF, eftersom naturliga omständigheter, som solljus, vågor, salinitet med mera i allmänhet påverkar mängden upplösta kolväten i vattenkolumnen då oljeutsläpp inträffar. Dessutom påverkar storleken av utsläppet och typen av olja mycket de molekylära komponenterna som upplöses (Wernersson et al. 2004). Erhållna värden från vattenkolumnen i samband med riktiga oljeutsläpp går ändå i samma riktning som den tillverkade syntetiska 30 volymprocent WSF-lösningens värden. Till exempel Pécseli et al. 2003 erhöll halter från 0,641 till 12,833µg /l vid den danska kusten i samband med en olycka där 2400 ton råolja rann ut i havet. De motsvarande värdena i examensarbetet i fråga är troligen inte mycket högre än de som Pécseli et al. 2003 dokumenterade. Detta beror på att samplet av den syntetiska 30 volymprocent WSF-lösningen i denna studie konserverades direkt, medan PAH-föreningarna fick fritt avdunsta i samband med det riktiga spillet tills Pécseli et al. 2003 nådde olycksområdet. Ifall samplet av den syntetiska 30%-V WSF lösningen i denna studie låtits avdunsta lika länge hade de erhållna värdena antagligen varit rätt lika de Pécseli et al. 2003 erhöll.

Den observerade minskningen av variation gällande individuell syreförbrukning till följd av en kraftig kolväteexponering kunde betyda att kraftigt exponerade individer och deras

avkomma inte lägre kan justera sin metabolism enligt förändringar i sin levnadsmiljö. Detta är en intressant synpunkt som möjligen skulle löna sig att studera vidare för att underlätta sammanställning av framtida riskbedömningar gällande marina oljeutsläpp i Östersjön.

Nyckelord: Kolväten i råolja, transgenerationella effekter, *Daphnia magna*, Östersjön, råoljeutsläpp

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1 Introduction

Crude oil is an important natural resource in the production of everyday products such as fuel and plastics (Harayama et al. 1999). It is a flammable liquid mainly consisting of a mixture of diverse molecules called "hydrocarbons" (Rudzinski & Aminabhavi 2000). Some of these hydrocarbons are toxic to living organisms. Therefore, crude oil is considered hazardous to the environment (Almeda et al. 2014). Crude oil is regularly spilled in nature as a result of ignorance or accidents. Spills occur even though there are strict regulations considering crude oil transportation, usage and disposal (HELCOM 2016). Especially aquatic environments are vulnerable regarding oil spills. Crude oil spreads along the water column horizontally and does not remain locally on the spill site (Harayama et al. 1999). This way the oil-affected area becomes larger compared to spills on land and can harm considerably large areas in aquatic ecosystems (EPA 1999).

In the Baltic Sea, the risk for marine oil spills is high due to heavy trafficking and challenging shipping lanes. Hence, understanding the environmental impacts of oil spills in countries located at the coast of the Baltic Sea is essential (Suzdalev et al. 2014). Diverse acute consequences to living organisms have been observed previously after oil spills, but little is known about the long-term effects of these events on organisms at spill sites (Elmgren et al. 1983). This thesis aims to investigate possible long-term effects by focusing on effects which are passed over generations (transgenerational) following an aquatic crude oil hydrocarbon exposure.

Three parental populations (F0) of *Daphnia magna* were reared and exposed to crude oil hydrocarbons in the current study. Two types of hydrocarbon exposures were used, a light and a heavy crude oil hydrocarbon exposure, also a control treatment where the individuals were not exposed to any hydrocarbons was conducted. To expose the crustaceans for crude oil hydrocarbons, a water-soluble fraction (WSF) of crude oil was prepared by diluting crude oil in water, and this fraction was used as the source of hydrocarbons in the exposures. The cultivation medium of the lightly exposed Daphnia contained 10%-V WSF and the medium of the heavily exposed contained 30%-V WSF during the 48-hour exposure periods.

Selected parameters were measured from the exposed populations and the control population after the exposure period to indicate possible effects of the hydrocarbon exposure. The offspring originating from the differently treated parental populations (F0) were then studied in the following two generations, F1 and F2, without exposing them to hydrocarbons. The filial generations originating from different parental populations (10%-V WSF, 30%-V WSF, control) were studied further to see if any possible effects of the original parental hydrocarbon exposures could be seen in the offspring of the exposed populations indicating transgenerational effects.

The parameters measured during the study were the individual oxygen consumption rate ($\dot{M}O_2$) and reproduction success of the hydrocarbon-exposed *Daphnia magna*. These parameters were chosen, since metabolic responses (Christiansen et al. 2010) and changes in reproduction success (Geiger & Buikema 1982) have been recognized as acute consequences of crude oil hydrocarbon exposures conducted in the laboratory. Metabolic responses are generally reflected by changes in individual $\dot{M}O_2$, because metabolism is oxygen-requiring in the long run. The metabolic activity and reproduction success of an individual are both also ecologically important parameters associated with the overall fitness of an individual (Kleiven 1992).

1.1 Oil spills in the Baltic Sea

Oil spills caused by intentional discharges have decreased significantly in the Baltic Sea during the past decades due to effective aerial surveillance combined with substantial fines and penalties (Aigars et al. 2011, Haapasaari et al. 2013). The governing commission of Baltic Sea welfare, HELCOM (Baltic Marine Environment Protection Commission - Helsinki Commission), reported in 2016 that only 82 discharges were detected during aerial surveillances in 2015 and that the amount of oil released was estimated to be less than 50 m^3 during the entire year. This is a significant decrease in detections reported. The corresponding number of oil releases detected in 1995 was documented to be 649 and the amount of oil released during that year was estimated to reach almost 600 m^3. However, it should be noted that these numbers describe only the numbers of detected discharges in the Baltic area and may give an imperfect picture of the situation. When inspecting the surveillance data published by HELCOM (2016) one can, for example, notice that aerial surveillance over Russian parts of the Baltic has been infrequent.

Due to the observed reduction of intentional discharges (Aigars et al. 2011, Haapasaari et al. 2013), the main risk of marine oil spills in the Baltic Sea is created by the possibility of marine trafficking accidents. The report of Sub Regional Risk of Spill of Oil and Hazardous Substances in the Baltic Sea (BRISK) (2013) estimated that over 250 million tons of crude oil and fuels refined from crude oil are shipped each year over the Baltic Sea region. This amount corresponds to 11% of all the crude oil, and related fuel, shipping on a global scale (Brusendorff et al. 2012). Besides heavy trafficking, the Baltic Sea shipping lanes have many shallow and narrow sections, underwater rocks and challenging weather conditions, such as ice-coverage during wintertime. These factors lead together to the fact that the Baltic Sea is a high-risk zone for marine oil spills caused by shipping accidents, such as groundings or collisions (Haapasaari 2013). Oil platforms and terminals are also possible sources for accidental oil spills in the Baltic Sea (Suzdalev et al. 2014). The Kravtsovskoye oil extraction platform, located near the Curonian Spit, a UNESCO World Heritage site (Aigars et al. 2011), for example, has been reported extracting and transporting approximately 700 000 tons of oil yearly (Lukoil 2010). Several spills have already been registered to occur at this platform, some of these notable in size (Suzdalev et al. 2014).

The estimated time interval for large scale oil spills, releasing more than 5000 tons of oil in the Baltic Sea, has been estimated to be 26 years. However, the interval for smaller spills, releasing 3000-5000 tons of oil, has been estimated to be merely four years (Brusendorff et al. 2012). Ship traffic in the Baltic Sea has been expected to increase by 64% from the situation in 2014 by the year 2020. The increase results from the construction of new Russian oil terminals and the expansion of Lithuanian terminals at Klapeida and Butinge. Moreover, the size of tankers is expected to grow, contributing to the possibility of significant oil spills (Suzdalev et al. 2014).

1.2 Crude oil chemistry

Different crude oils vary in molecular composition depending on the source and origin of the oil, but generally, 50-90% of the liquid consists of hydrocarbons (Jiang et al. 2010). Hydrocarbons are a diverse group of different molecules that consists of a carbon (C) chain and hydrogen (H) bound to the carbon core. Different hydrocarbons can vary to a great extent in molecular weight and structure, giving them varying properties (Almeda et al. 2014).

Carbon and hydrogen together build electrically neutral, nonpolar molecules. The non-polarity makes hydrocarbons hydrophobic. The hydrophobicity is one of the reasons why crude oil creates a horizontally spreading slick on the water surface when spilled into aquatic environments (EPA 1999). The nonpolar hydrocarbons are fat-soluble and many of them, therefore, can penetrate the fatty-acid layer of a cell wall and cause cellular damage when interacting with living organisms in aquatic environments (Sikkema et al. 1995). Non-hydrocarbon substituents of hydrocarbons increase the electrical polarity of hydrocarbons and, thus, increase the solubility of hydrocarbons in the water column (Mannio et al. 2011).

The hydrocarbons which are considered as the most toxic components in crude oil are called "Aromatic hydrocarbons". Aromatic hydrocarbons contain at least one Benzene ring which is the simplest aromatic compound. Approximately 1-20% of crude oil hydrocarbons belong to the aromatic fraction (NRC 2003). Non-aromatic hydrocarbons are referred to as "Aliphatic hydrocarbons" (Kang et al. 2014) and they are less toxic (Sikkema et al. 1995).

Benzene is a highly toxic circular molecule that consists of six carbon atoms (Philip 2013). The structure of benzene contains special alternating single and double bonds between all the carbons that make the molecular structure very persistent (Albers 1995). Benzene and other compounds that contain one benzene ring bound with non-aromatic substituents are called "monoaromatic compounds". Benzene and three alkylated monoaromatic compounds, toluene, ethylbenzene and xylene, are found commonly in crude oil. These compounds are generally referred to with the acronym "BTEX" due to their abundance in crude oil and related substances (NRC 2003, Kang et al. 2014).

Molecules that consist of one, or more, benzene units are called "Polycyclic Aromatic Hydrocarbons" (PAHs). The PAHs consisting of two or three benzene rings are the most common PAHs found in crude oil. As much as 90% of the PAHs present in crude oil usually belong to this group of molecules (NRC 2003). Much like the monoaromatics, PAHs can be substituted by various non-aromatic molecule groups. Naphthalene is the simplest PAH built by two combined benzene structures and no substituents. The compound consisting of three benzene structures and no substituents is called phenanthrene (Menon & Menon 1999).

Other, non-hydrocarbon, molecules found in crude oil are for example mineral salts, trace metals, and organic molecules which are built of a hydrocarbon structure where other elements, such as oxygen (O), nitrogen (N) and sulfur (S), are incorporated. Well-known groups of these complex organic molecules are resins and asphaltenes (Albers 1995).

Molecules containing other elements, apart from hydrogen and carbon, often possess a charge and are, thus, water-soluble compared to pure hydrocarbons (Stanford et al. 2007).

1.3 Behavior of spilled crude oil in aquatic environments

When crude oil is spilled in the aquatic environment, the oil becomes modified by the powers of nature. These physical modification processes are referred to as "weathering" (Wernersson 2004). Weathering happens, for example, when waves break the oil column and mix the oil with water creating water-in-oil emulsions. This also dissolves crude oil molecules in the water column (Albers 1995).

The molecules that dissolve to water are called "Water-soluble Fraction" (WSF) of the crude oil (Martínez-Jerónimo et al. 2003). The WSF is a mixture of different crude oil components: PAH-compounds, heterocyclic compounds, resins and asphaltenes (Akaishi et al. 2004). The hydrocarbons dissolved in water are highly bioavailable and some of them very toxic for aquatic organisms (NCR 2003). The quantity of hydrocarbons that dissolve in water after oil spills vary considerably depending e.g. on weather conditions and sampling time. For example, concentrations of Polycyclic Aromatic Compounds (PAC) varied between 641 and 2833 ng/l at different sites on the Danish coast that were affected by a spill where 2400 tons of heavy bunker oil was released in 2001 (Pécseli et al. 2003).

Together with the environmental conditions, such as pH and amount of sunlight (Wernersson 2004), the molecular composition of crude oil affects the outcome of weathering processes. A significant fraction of the released hydrocarbons is usually evaporated within a couple of days following an oil spill. Especially molecules having low molecular weights evaporate effectively (Albers 1995). For example, in a 4.3 m^3-sized crude oil spill in the North Sea, more than 50% of the light hydrocarbons (<17C) were lost from the oil slick within 25 hours (Gros et al. 2014). However, parts of the oil do not dissolve, but create small oil droplets which are dispersed as free particles in the environment (Almeda et al. 2014). Complex PAH compounds that do not dissolve easily in the water column, due to high hydrophobicity, can also be absorbed in the bottom sediment (Menon & Menon 1999).

1.4 The effects of oil spills on the marine life of the Baltic Sea

Oil spills affect the living organisms in aquatic environments in various ways. Mortality is caused by hydrocarbon toxifications, but also by a plain physical disturbance that the oil causes. For example, seabirds and mammals lose the heat isolation of their coatings if covered in oil. This, in turn, can lead to death caused by hypothermia (Hartung 1976). The Baltic Sea is no exception regarding harmful consequences, as local mass deaths of oil-covered sea birds have been detected at the occasions of oil spills (Žydelis et al. 2006).

Investigations of the effects after local oil spills in the Baltic Sea have documented significant reductions in the marine macrofauna populations. In October 1977, 1000 tons of fuel oil was spilled in the Swedish archipelago, resulting in a drastic reduction of the macrofauna abundance in the oil-contaminated observation site, only 16 days following the spill. Species such as the amphipod *Pontoporeia sp*. and the polychaete *Harmothoe sarsi* vanished almost completely from the macrofauna samples. The crash in population densities was, however, recovered within the two following years (Elmgren 1983). Similar back and forth oscillations in the population densities of macrofauna have been documented by several other case studies regarding oil spills in the Baltic Sea (Bonsdorff 1981, Notini 1978). Population oscillations following an oil spill could be caused by mass deaths following a hydrocarbon exposure and thereby, small reproduction capacity of a reduced population or by a reduced reproduction capacity of the individuals that remain alive following an oil spill (Rousi & Kankaanpää 2012).

In addition to mass deaths, developmental and reproduction disturbances have been detected in the case studies of Baltic oil spills. For example, the production rate of abnormal eggs increased for the planktonic copepod *Monoporeia affinis* following a spill in the Swedish archipelago (Elmgren 1983). The abundance of abnormal herring (*Clupea harengus membras*) larvae also increased following a crude oil spill in 1987 on the Finnish coast where 5000-6000 tons of oil was released (Rahikainen et al. 2017).

Genotoxic effects of oil exposure have been documented, for example, in two oil spills at the Butinge oil terminal, Lithuania. In November 2001, more than 10 tons of oil was spilled in the sea at the Butinge terminal (Tracevskis & Bradley 2001). Micronuclei (MN) are small fragments of DNA which are abnormally found outside the nucleus in cells. The high occurrence of MN indicates a disturbed chromosomal division usually due to exposure to

genotoxic substances (Luzhna et al. 2013). In the Butinge terminal, the numbers of MN were 1.7-2.5 times higher two years after a spill in 2001 compared with an uncontaminated reference site (Baršienė et al. 2006). Increases in MN of *M. edulis* cells were also observed at the same site following an additional oil spill in February 2008 when 6.5 tons of crude oil was spilled (Suzdalev et al. 2014). The frequency of MN was 5.6 times higher than the reference value six months following the second oil spill, indicating quite long-lasting effects of a single spill at individuals' cellular level (Baršienė et al. 2012). Carcinogenic effects caused by the genotoxicity of crude oil have been suggested to be one of the major long-term effects of oil spills. The occurrence of carcinogenic effects in nature is challenging to monitor systematically and, thus, the idea remains hypothetical (Bonsdorff 1981, Elmgren 1983). Acute hydrocarbon exposures are not expected to create genetical differences between the individuals, since genotoxic effects are associated with chronic hydrocarbon exposures, lasting for months (Baršienė et al. 2006, Kreitsberg et al. 2010, Baršienė et al. 2012).

Oil spills have been shown to influence the metabolism and enzyme activity of animals living in the Baltic Sea. For example, increased excretion of hydrocarbon metabolites was documented in the harbor of Gothenburg, Sweden, when bile liquids of local eelpout (*Zoarces viviparus*) individuals were analyzed for two weeks following a 10-100 ton oil spill (Sturve et al. 2014). Living in brackish water demands metabolic adaptation of both saltwater- and freshwater-originating species and creates an underlying metabolic stress for the organisms living in the Baltic Sea. The release of crude oil-derived molecules in the water column is increasing the metabolic stress of the living organisms, since the organisms must detoxify many of the lipophilic substances penetrating the cell walls. Considering the high basal rates of metabolic stress in Baltic fauna, pollution-caused metabolic stress can, thus, be regarded especially challenging for organisms living in the Baltic Sea. An increase in the metabolic rate due to oil exposures following spills could reduce the energy available for other vital functions of organisms (Rousi & Kankaanpää 2012).

1.5 Toxicological effects of the Water-Soluble Fraction (WSF)

Effects of acute exposure to crude oil WSF have been investigated by several researchers in laboratory experiments using many different organisms. Acute exposures to the sub-lethal concentrations of hydrocarbons have been associated with the induction of many different physiological responses in aquatic organisms, such as decreased heart rate or arrhythmia

(Lipschitz et al. 1982), increased rate of opercular movements in fish (Thomas & Rice 1975), decreased swimming activity (Carls & Rice 1988) and even narcosis (Lari et al. 2016). Most of the responses listed above have also been documented to recover within 24 hours in a pollution-free environment (Thomas & Rice 1975, Lipschitz et al. 1982, Carls & Rice 1988, Lari et al. 2016).

Some damage following acute exposure to hydrocarbons can, however, persist throughout the life cycle of an organism. Life stages at an early age are considered the most vulnerable when individual long-term effects following hydrocarbon exposure are considered. Exposures to PAH compounds during embryonic development can cause severe physiological and neurological malformations (Hodson 2017) and decrease the growth rates of the exposed individual for a long time (Gilliers et al. 2012). Acute hydrocarbon exposure was, for example, observed to depress the growth rate of *Daphnia pulex* and to cause a decrease in the number of broods produced in a study by Geiger & Buikema (1982). These depressions were significant in the exposures of phenanthrene in 0.36 mg/ L. The phenanthrene-exposed animals also reached reproductive age approximately three days later than control animals. The reductions were assumed to be caused by a depression in metabolic activity. Similar results considering metabolic depressions after hydrocarbon exposure have also been observed in the study by Crider et al. (1982). In their study, *Daphnia magna* was exposed to stepwise increasing concentrations of a naphthalene solution. The exposure lasted 24 hours and the $\dot{M}O_2$ was reduced by half at concentrations over 8 mg/l.

Decreased metabolic rates following hydrocarbon exposures have been documented also in fish. Christiansen et al. (2010) investigated the effects of a crude oil WSF exposure on immature, approximately three-year-old, polar cod (*Boreogadus saida*). The concentration of PAH compounds during a 60 min exposure was 4.3 µg/ L and this type of acute exposure caused the mass-specific $\dot{M}O2$ to decrease by 58% when compared with control individuals.

The decreasing effects of a hydrocarbon WSF on metabolism have, however, not been observed in all studies. For example, Geiger & Buikema (1981) did not find any significant differences between the $\dot{M}O2$ of exposed and non-exposed *D. pulex* when they studied organisms that were exposed to varying concentrations of WSF. In a following experiment, the same authors (Geiger & Buikema 1982) observed a contrasting effect following lifelong chronic (< 0.6 mg/ L) naphthalene exposures. The prolonged exposure was documented to increase the amount of offspring produced. Organisms exposed to low naphthalene

concentrations lived longer and reproduced earlier than the other studied groups. In light of this observation, small enough concentrations of aromatic hydrocarbons were assumed to stimulate, not to depress, metabolism.

1.6 Aim of the study

The current study aimed to investigate the possible transgenerational effects of an acute (48 hours) crude oil-derived hydrocarbon exposure using the $\dot{M}O_2$ and reproduction success of *D. magna* as parameters. Acute exposures have been suggested to mimic natural exposure-time for water-living organisms following the events of oil spills (Lipschitz et al. 1982) since the hydrocarbons are naturally diluted within a short time due to evaporation and other weathering processes (Martínez-Jerónimo et al. 2003). The liquid containing the hydrocarbons used in the exposure was prepared by mixing oil with water to simulate a natural dissolution process resulting in a crude oil water-soluble fraction (WSF).

My interest was to investigate if individual changes, following an acute crude oil WSF exposure, could be passed further to the offspring of the affected individual. The $\dot{M}O_2$ and reproduction success were for this reason investigated by examining hydrocarbon-exposed individuals (F0) and their offspring in two generations (F1 and F2). Three populations were created for the study purpose: one population originated from heavily exposed (30%-V WSF) parents, another population originated from lightly exposed (10%-V WSF) parents and one from non-exposed parents used as controls.

The current idea, to investigate if transgenerational changes can be observed following an acute crude oil hydrocarbon exposure, is inspired by research where no traditional genetic changes are passed on between generations, rather the observed effects in offspring of exposed individuals are generated epigenetically (Vandegehuchte et al. 2014). *Daphnia magna* was selected as a model organism due to it being a well-studied cladoceran and a commonly used test subject in ecotoxicological (Adema 1978, OECD 2004) as well as epigenetic (Harris et al. 2011) research. Normally, *D. magna* reproduces asexually in laboratory circumstances (Kleiven et al. 1992). The asexual reproduction leads to the offspring being genetic copies of the parental organism (Harris et al. 2011). The genetic variation between a parthenogenetic *D. magna* and its clonal offspring has experimentally been proven to be very low by Dukić et al. (2019) who screened for genetic variation

between asexually generated clonal generations of *D. magna*. The assumption that genetic variation between individuals would manipulate the results of an experimental exposure, such as the one in question, can be reduced using one single *D. magna* to create a clonal population of model organisms to be used in the experiment.

The study hypothesized that the $\dot{M}O_2$ and the reproduction success of the parental populations would differ between the hydrocarbon-exposed groups and the non-hydrocarbon-exposed control group since the hydrocarbon agents of crude oil have been recognized as biohazards that alter the well-being of living organisms (Elmgren 1983, Carls & Rice 1988, Rousi & Kankaanpää 2012, Rahikainen et al. 2017). Also, it was expected that these differences might persist in the offspring of the differently treated parental populations because transgenerational effects have been detected in preceding studies of hazardous exposure (Vandegehuchte et al. 2014, Trijau 2018). The alternative that the changes would be indicated by other than direct significant differences in the means of measured parameters was also considered as a noteworthy alternative. For example, changes in variation without changes in mean have been recognized as important responses following environmental contamination (Nikinmaa & Anttila 2019).

Indications of transgenerational effects would mean that an acute hydrocarbon exposure following an oil spill can affect the well-being of marine organisms in a longer perspective than a single individual life cycle. This point of view is not often considered in risk assessments regarding Baltic Sea oil spills (Rousi & Kankaanpää 2012). Finding the evidence of transgenerational effects would also motivate further investigations of possible epigenetic changes following a crude oil hydrocarbon exposure, since epigenetic changes have already been associated with other PAH-exposure (Weis et al. 1998, Huang et al. 2016).

2 Materials and methods

2.1 Laboratory preparations and experimental animals

A stock culture of *D. magna* was received on 8th June 2017 from the Laboratory Centre for Ecotoxicology and Risk Assessment of the Finnish Environment Institute (SYKE) (Jyväskylä, Finland). After the arrival, the daphnids were cultivated at the University of Turku in a laboratory of the section of Physiology and Genetics. The cultivation was done in a 7.5L glass aquarium provided with an air stone and filled with the growth medium M7 (Appendix 1). The growth medium had been prepared following the instructions of the OECD Guideline for testing of chemicals (OECD 2004).

The photoperiod of the laboratory was fixed on 16 h light and 8 h dark. The light bulbs used were OSRAM Relax 15W 2700 K (Ledvance, Augsburg, Germany). The room temperature was controlled to be within the range of 18–22 °C by measuring the temperature of the M7 in the cultivation aquarium of the daphnids. The measurements were carried out using a handheld meter pHenomenal® OX4100H (VWR, Radnor, Pennsylvania, U.S.). The same meter was used to measure the oxygen saturation of the M7. The oxygen saturation was controlled to be above 86% of air saturation (Limits for normoxic conditions following Seidi et al. (2005)). The pH of the medium in the aquarium was monitored twice a week with a Tetra® pH test (Tetra Fish, Melle, Germany) and maintained within 7.0–7.5. The cleanness of the aquarium was controlled during the measurements and the aquarium was replaced when needed. The daphnids were fed by adding algal *Pseudokirchneriella subcapitata* (Appendix 2). The algae were added to the aquariums three times a week, so the M7 held a green color throughout the cultivation.

For creating a genetically similar lineage of daphnids, one full-grown individual was randomly chosen from the stock aquarium on 16th June 2017 and transferred into a separate aquarium where it could reproduce freely. The conditions regarding nutrition, light, pH, and temperature were adjusted as for the stock aquarium. All the individuals used in experiments originated from this aquarium, which had the clones of the chosen mother individual.

The animals in this experiment were always transferred carefully using clean Pasteur pipettes. daphnids were individually collected by sucking them up from the original location and releasing them into the desired location. On occasions, when a specific number of daphnids of specific age were replaced, the individuals were released from the pipette on a FALCON® 40 µm cell-strainer (Corning Inc., Corning, New York, U.S.) that was submerged in the growth medium in the location of placement. The filtering of growth medium along the study was also conducted by letting the M7 run through a FALCON® 40 µm cell-strainer into sterile beakers, repeatedly for three times before pouring the filtered medium to the desired location.

2.2 Pilot experiments

A series of pilot experiments were conducted to determine the most suitable design for the study of the transgenerational effects. The intention of the first pilot experiment (paragraph 2.2.1) was to verify the age that daphnids started to carry eggs in the present laboratory conditions to avoid exposing the eggs to the WSF. Exposing the eggs to hydrocarbons could affect the developing embryo negatively (Incardona et al. 2004) and, thus, skew the $\dot{M}O2$ or reproduction success in generation F1. However, it should be noted that avoiding the exposure of visible eggs is not ensuring that the individuals in the F1 generation were not exposed to hydrocarbons as primordial cells (Trijau et al. 2018) and therefore the F1 generation cannot be considered as strictly unexposed.

The second pilot was about determining a suitable age for the model organisms (paragraph 2.2.2). Newborn daphnids are sensitive and might suffer damage when transferred from an aquarium to another (Hoang & Klaine 2007) and, thus, a lower limit for handling daphnids was determined. The second pilot was conducted to avoid the situation where the dysfunction of the studied organisms could have been caused by the study process itself, rather than the exposure to hydrocarbons.

The purpose of the third pilot experiment was to monitor mortality and the $\dot{M}O_2$ of *D. magna* following exposure to different concentrations of a WSF derived from crude oil (paragraph 2.2.3). The ideal concentration would be non-lethal for the daphnids, but significantly affecting the $\dot{M}O_2$ so that a clear effect of the exposure was indicated.

2.2.1 Maturity for carrying eggs

The individuals for this experiment were obtained by transferring one parental individual to a 500 mL beaker where it reproduced for 24 h and was removed afterward, so no new individuals were born to the aquarium. The age of the born offspring was defined as 24 h when the parent was removed. Five individuals were selected from the brood for analyzing at which age the daphnids reach reproduction maturity. These individuals were studied during 24hour intervals until each individual had developed visible eggs and the individuals could be interpreted as mature for reproduction.

Individual offspring was studied by dropping the individual on a microscope slide glass with a Pasteur pipette and examine the individual with an Olympus SZ-CTV microscope (Olympus, Shinjuku, Tokyo, Japan), using magnification 2.0. After the examination, all the individuals were returned to the original beaker until another 24-hour interval had passed and the next examination began. The day when individuals started to carry eggs was noted and determined the endpoint of the observation period.

All the individuals displayed visible signs of maturation on the fifth day of the observations, since clear formation of eggs could be spotted in the posterior cavity of the body. The age of five days was, thus, defined as the absolute end of exposing the individuals to crude oil hydrocarbons.

2.2.2 Tolerance of experimental procedure-related stress

Two groups of different-aged daphnids were raised to examine differences in stress tolerance between age groups. The older group was generated 24 hours before the younger group by transferring one full-grown individual from the parent aquarium into an unoccupied 7.5L glass aquarium filled with M7 growth medium and algae. After the individual had been in the aquarium for 24 hours, all newborn daphnids were caught by filtering the M7 through a sterile cell strainer. The caught individuals were transferred into a 300 mL beaker filled with 200 mL of growth medium filtered from the aquarium where the offspring were born. The mother individual was transferred into a new aquarium and left there for another 24-hour period. The 24 hours younger clutch was similarly caught and transferred into a 300 mL beaker. Twenty individuals from each age group (24 hours and 48 hours) were then transferred into new beakers representing a simulated WSF solution, containing 250 mL fresh M7 medium and 250 mL filtered medium from the aquaria were the individuals originated from.

The transferred daphnids were left to acclimatize into the new environment for 48 hours (this corresponds to the time of WSF exposure in the experiment investigating transgenerational effects). After 48 hours the mortality in the different groups was documented and compared to find out possible sensitivity to transfer or environmental change-related stress. The number of dead individuals in each treatment was compared with the original number of living individuals (20) by division and this product represented the percentage of mortality in each treatment.

$$\frac{Number\ of\ dead\ individuals}{Original\ number\ of\ individuals} \times 100\% = Mortality\ percentage$$

The group of younger daphnids (24 h) was clearly found to be more sensitive to transfer and environmental change-related stress. The mortality percentage was 25% in the younger group, since five individuals were found dead after the treatment. Only one was found dead in the group of older daphnids (48 h), resulting in a 5% mortality percentage. Therefore, 48-hour-old individuals were used for further exposures. Considering these requirements, the age of the exposed daphnids would be below the age defined for maturation (Paragraph *2.2.1*).

2.2.3 Determination of suitable WSF exposure concentration

On the starting day of an experiment, a set of 20 full-grown individuals were transferred in a 7.5L aquarium filled with M7 and algae for reproduction. After 24 hours, the full-grown individuals were removed from the aquarium using a strainer. All newborn individuals in the aquarium were transferred back to the aquarium and left to grow for 24 h, before the beginning of the exposure.

The preparation of a 100%-V WSF liquid began two hours before the newborn individuals were separated from the parental individuals (see Appendix 3 for detailed preparations). Briefly, 100 g of crude oil (Russian Export Blend medium crude oil, Neste Oil, Naantali, Finland) were mixed with 900 mL of fresh M7 for 24 h, where after the water fraction was collected. The watery fraction was considered as 100%-V WSF. The test liquids used in exposures were prepared immediately after the 100%-V WSF liquid was collected. A fresh 100%-V WSF was prepared for each experimental set. The finalization of the test media lasted approximately 2 hours and the full 24 hours of the maturation period was completed during this time, resulting in 48-hour-aged individuals.

The test liquids were prepared from the 100%-V WSF in a fume hood into 1000 mL beakers by measuring and mixing assigned volumes of the freshly prepared WSF and M7 medium filtered from the stock aquarium. The control liquid was prepared like the WSF containing test liquids except that no 100%-V WSF liquid was added. Instead of adding WSF to the control treatment, a defined volume of fresh M7 medium was added to stimulate an environmental change corresponding to the replacement of M7 with WSF.

For the first set of pilot experiments, a control, a 50%-V, a 30%-V, and a 10%-V WSF liquid were prepared. For the second set of pilot experiments, a control, a 10%-V, 15%-V, and a 20%-V WSF liquid were prepared (Table 1).

Table 1 The assigned volumes of WSF and M7 medium used to create different concentrations of WSF to the exposure liquids. The final volume of each test-liquid was 500 mL. The entire M7 medium, except the fresh M7, used was filtered from the aquarium where the test organisms were stored before the start of the exposure. Fresh M7 was added to the control treatments to stimulate similar stress caused by the environmental change that was caused by the addition of the 100%-V WSF liquids to the exposure liquids.

Pilot experiment set	Percentage of WSF in liquid	WSF volume (mL)	Filtered M7 volume (mL)	Fresh M7 volume (mL)	Total volume (mL)
1	50%-V	250	250	-	500
1	30%-V	150	350	-	500
1	10%-V	50	450	-	500
1	Control (0%-V)	0	250	250	500
2	10%-V	50	450	-	500
2	15%-V	75	425	-	500
2	20%-V	100	400	-	500
2	Control (0%-V)	-	400	100	500

Clean air stones were added to each beaker with test liquids. The flow of air was fixed to a modest level so that only small bubbles were popping up to the surface of the test liquid, but no intense water movement was observed. Twenty daphnids (48 h) were transferred to each WSF exposure solution with strainers. The adding of daphnids to all the test liquids was performed within a 15-minuteperiod. The individuals were left to the beakers for a 48-hour exposure period in a fume hood where the light period was fixed as previously. The temperature in the hood was not manipulated but followed the room temperature. No pH adjustments were performed on the final test liquids and algae were not added to the test liquids during the exposure period.

After the exposures, the $\dot{M}O_2$ and mortality in each group were calculated. The $\dot{M}O_2$ was calculated using ten individuals from each treatment. The measurement was conducted with a Loligo® 24-channel optical fluorescence, oxygen-sensing microplate (Loligo systems, Viborg, Denmark) connected with sensor dish® reader (SDR) (Presens Precision Sensing

GmbH, Resenburg, Germany). The Loligo® microplate had 24x80 µL measuring chambers where individual daphnids could be placed to measure the individual $\dot{M}O_2$ within each chamber. The measurements of $\dot{M}O_2$ were performed in two lots, since the total number of the daphnids to be studied exceeded the number of the chambers available. In each measurement, a set of four chambers were left empty to control for any background effects on the $\dot{M}O_2$.

A volume of 85 µL of fresh oxygen saturated M7 was pipetted to each measurement chamber 20 minutes before the daphnids were placed in the chambers. The freshly prepared M7 had been saturated with oxygen in a batch of 30 mL by enclosing the liquid in a 50 mL falcon tube and shaking the tube vigorously for 2 minutes. After the shaking, the cap was released and the M7 was left standing within the tube until used.

The daphnids were rinsed from algae and the test liquid before placing them in the chambers by submerging the individuals twice in fresh M7. The rinsed individuals were transferred to Loligo® oxygen-sensing microplate so that one individual from each test solution was placed on the plate in alternative order so that individuals from each solution would spend about equal time on the plate before measurement (5 individuals per WSF treatment per measurement set). After transferring daphnids to chambers, the outlets of the chambers were sealed with approximately 0.7×0.7 cm square pieces of Parafilm M (Sigma-Aldrich, St. Louis, Missouri, US) with clean tweezers. No air bubbles were left between the Parafilm M and the M7 in the chambers. A silicon panel and a weight were carefully placed on the microplate and the measurement system was covered with black plastic bags before the measurement was started.

The measurements were done with SDR v4.0.0 software (PreSens, Resenbourg, Germany) and for the software temperature, air pressure, chamber volume and the desired measurement interval (for the first set of the experiment once per 3 minutes, but in all the rest of the measurements once per minute) were set for each measurement. The temperature of M7 was measured with HI 145 Digital Thermometer (Hanna Instruments, Woonsocket, Rhode Island, U.S.). The current air pressure was obtained from the Finnish Meteorological Institute by checking the local air pressure of the weather station nearest to the University of Turku (Artukainen, Link 1).

The $\dot{M}O_2$ was read until air saturation inside the chambers decreased to 50%-V. The daphnids were removed and the microplate was rinsed between the measurements first with hot tap

water, then with filtered milli-Q water and last with fresh M7. The plate was then dried by vigorous shaking. The next set of individuals were then measured as described above. A waiting time of 10 min was implemented before adding the daphnids.

The sample sizes of different groups were equal in the two sets of pilot experiments (10 individuals measured) except for the 15%-V WSF exposure were individual daphnids had adhered on the glass walls of the beaker. This disturbance caused the death of 11 individuals, therefore, the $\dot{M}O_2$ was measured only from nine individuals.

The remaining individuals alive in each beaker were counted after conducting the $\dot{M}O_2$ measurements. The mortality rate was calculated as before (paragraph 2.2.2).

The reported percentages for the 50%-V WSF exposure and the 30%-V WSF exposure originate from the first set of the pilot experiments and the reported percentages for the 20%-V WSF exposure originate from the second set of the pilot experiments. The 10%-V WSF exposure and the control treatment were performed in both experimental sets and the mortality percentages reported by the results are based on the average of these values (See Results, Paragraph 3.1). Due to a problem in the procession of the 15%-V WSF exposure, the percentages reported originate from a separately conducted mortality test that was accomplished after the two first sets of pilot experiments, but still following the same scheme.

Suitable concentrations of the WSF to be used in the actual WSF exposures were determined based on the mortality within the different concentrations of WSF. This choice was made since no statistically significant effects of the hydrocarbon exposures in the two sets of pilot experiments could be seen when comparing the $\dot{M}O_2$ of the exposed animals and the control treatment of each set (see Results, Paragraph 3.1.2).

The 10%-V WSF exposure was not observed to cause mortality in relation to the control treatment. Due to this, 10%-V WSF exposure was selected to represent a low-concentration exposure causing no mortality in the experiment investigating transgenerational effects. The 30%-V WSF exposure was chosen to represent a more harmful exposure, since this treatment caused up to 50% mortality in the pilot experiments. (Results 3.1, Figure 1).

The temperature during the measurement of the first experiment 06.07.2017 was 19.8 °C and air pressure 1013 hPa. The temperature during the second measurement 20.07.2017 was 20.2

°C and air pressure 1014 hPa. See Appendix 4 for the illustrated temperature curve throughout the experimental period of pilots and the main experiment.

2.3 Transgenerational effects of WSF exposure

All the procedures in final WSF exposures were done similarly as in pilot experiments. The WSF concentrations for exposures were 0%-V (control), 10%-V and 30%-V as determined in paragraph 2.2.3. The number of daphnids used in exposures is given in Table 2. Four batches of the 100%-V WSF solution were prepared for the final experiments following Appendix 3. Also, a chemical analysis of the exposure liquid was performed on a 30%-V WSF sample following Appendix 3. The exposures were conducted using 7.5L aquaria. The ratio of animals per volume of test medium was kept the same as in the pilot experiments (20 dapnids/500 mL of test medium) and the needed volumes of test media were counted using this ratio (Table 2).

Table 2 The volumes of 100%-V -WSF and M7 medium used to create a total volume of a 10%-V and a 30%-V WSF exposure medium are presented on separate rows. No WSF was added to the control treatment. Also, the number of daphnids designated for each treatment is presented on the uppermost row.

Treatment	**30%-V WSF exposure**	**10%-V WSF exposure**	**Control treatment 0%-V WSF**
Number of daphnids	90	50	50
Volume (L) of 100%-V WSF added	0.675L	0.125L	-
Volume (L) of filtered M7 with algae added	1.575	1.125	0.575
Volume (L) of fresh M7 added	-	-	0.675
Total test liquid volume	2.250	1.250	1.250

The aquaria were placed in a fume hood and clean air stones were added to each aquarium. The daphnids were transferred to each aquarium with a strainer. The start of the exposure was the point when all the aquariums had received age selected (48 h, like Paragraph 2.2.3) daphnids. The exposure period was 48 hour long.

2.3.1 Measuring the $\dot{M}O_2$ of the exposed F0 individuals

Measurement of individual $\dot{M}O_2$ of the exposed daphnids begun immediately after the exposure period had finished. The measurements were done as in the pilot experiment but using 21 individuals from each differently treated population. Therefore, the measurements were conducted in three sub-sets. During each measurement, the $\dot{M}O_2$ of seven individuals from each population was measured. Three measurement chambers were left empty to control for possible background effects on the $\dot{M}O_2$. The transferring of daphnids to the cambers was performed as previously.

The temperature during the measurement of the F0 generation 21.08.2017 was 21.4 °C and air pressure 1012 hPa. See Appendix 4, Figure 4.

2.3.2 Transferring F0 daphnids for further reproduction and collecting the offspring representing F1 generation

Twenty individuals were picked randomly from the remaining individuals in the exposure tanks and the control population. The chosen 20 individuals per population were transferred into three separate 900 mL glass beakers filled with fresh M7, provided with algae and air stones. The beakers were placed in the same fume hood where the exposures had been conducted. The individuals in the beakers were let to mature and reproduce for 11 days. The number of born offspring per day was recorded during this period.

The number of offspring was recorded by filtering the water from a beaker through a cell strainer to an additional 900 mL beaker and counting the number of newborn animals caught in the net. After all the individuals had been caught within the cell strainer it was dipped into a 300 mL glass beaker containing fresh M7 so that the caught daphnids were released into the liquid. The parental individuals were further transferred into the 900 mL beaker containing the filtered M7 from the maturation beaker. The offspring in the smaller beaker were discharged. This routine was repeated during the maturation period at 24-hour intervals.

The clutches born on the 11th day of the maturation period were caught and counted, but the offspring were not discharged before 50 individuals representing the offspring of each population were collected representing 24-hour-aged daphnids. The chosen offspring were transferred into three new 900 mL beakers provided with air stones, fresh M7 and algae and let to grow in these containers, representing generation F1. The parental organisms (F0) were discharged.

2.3.3 Measuring the $\dot{M}O_2$ of the F1 and F2 individuals and documenting their reproduction success

The newborn individuals in the beakers were let to grow an additional 72 hours in the beakers, beakers, so the age of 96 hours was reached by the F1 individuals. The age of 96 hours equaled the age when the $\dot{M}O_2$ of the exposed parental organisms in the F0 generation had been measured. Measurement of $\dot{M}O_2$ of 21 individuals per population was performed in three sub-sets on the F1 individuals as previously done for the parental F0 individuals.

Since the possible influence of the temperature on the $\dot{M}O_2$ had been noticed during the pilot experiment, temperature adjustments were done 12 hours before starting the measurement so that the temperature was fixed to what it had been during the measurement of the first generation. The temperature was fixed by heating the laboratory with an electric radiator and observing the temperature change with a handheld HI 145 Digital Thermometer (Hanna Instruments) from a 50 mL Falcon tube containing a fresh M7 medium.

The temperature during the F1 generation measurement 04.09.2017 was 21.8 °C and air pressure 1012 hPa. The temperature during the F2 generation measurement 19.09.2017 was 21.4 °C and air pressure 1010 hPa. See Appendix 4, Figure 4.

After the $\dot{M}O_2$ measurement was completed 20 remaining individuals from each beaker was further transferred into three new 900 mL glass beakers supplied as previously. Overlapping daphnids were discharged. Twenty individuals in each beaker could mature and reproduce for one day more than the previous generation since the needed clutch size was not reached on the 11th day. The number of born offspring was recorded following the same 11-day period and 24-hour interval as in the F0 generation.

Fifty individuals representing the second-generation offspring (F2) from each population were collected on the 12th day and transferred into three new 900 mL beakers and left to grow for 72 hours as previously. The excess organisms were discharged. The $\dot{M}O_2$ of 21 individuals from F2 generation per each beaker was recorded when these individuals had reached the age of 96 hours. Temperature adjustments were done similarly as in the F1 generation. After the measurements, 20 individuals from each beaker were further transferred into 900 mL maturation beakers for the reproduction success observation of F2. The reproduction was observed following the 24-hour interval for 11 days.

2.4 Mathematical treatment of the $\dot{M}O_2$

The SDR reader measures the amount of oxygen present in the Loligo® microplate chambers using an optical fluorescence oxygen sensing technology. A sensor measures the phase angles of light penetrating the microplate chambers with set intervals. The phase angle is dependent on oxygen saturation (Link 2). The phase angles for the M7 medium in the chambers were documented by SDR v4.0.0 (Presens Precision Sensing GmbH, Resenburg, Germany) software. The documented phase values at each measuring point were transformed into ṀO2 using the Microplate analysis tool v1.0.0 provided by Loligo® Systems.

The analysis tool is based on a Stern-Volmer (S-V) equation for defining oxygen concentration in percentages. In the equation, the phase angles of oxygen-free water are divided with the measured phase angle in the medium at a certain measuring point. The ratio of the phase angles is then used to count the oxygen content in the chamber at the measure point by dividing it with the S-V constant added with one (see equation below). The S-V constant is a value within the analysis tool possessed by Loligo®.

$$\frac{\tan\Phi_0}{\tan\Phi} = 1 + K_{SV} \cdot [O_2]$$

Φ_0	phase angle of oxygen-free water
Φ	measured phase angle
K_{SV}	Stern-Volmer constant
$[O_2]$	oxygen content in % air-saturation

The used phase angle of oxygen-free water was 54.45 °. This value was originating from a calibration dataset for liquids at 25 °C, received from Loligo®-software team.

The values for percent air saturation counted with the Stern-Volmer equation were plotted by time for each chamber. Only the values from 95% to 70% of air saturation were used in calculations. Values measured during the first 180 seconds, when the daphnids were in the chamber, were discharged from the analysis of the $\dot{M}O_2$ in the experiment where transgenerational effects were studied. A wait-time was not implied to the analysis of the $\dot{M}O_2$ of the pilot experiments. An $\dot{M}O_2$ slope, describing the individual oxygen consumption in each chamber by O_2/individual/min, was transposed by the analysis tool provided by Loligo® Systems. These slopes were used to compare individual ṀO2 between different groups and generations in the statistical analysis of data. Unsuccessful measure results were

excluded from the analysis and this reduced the sample size in some of the analyses (Appendix 4, Table 9).

2.5 Statistics

The analysis software IBM SPSS Statistics 23.0 (IBM Corporation, Armonk, New York, U. S.) was used in all the statistical treatments of the data. The data sets were always checked for homogeneity with Levene's test for variance and a Shapiro-Wilks test was used to check for a normal distribution within the groups within each data set.

If the p-values of the two tests listed above were non-significant (>0.05), the assumptions of homogeneity and normal distribution were not violated and a One-way Analysis of Variance (One-way ANOVA) was used to analyze differences between the $\dot{M}O2$ (picomoles/individual/ min) within the different populations. In cases where the analysis was showing a p-value <0.05, and expressing a significant difference between the populations, a post-hoc Tukey test was conducted.

In cases where the data set was not found homogenous or normally distributed the comparisons of the grouped values were completed with a nonparametric Kruskal-Wallis test. In cases where the nonparametric Kruskal-Wallis test was indicating a p-value <0.05, and so expressing a significant difference between the populations, a pairwise Kruskal-Wallis comparison was performed.

The differences in $\dot{M}O_2$ between the common treatment (the 10%-V -WSF) of pilot experiments were analyzed with a One-Way ANOVA and a Post Hoc Tukey-test. The populations (50%-V WSF, 30%-V WSF, 10%-V WSF and control) of the first set of pilot experiments were compared with each other and the populations (20%-V WSF, 15%-V WSF, 10%-V WSF and control) of the second set of pilot experiments were compared with each other. Both comparisons were performed using a One-Way ANOVA.

The results of the main experiment were compared in the inter-generational and transgenerational ways. In the inter-generational comparison, Independent Samples Kruskal-Wallis test was used to compare the different populations within each generation (each generation: F0, F1, and F2 tested separately). In the transgenerational comparison, the independent Samples Kruskal-Wallis test was used to compare the differences between

generations within each population (30%-V WSF, 10%-V WSF, and control tested separately).

The number of born daphnids per day was compared between the populations within each generation with Kruskal-Wallis (inter-generational comparison only). Different generations were not compared with each other.

3 Results

3.1 Pilot experiments

3.1.1 Comparison of the corresponding treatments in the two sets of pilots

According to Levene's test (F = 0.123 p = 0.730), the variances in the $\dot{M}O_2$ of daphnids were equal between both the first and second 10%-V WSF pilot experiments conducted. The normal distribution was also confirmed in both set values by a Shapiro-Wilks test p > 0.05.

The conducted One-Way ANOVA indicated that the $\dot{M}O_2$ significantly differed between the first and the second set of 10%-V WSF performed (F = 7.233, p = 0.015). The $\dot{M}O_2$ of daphnids of the second set was 65.3% higher in comparison with the daphnids from the first set of pilot experiments.

3.1.2 Comparing $\dot{M}O2$ between treatments in the first set of pilot experiments

The first set of pilot experiments included 50%-V, 30%-V,10%-V WSF exposure, and the control treatment where 50%-V of the filtered M7 was replaced with a fresh medium. The $\dot{M}O_2$ data within each treatment was normally distributed (Shapiro-Wilks p > 0.05) and variances were equal between the treatments (Levene's test F = 2.199, p = 0.105). One-Way ANOVA did not show significant differences (F = 2.532, p = 0.072) between $\dot{M}O2$ of individuals exposed to different concentrations of WSF or the control M7. The average $\dot{M}O_2$ recorded in the treatments are shown in figure 1, paragraph 3.2.5.

3.1.3 Comparing $\dot{M}O_2$ between the treatments in the second set of pilot experiments

The analyzed treatments of the second set of pilot experiments included the 20%-V, 15%-V, 10%-V WSF exposures, and a control treatment where 20%-V of the filtered M7 was replaced with fresh medium. The $\dot{M}O_2$ data was normally distributed (Shapiro-Wilks p > 0.05) in all other populations except the 15%-V WSF (Shapiro-Wilks F = 0.792 p = 0.019) and variances were equal between the populations (F = 1.817 p = 0.162). Independent-Samples Kruskal-Wallis Test did not show significant differences (F = 2.589, p = 0.460) between the $\dot{M}O_2$ of the individuals exposed to different consecrations of WSF control-

treated individuals. The averages of the recorded ṀO2 are shown in figure 1, paragraph 3.2.5.

3.1.4 Mortality observed in the different WSF exposures

The mortality percentages from each exposure concentration are shown in figure 1 together with the means of the recorded ṀO2. The highest mortality was observed in the 30%-V WSF exposure, where 50%-V of the exposed daphnids were dead after the 48-hour exposure period. The observed mortality pattern in both conducted 10%-V WSF exposures and the control treatments was the same. Mortality kept rising stepwise at higher WSF concentrations.

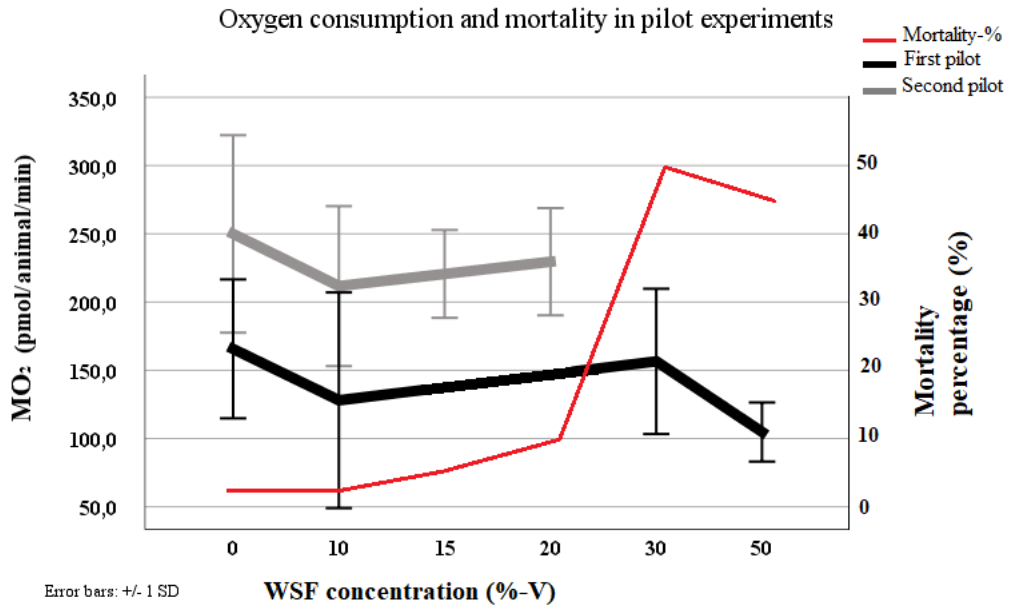

Figure 1 The average values of the ṀO2 rates in the first and second sets of pilot experiments are shown with the grey and black lines. The values of $\dot{M}O_2$ are shown on the left-side Y-axis. The mortality is presented by the red line and the mortality percentages are shown on the right-side Y-axis. WSF concentrations of each measurement point are given on the X-axis. N=10 per $\dot{M}O_2$ measurement, except the 15%-V WSF where the N=9. N=20 in observation of mortality.

3.2 Chemical analysis of the 30%-V WSF samples

The chemical analyses of the 30%-V WSF solution showed that the total amount of PAH-compounds decreased 93%-V from the sample that was immediately conserved to the sample that was conserved after the 48-hour evaporation period. The documented concentrations of PAH's can be seen in Table 3. Most of the singularly detected PAH-compounds had decreased below the detection levels in the latter conserved sample, only naphthalene being detectable as a separate PAH-compound.

Table 3 The measured contents of aromatic hydrocarbon compounds present in the prepared samples of a 30%-V WSF. The first column with numbers on the right side presents the contents in the immediately conserved sample and the second presents the contents in the sample that was conserved after a 48-hour evaporation period.

Aromatic hydrocarbons	Immediately conserved (µg/l)	After 48-h conserved (µg/l)
Naphthalene	25	1.7
Acenaphthylene	0.2	<0.1
Fluorene	0.5	<0.1
Phenanthrene	0.6	<0.1
PAH-compounds in total	27	1.9

A decreasing trend was observed for light hydrocarbons, but not seen in the case of heavy hydrocarbons in the comparison of the immediate and the later conserved sample. The later conserved sample showed higher contents of total hydrocarbons than the earlier conserved sample as seen in Table 4.

Table 4 Hydrocarbon compounds present in the prepared samples of a 30%-V WSF. The first column with numbers on the right side presents the contents in the immediately conserved sample and the second presents the contents in the sample that was conserved after a 48-hour evaporation period.

General hydrocarbons	Immediately conserved (mg/l)	After 48-h conserved (mg/l)
Light hydrocarbons (C5-C10)	3.2	2.6
Middle heavy hydrocarbons (C10-C21)	0.25	0.95
Heavy hydrocarbons (C21-C40)	0.11	1.2
Total hydrocarbons (C5-C40)	3.6	4.7

3.3 Results of the experiment investigating transgenerational effects

3.3.1 Intergenerational comparisons

The variances in the $\dot{M}O_2$ of the exposed and control individuals within the F0 generation were found unequal by Levene's Test for Homogeneity of variances ($F = 5.332$, $p = 0.008$). The $\dot{M}O_2$ within each group (control, 10%-V WSF and 30%-V WSF) was found normally distributed using Shapiro-Wilks ($p > 0.05$). Independent-Samples Kruskal-Wallis Test indicated no significant differences in the $\dot{M}O_2$ data between the different groups in the parental generations ($H = 0.672$, $p = 0.715$).

Variances in the $\dot{M}O_2$ of the individuals from the first filial generation (F1 generation) were found equal by Levene's Test for Homogeneity of variances ($F = 2.726$, $p = 0.072$). The $\dot{M}O_2$ within the populations of individuals originating from parents exposed to 10%-V WSF or 30%-V WSF was found normally distributed (Shapiro-Wilks $p > 0.05$), but the $\dot{M}O_2$ of individuals originating from the control-population was not normally distributed in Shapiro-Wilks, $p = 0.000$. Significant differences in the $\dot{M}O_2$were seen in the offspring originating from differently treated parental populations (Independent-Samples Kruskal-Wallis Test $H = 27.927$, $p = 0.000$). The F1 offspring of the population that was exposed to 30%-V WSF had 48.1% (Pairwise comparison Kruskal-Wallis $H = -25.3$ $p = 0.000$) higher $\dot{M}O_2$ than the offspring of the control individuals. The F1 offspring of the 30%-V WSF exposed population had also 61.3% higher (Pairwise comparison Kruskal-Wallis $H = -25.250$ $p = 0.000$) $\dot{M}O_2$ than the F1 offspring of the 10%-V WSF exposed population. Any significant differences were not indicated between the F1 offspring of the control population and the 10%-V WSF-treated population. The results are visualized in figure 2 in paragraph 3.3.2.

The variances within the second filial generation (F2) $\dot{M}O_2$ data were found equal by Levene's Test for Homogeneity of variances ($F = 2.152$, $p = 0.125$). The $\dot{M}O2$ of the F2 offspring originating from the exposed populations (10%-V and 30%-V WSF) were normally distributed (Shapiro-Wilk's test $p > 0.05$), but the individuals originating from the control population did not show normally distributed values of $\dot{M}O_2$ (Shapiro-Wilk's test $W = 0.889$, $p = 0.022$). Significant differences, between the F2 offspring with different origins, were pointed out by an Independent-Samples Kruskal-Wallis Test ($H = 20.639$, $p = 0.000$). The lowest mean of the $\dot{M}O_2$ regarding the F2 generation was recorded for the offspring originating from the 10%-V WSF exposed population, as seen in figure 2.

Individuals of the F2 generation that originated from the control population (Pairwise comparison Kruskal-Wallis H =15.000 p = 0.024) had a significantly (29.3%) higher $\dot{M}O_2$ than the F2 offspring of daphnids exposed to 10%-V WSF. The F2 offspring of the population exposed to 30%-V WSF (Pairwise comparison Kruskal-Wallis H = -25.571 p =0.000) had a 57.3% higher $\dot{M}O_2$ than the F2 offspring of daphnids exposed to 10%-V WSF. No significant difference was found between the F2 offspring of the control population and the F2 offspring of the 30%-V WSF exposed population (Pairwise comparison Kruskal-Wallis H = -10.571 p =0.185). Specific $\dot{M}O_2$ means and standard deviations for each generation within the population are given in Appendix 4, Table 9.

3.3.2 Transgenerational comparisons

Examining the population exposed to a 30%-V WSF, the variances of the $\dot{M}O_2$ in the parental generation (F0) were found equal with the two filial generations (F1 & F2) when tested with Levene's Test for Homogeneity of variances (F = 0.656, p = 0.523). The values within each generation were found normally distributed (Shapiro-Wilks p >0.05). There were no significant differences in the $\dot{M}O_2$ between the generations representing the population generated from the parents exposed to a 30%-V WSF when the generations were compared with an Independent-Samples Kruskal-Wallis Test (H = 3.537, p = 0.171).

The different generations presenting the population originating from the parents exposed to a 10%-V WSF did not show equal variances in the $\dot{M}O_2$ between generations (Levene's Test for Homogeneity F = 17.598, p = 0.000). The values within each generation were found normally distributed (Shapiro-Wilk's p > 0.05). Significant differences between the different generations in the population were indicated by an Independent-Samples Kruskal-Wallis Test (H = 11.290, p = 0.004). Kruskal-Wallis pairwise comparisons indicated significant differences between the F1 and F0 generation (H = 13.856 p = 0.039). The F0 had 44.1% higher $\dot{M}O_2$ than the F1 generation. Significant differences between the F2 and F0 generation were also indicated by the Kruskal-Wallis pairwise comparison (H = 17.865 p = 0.004). The F0 had 53.2% higher $\dot{M}O_2$ than the F2 generation. No significant differences were indicated between the F1 and F2 generation representing the population originating from the parents exposed to a 10%-V WSF, as seen in figure 2.

The $\dot{M}O_2$ data from the control population was not found to have equal variances in $\dot{M}O_2$ of different generations when tested with Levene's Test for Homogeneity of variances (F = 8.027, p = 0.001). The values within the populations were found normally distributed in the

F0 generation (Shapiro-Wilk's test p >0.05), but not in the F1 generation (W = 0.957 p= 0.489) or the F2 generation (W = 0.889 p = 0.022). Independent-Samples Kruskal-Wallis Test indicated significant differences between the generations (H = 7.408, p = 0.025). Pairwise comparisons gave a significant difference in the $\dot{M}O_2$ (H = 15.050 p = 0.022) between the F0 generation and the F1 generation, F0 having 34.4% higher $\dot{M}O_2$. No significant differences were found between F0 and F2 generation or F1 and F2 representing the offspring of unexposed controls.

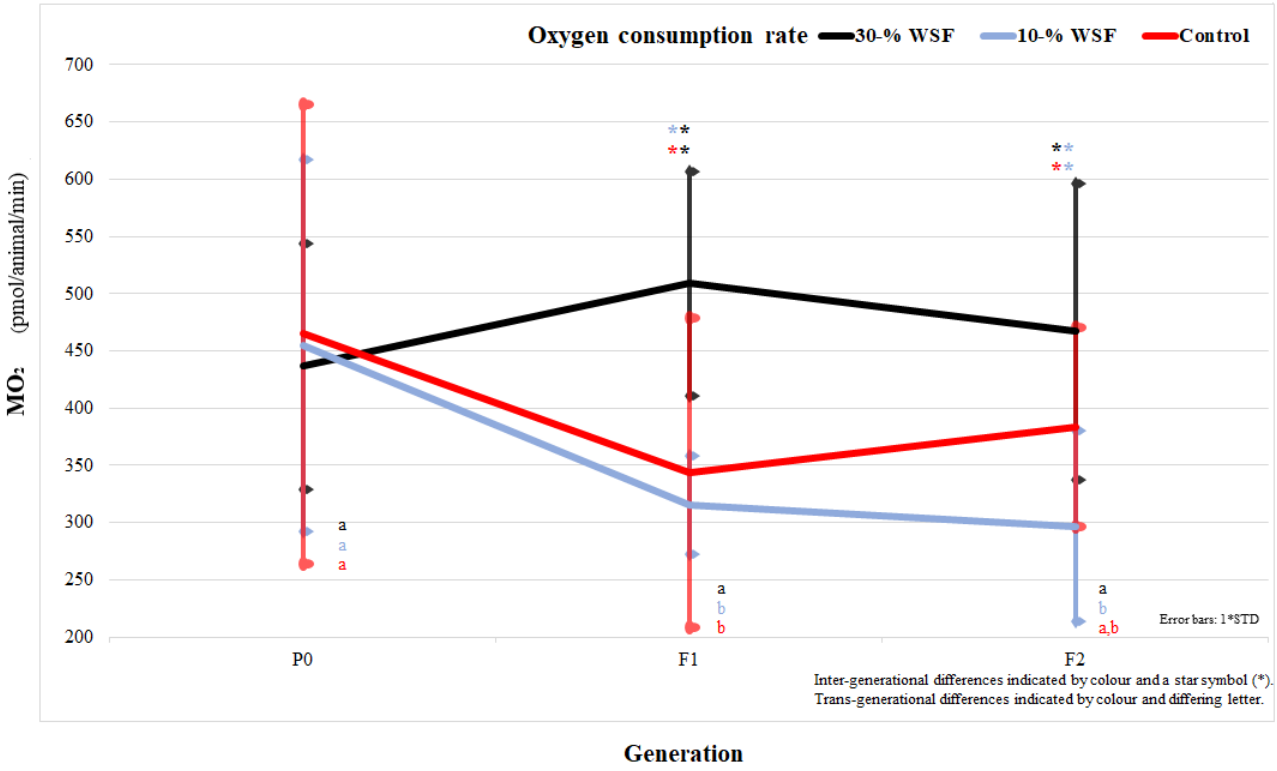

Figure 2 The mean and the SD of $\dot{M}O_2$ (Y-axis) for each generation (X-axis). The different populations are represented by different colored lines. Star symbols (**) at the top of the figure indicate significant differences between the populations within the generation in question. The color of the stars indicates between which populations the differences are found. Ex. A blue star next to a black star at the point of generation F1 indicates that there is a significant difference in the $\dot{M}O_2$ of the individuals originating from the 10-% WSF-treated population and the individuals originating from the 30-% WSF-treated population. Letters at the foot of the figure stand for differences between the generations within each population. The color of each letter indicates which population it is presenting and a change of letter between generations means that there is a significant difference in the $\dot{M}O_2$ between these generations. If a generation is presented by two letters, it is indicating that the generation is similar to both generations presented by the letters. Ex. The $\dot{M}O_2$ of the F2 generation (a, b) in the Control-population is statistically resembling both F1 (b) and F0 (a), but the $\dot{M}O_2$ of the F1 and F2 significantly differ from each other, since represented by different letters. N=21 at each point of $\dot{M}O_2$ measurement.

3. 4 Reproduction success

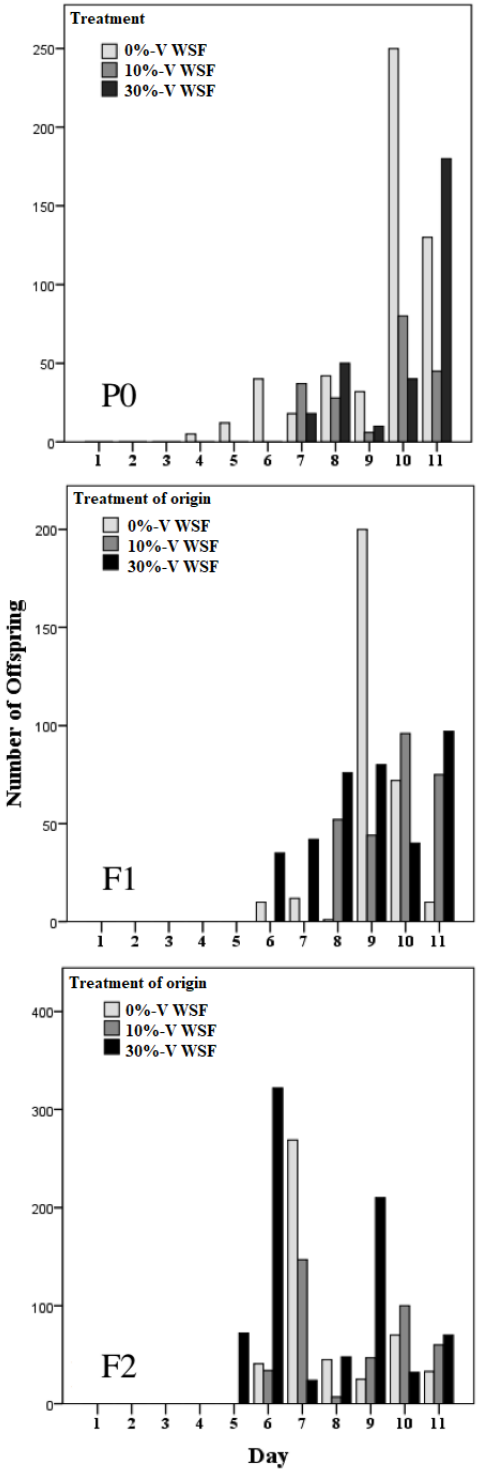

Figure 3 The number of offspring (Y-axis) borne per day (X-axis) for each population during an 11-day observation period. The different generations P0, F1, and F2 are included in different boxes and different populations are represented by different colored staples.

Trends of reproduction success can be seen from Figure 3, where the bars are representing numbers of offspring at each day throughout the 11-day observation period. Without including statistical evidence, it can be seen in the diagram representing the P0 generation that the WSF exposed organisms showed a three-day delay in reproduction. The WSF exposed individuals of both treatments in the generation P0 gave birth to offspring first on day 7 when the control group of the P0 generation reproduced on day 4 of the observation period.

The clutch sizes were not even throughout the 11-day observation periods but varied from zero up to the highest recorded clutch consisting of 322 individuals (generation F2, the heavily exposed population). The largest clutch sizes occurred especially in the latter days of the observation period in all the generations and populations. Only one exception can be observed in this trend when studying Figure 3: the F2 generation of the population originating from the heavily exposed population had the largest clutch born already on day six. Also, the largest clutches were born on day 7 for the control population and the lightly exposed population. Larger clutches were not observed at later days during the observation period in the F2 generation.

In the control population, the mean value for the number of offspring per day in the F0 generation was 48 (SD. 76.7) and the total number of offspring was 529. The individuals exposed to a 10%-V WSF liquid had 196 offspring in total. The mean value for the number of offspring per day was 18 (SD. 26.7). The individuals exposed to a 30%-V WSF liquid had a total number of 298 born offspring. The mean of born offspring was 27 per day (SD. 53.71).

Considering the clutch sizes of different populations in the F0 generation the variance in clutch size appeared equal (Levene's test for homogeneity $F = 0.91$ $p = 0.$). The clutch sizes were not found within normal distribution (Shapiro-Wilk's test of normality $p < 0.05$ for each population). A Kruskal-Wallis Test ($H = 1.60$, $p = 0.22$) indicated no significant differences in the clutch sizes between the differently treated population in the F0.

The offspring of the 30%-V WSF exposed individuals and the control individuals in the F1 generation both gave births to offspring on day 6. The offspring of the 10%-V WSF exposed individuals gave birth to their first clutch two days later. The mean value for the number of offspring born per day in the population originating from the 10%-V WSF exposed founders was 24 (SD. 36.6) and the total number of born offspring was 267. The mean value for the number of offspring born per day in the population originating from the 30%-V WSF exposed founders was 34 (SD. 37.1) and the total number of born offspring was 370. The mean of born offspring per day was 28 (SD. 60.9) for the individuals originating from the control population and the total number of born offspring was 295.

Variance in clutch size appeared equal in the F1 generation (Levene's test for homogeneity of variances $F = 0.316$ $p = 0.731$). Clutch sizes were not normally distributed in any group (Shapiro-Wilk's test of normality $p < 0.05$). A Kruskal-Wallis Test ($H = 0.73$, $p = 0.69$) indicated no differences in the clutch sizes between the different populations in the F1 generation.

The individuals in the F2 generation broke the earlier trends so that the individuals from the exposed populations reproduced before the individuals in the control population. The population originating from individuals exposed to a 30%-V WSF liquid started to reproduce at day five, whereas the control population started to reproduce at day six, simultaneously with the population originating from individuals exposed to the 10%-V WSF. The mean value for the number of offspring born per day in the population originating from the 10%-V WSF exposed founders was 36 (SD. 49.4) and the total number of born offspring was 395. The mean value for the number of offspring born per day in the population originating from

the 30%-V WSF exposed founders was 71 (SD. 103.3) and the total number of born offspring was 778. In the control population, the mean of born offspring per day was 44 (SD. 78.4) and the total number of born offspring was 395.

Variance in clutch size appeared equal (Levene's test for homogeneity of variances $F = 1.01$ $p = 0.38$). Clutch sizes per day were not within normal distribution (Shapiro-Wilk's test of normality $p < 0.05$) and the Kruskal-Wallis Test ($H = 1.66$, $p = 0.44$) indicated no differences in the clutch sizes between the different populations in the F2 generation.

4 Discussion

The current study provides an interesting viewpoint to transgenerational effects caused by acute and heavy hydrocarbon exposure, an event that can follow marine oil spills (Rousi & Kankaanpää 2012). The main result of this thesis was that the $\dot{M}O_2$ in the population originating from the heavily exposed F0 generation (30%-V WSF) did not change throughout generations in contrast to the two other populations:10%-V WSF and control. Generally, the $\dot{M}O_2$ was lower in the F1 and F2 generations compared with the parental generation (F0) in the two other populations that demonstrated differences between generations within each population. The variances in $\dot{M}O_2$ were also equal in the F0, F1, and F2 generations of the heavily exposed population. Contrastingly, the variances were not equal between the different generations in the lightly exposed population or the generations in the control population indicating that the heavy hydrocarbon exposure caused the reduction of variation in the $\dot{M}O_2$ throughout the generations that followed.

The stability of the $\dot{M}O_2$ throughout generations and the low variances of individual $\dot{M}O_2$ documented in the F0 generation of the heavily exposed population could indicate that a heavy hydrocarbon exposure can cause the deterioration of the ability of an individual to change its $\dot{M}O_2$ in a changing environment. This defect is possibly carried over generations since the indication of a stable $\dot{M}O_2$ could be seen in the later generations of offspring. A perspective like this is often forgotten in the risk assessments of oil spills (Elmgren 1983, Rousi & Kankaanpää 2012, BRISK 2013, Nikinmaa & Anttila 2018) and makes the current results very important. The possible explanation behind this phenomenon will be discussed on a hypothetical level later in the discussion.

The trend of $\dot{M}O_2$ stability throughout generations in the heavily exposed population was not reflected on the reproduction success, since the clutch sizes during the observation period displayed equal variances in all the populations at every generation. Also, no significant differences were documented in the clutch sizes between the different populations in any of the generations, indicating that all the populations performed similarly in reproduction.

4.1 The chemical components in the WSF

As described in the introduction, the dissolving of molecular components from crude oil in the water column depends greatly on environmental conditions as well as the amount and type of oil released (Wernersson et al. 2004). Due to this, it is difficult to directly compare the chemical composition of the water column following an actual spill with the created WSF that was used in the current study. However, the PAH concentrations in the 30%-V WSF exposure seemed to lie within the range of PAH concentrations that have been documented following previous marine oil spills. The PAH concentration of the immediately conserved 30%-V WSF was found to be 27µg/l when again the concentration of corresponding molecules named "Polycyclic Aromatic Compounds" (PAC) varied between 0.641 and12.833µg /l at different sites in parts of the Danish coast where 2400 tons of heavy bunker oil was spilled (Pécseli et al. 2003). Oil spills of this scale have been estimated to occur in the Baltic Sea in intervals of four years (Brusendorff et al. 2012). The PAH concentrations of the immediately conserved WSF seem somewhat higher than the PAC values documented by Pécseli et al. (2003), but the difference is not presumably very dramatic. Thus, it is known that a significant fraction of the water-diluted hydrocarbons usually evaporates within a short time following the release of oil into the water column (Albers 1995). The quick reduction of PAH compounds is seen in the sample that was left to evaporate for 48 hours: only 1.9µg of PAHs in one liter was present in the evaporated sample. If the analyzed sample of WSF had not been conserved immediately, it would probably have reached exactly corresponding values with Pécseli et al. (2003) following a couple of hours of evaporation.

The total hydrocarbon concentration in the created 30%-V WSF was high (3.6 mg/l), but only PAHs in crude oil are generally considered as directly harmful components for living organisms (Sikkema et al. 1995) and, thus, the effects of a crude oil hydrocarbon exposure are associated specifically with the PAH concentration during the exposure. In the following part, the major PAHs found in the WSF will be discussed due to their importance as disruptive chemicals. Even small acute concentrations of PAHs have been noted to result in biological uptake. For example, Corner et al. (1976) exposed adult copepod *Calanus helgolandicus* for 24 hours to 10µg/l of naphthalene and observed a mean uptake of 3.6 pg of naphthalene in the tissue of each exposed copepod. When considering the possibility of biological uptake, it should also be noted that the algal *P. subcapitata* was also present in the

exposure tanks during the exposure period. It is most likely that the algae did perform biological uptake of the harmful hydrocarbons during the highest peak of concentration and,_ thus,_ created an additional intake path of hydrocarbons to the *D. magna* in the tanks. Even though most of the water-diluted PAHs had evaporated from the later WSF sample, *D. magna* possibly took up PAHs in meaningful concentrations throughout the 48-hour period while consuming the *P. subcapitata*. Regarding these factors, it is assumed that significant uptake of PAHs, by exposing *D. magna* to WSF of crude oil, most likely was happening even though the concentrations of PAHs were mild during the 48-hour exposure period of the parental generation.

In the current exposure, the concentration of single PAHs was lower than what has been reported in studies where the harmful effects of a hydrocarbon exposure have been documented previously (Crider et al. 1982, Geiger & Buikema 1982, Schirmer et al. 1998). However, the effects of a hydrocarbon exposure should most likely not be associated with any single PAH present in the WSF, since the effects are more likely a matter of a synergistic action of all the components in the WSF. This assumption is based on results where effects following exposure to a single PAH have been documented to be milder than effects following exposure to a mixture of hydrocarbons. For example, Moles & Rice (1983) noted that 40-day chronic exposures to pure naphthalene (<0.87mg/l) caused significantly milder reductions in the growth rate of juvenile pink salmon (*Oncorhynchus gorbus*) than the corresponding concentrations of mixed hydrocarbons from crude oil WSF. Berdugo et al. (1977) derived a WSF from aromatic heating oil and pure naphthalene. Naphthalene alone did not cause any mortality at concentrations 1-2mg/l following a 24-hour exposure of the copepod *Eurytemora affinis*, but 100% of the test organisms died within six hours following an exposure to WSF of heating oil where the naphthalene concentration was between 252 and420µg/l.

In the created WSF, 92% of the identified PAHs were naphthalene. The concentration of naphthalene was 25µg/l at the starting point of the experiment and decreasing to 1.7µg/l during the 48-hour period. Phenanthrene, fluorene, and acenaphthylene were also found in the sample conserved at the starting point, but all of them in lower concentrations than 1µg/l. The PAHs in question are all light molecules containing two to three benzene molecules. The presence of these compounds could be expected, since light PAHs are commonly found in

crude oil (NRC 2003). These light PAHs are also regarded as more water-soluble than the heavier PAHs containing more benzene units (Albers 1995).

Naphthalene is formed by two combined benzene units without any substituents (Sikkema et al. 1995). Studies using bacterial cells have shown that naphthalene can penetrate the cell membrane passively and cause cellular malfunction following the uptake (Bateman et al. 1986). Naphthalene is well bioavailable in aquatic environments due to being relatively water-soluble (Albers 1995). This also explains the high occurrence of naphthalene in the created WSF. Significant effects of naphthalene on the $\dot{M}O_2$ have been seen in previous research, but only when the exposure concentrations of naphthalene have been at least 40 times higher than in the current experiment. Darville & Wilhm (1983) noted a trend of significant decrease in the $\dot{M}O_2$ of the *Tanytarsus dissimilis* and *Chironomus attenuates* when the organisms were exposed stepwise to rising concentrations (1- 12mg/l) of naphthalene during one hour per each exposure concentration. Lower concentrations than 1 mg/l (24 h) of naphthalene were not found to cause any significant effects on the $\dot{M}O_2$ of *D. magna* (Crider et al. 1982). The low concentrations of naphthalene could, thus, explain that no differences in the ṀO2 of *D. magna* were discovered between differently treated individuals in the F0 generation.

Phenanthrene is built from three benzene units with no subunits (Hawkins et al. 2002). Phenanthrene can accumulate in fish tissues and disrupt of the cell membrane that leads to apoptosis (Sikkema et al. 1995). Abnormal mitochondrial functioning has also been identified to be one of the mechanisms behind phenanthrene-caused cell death. A significant decrease in mitochondrial membrane potential was accompanied by a significant amount of cell death following a 48-hour exposure (53.5mg/l) to phenanthrene in adult Chinese rare minnows (*Gobiocypris rarus*) (Hong et al. 2017). Mitochondrial dysfunction would probably lead to lowered $\dot{M}O_2$, but to achieve this effect the concentration of phenanthrene would need to be considerably higher than the concentration of phenanthrene in the currently created WSF. Phenanthrene exposure has also been associated with decreased developmental rates and reproduction rates in experiments where *Daphnia pulex* has been exposed to concentrations of 0.11 mg/l and above of phenanthrene during lifelong exposure (Geiger & Buikema 1982).

Fluorene is a three-cyclic compound where two outermost cyclic structures are benzene rings and the one in the middle is a cycloalkane (NCBI 2018a). Acenaphthylene is a molecule that is built up by a naphthalene unit combined with a cycloalkane structure on one of the

horizontal sides of the naphthalene unit (NCBI 2018b). Exposure to both fluorene and acenaphthylene has been shown to cause cell membrane perturbation when rainbow trout (*Oncorhynchus mykiss*) gill-cells have been cultivated together with these contaminants for only two hours at concentrations below 100µmol/l (Schirmer et al. 1998). These concentrations correspond to approximately 16.6 mg/l for fluorene and 15.2 mg/l for acenaphthylene and, thus, represent significantly higher concentrations of the substances than what were present in the currently used WSF.

Part of the toxicity of PAHs is due to reactive metabolic products which cause cellular damage. Metabolic processing of fluorene (Kopecka-Pilarczyk 2009), acenaphthylene (Shimada et al. 2015), naphthalene (Brausch & Smith 2009) and phenanthrene (Yin et al. 2007) has been associated with up-regulated expressions of enzymes from the family cytochrome P450. The P450 enzymes catalyze the detoxification processes of xenobiotics by oxidizing the compounds. Oxidized PAHs can form reactive molecules that disrupt the structure and function of essential proteins of the cell (O'Brien 1991) or give rise to reactive oxygen species (ROS) that can disrupt biomolecules within the cell (Hawkins et al. 2002). Shi et al. (2005) observed membrane disruption due to the lipid peroxidation of the cell membrane fatty acids with a 150% increase in ROS prevalence in goldfish (*Carassius auratus*) following a 24-hour exposure to naphthalene 0.5mg/l. ROS formation following a phenanthrene exposure was also indicated in a study by Yin et al. (2007) by increasing the rates of oxidative stress on the liver of the goldfish after a 4-day exposure to 50µg/l. Enzymes belonging to the P450 family are recognized from nearly all domains of life, also daphnids (Plopper et al. 2001, Brausch & Smith 2009). Thus, it is likely that harmful metabolites did form in the metabolism of the model organism used in the current study and that levels of oxidative stress would have been a suitable additional parameter to measure when considering the effects of the WSF exposure in the F0 generation.

4.2 Effects seen in the F0 generation

The WSF exposures were not observed to directly shift the $\dot{M}O_2$ of the exposed individuals in the pilot experiments nor the main experiment as expected. The lack of the significant differences in the $\dot{M}O_2$s of the differently treated populations is probably due to this parameter being affected by many other factors in addition to the exposure to oil (Clarke & Fraser 2004) and a more perfectly defined environment would have been needed to study the

direct effects of a WSF exposure on the $\dot{M}O_2$. However, the variances of $\dot{M}O_2$ by the differently treated populations in the F0 generation were unequal and the heavily exposed population displayed the lowest standard deviation of $\dot{M}O_2$. This reduced variance is thought to be a direct indicator of the effects of the heavy hydrocarbon exposure in the 30%-V WSF exposed population.

Another directly seen consequence caused by the WSF exposure, in the pilot experiments and the main experiment, is the increasing mortality following the increasing concentrations of WSF. Also, a noted difference between the populations in the F0 generation was that both the lightly and heavily exposed populations gave birth to their first brood three days later than the control population. The delayed birth-giving indicated that the WSF exposure affected the individuals by slowing down their sexual maturation. The delay of reproduction could be expected based on previous experiments where *Daphnia pulex* had been exposed to phenanthrene (Geiger & Buikema 1982). Exposure to PAHs has also been associated with hormone-disrupting functions in a study where crayfish (*Procambrus clarkia*) had been exposed to naphthalene (10 mg/l) for 48 hours and reduced ovarian development demonstrated (Sarojini et al. 1994).

Also, when discussing the lack of the significant differences of the WSF exposure on the $\dot{M}O_2$ of the exposed individuals at the parental generations it should be noted that only the exposure-surviving individuals were included in the $\dot{M}O_2$ measurements and possible shifts of $\dot{M}O_2$ could have been present in the individuals lost before the measurement.

4.3 Transgenerational effects in the F1 and F2

Based on the current results, it can be suggested that constancy of $\dot{M}O_2$ throughout generations was generated by the heavy WSF exposure. This is demonstrated by the insignificant differences of the $\dot{M}O_2$ between the generations within the heavily exposed population and by the fact that the heavily exposed population was the only population in which equality of variances was documented between generations using Levene's test for homogeneity of variances. Uniformity like this was not documented in the two other populations where significant differences in $\dot{M}O_2$ were noted between different generations within a population and equal variances were not present. Also, the F1 generation of the heavily exposed population presented significantly higher $\dot{M}O_2$ than either the control or the lightly exposed populations, simultaneously when differences between the F0 generation and

the F1 generation within the heavily exposed population were insignificant. Both additional populations had significantly lower $\dot{M}O_2$ in the F1 generation compared with the F0 generation but, for some reason, the heavily exposed population did not follow the same patterns of change throughout generations as the two additional populations.

No similar trends were seen in the reproduction success of the filial generations and no delays in the day for the birth of the first clutches was documented for the offspring of exposed populations indicating that reproductive disturbances demand direct exposure and are not passed over to filial generations.

In light of these results, it can be suggested that exposure to high enough concentrations of the crude oil WSF could affect the exposed organisms as well as their offspring by causing metabolic stability in the population originating from heavily exposed individuals. Since the light WSF exposure did not show a similar trend of stability in the parental generation nor following generations, it is assumed that a threshold of WSF concentrations should be exceeded before such transgenerational stability can be seen.

Even if the heavily exposed population presented significantly higher $\dot{M}O_2$ in the F1 generation compared with both additional populations, these significant differences were not present in the $\dot{M}O_2$ between the heavily exposed population and the control population any more in the F2 generation. The standard deviation of the heavily exposed population was also higher than what the standard deviation was in the two additional populations in the F2 generation. These results, thus, indicate that the transgenerational effects seen as metabolic stability disappeared after the first two generations had passed and that the transgenerational effects caused by a heavy hydrocarbon exposure could be dissipating within a certain recovery time. In future research, it would be interesting to study the effects of a similar exposure on even later filial generations (F3, F4, and F5). A study including additional generations, would show whether the reduced variation would be continuous in later filial generations F3, F4, and F5.

4.4 Reasons for the seen transgenerational effects?

The current study was performed to examine whether transgenerational effects following a crude oil hydrocarbon exposure could be found, but explanations for the documented phenomena can unfortunately not be provided by the current results. Considering contemporary biological thinking, the most likely explanation is that the heavily exposed individuals decreased the variation of $\dot{M}O_2$ in response to the hydrocarbon exposure. This assumption is based on the conception that genetically similar individuals, such as asexually produced clones, can display different phenotypes after being exposed to different environmental conditions (Pigliucci 2006). Even if the used model organism is a clone, it has been proved that individual *D. magna* can have high phenotypic plasticity (Sakwińska 1998) and, thus, differences in the $\dot{M}O_2$ could be assumed to occur naturally following environmental change. Natural plasticity of the $\dot{M}O_2$ is also indicated by the unequal variances of the $\dot{M}O_2$ and values not following normal distribution within the control and the lightly exposed populations. The lightly exposed and the control population showed higher standard deviation in the $\dot{M}O_2$ of the parental generation indicating that no factors reducing $\dot{M}O_2$ variation were present in the treatments of the parental individuals.

Phenotypic alternations of offspring due to parental exposure have also been seen in previous studies of clonal model organisms. Brausch & Smith (2009), for example, created naphthalene resistant offspring in *D. magna* by exposing <24-hour-aged individuals for 48 hours, generation after generation, to higher and higher concentrations of naphthalene. The LC50 of the first generation was 7.8µg/l, but during the study, the LC50 of the twelfth generation became three times higher. Already the fourth generation displayed significantly increased naphthalene tolerance due to the parental exposure to the PAH. The induced resistance was explained by increased P450-enzymatic activity and supports the idea of offspring changing their metabolic appearance following parental exposure to PAHs. The current results can also be seen as evidence of phenotypic alternations due to parental PAH exposure.

One explanation for the low standard deviation of the $\dot{M}O_2$ in the heavily exposed F0 generation could be that the heavy exposure-caused a type of metabolic shift of the exposed individuals that reduced the variation of $\dot{M}O_2$. The inability of PAH-exposed organisms to adjust the metabolism to changing oxygen contents and to cope with the demands of hypoxia

has been documented previously by Zou & Stueben (2006). In the study, the shrimp *Penaeus aztecus* was exposed to naphthalene (500µg/l, 19–21°C) within a respirometer and the response to stepwise lowering oxygen concentrations was recorded. To be able to maintain a normal function of oxygen-dependent metabolism the naphthalene-exposed shrimps needed 1.33mg/l more oxygen to be present in the environment compared with the non-exposed shrimps. Higher oxygen demand could explain the high $\dot{M}O_2$ in the F1 generation of the heavily exposed population, but the ability to perform oxy-regulation following PAH-exposure has not been studied from the offspring of the exposed organisms and the study of Zou & Stueben (2006) gives no outlook to the transgenerational nature of the phenomena.

Some effects of exposure to PAH-compounds have been explained with epigenetic changes induced by hydrocarbon exposure (Weis et al. 1998, Huang et al. 2016) and epigenetic mechanisms have also been offered as a cause behind phenotypic plasticity (Metzger & Schulte 2016). The documented phenomena of reduced variation throughout generations among the heavily exposed population could, thus, be further investigated with epigenetic markers to investigate whether the heavy hydrocarbon exposure changed variances of $\dot{M}O_2$ via this mechanism.

Epigenetic changes are chemical changes on the surface of the genetic material, deoxyribonucleic acid (DNA) that alter the way DNA is transcribed. Epigenetic changes can also be passed over to the offspring affecting also the following generations (Bird 2007). One of the best-known examples of epigenetic change is DNA methylation. Methylation is the addition of methyl groups on the DNA molecule so that the transcription of genes is changed (Metzger & Schulte 2016). Epigenetic changes can, thus, establish a significant difference even between genetically similar individuals (Bestor 1998).

Polycyclic Aromatic Hydrocarbon-exposure induced DNA methylation has been documented by Huang et al. (2016) who observed significant reduction in micro-ribonucleic acid(miR)-synthesis due to methylation of DNA encoding for a specific miR: the miR-133a. The effects were observed when rat H9C2 cells were cultivated with 0.05µM of phenanthrene for 24 hours. The cells where methylation had occurred represented significant enlargement in size and a twice more active protein synthesis compared with the non-exposed control cells. The enlargement of the cultivated cells was coupled with the reduction of the miR-133a that is normally inhibiting over-producing proteins. Interestingly, also the F1 generation of the heavily exposed population displayed higher $\dot{M}O_2$ than the two additional populations. The

elevated $\dot{M}O_2$ could hypothetically indicate a higher phase of protein synthesis in the offspring originating from the heavily exposed parents.

When discussing the possibility of epigenetic changes, the research of Trijau et al. (2018) should be noted, since the study screened for transgenerational DNA methylations in three generations following exposure of a parental population of *D.magna* for γ-radiation. Trijau et al. (2018) found that methylations that occurred in germline cells, not somatic cells, could be transmitted to the following generations. In the current research, the individuals representing the F1 generation were exposed to hydrocarbons as primordial cells and, thus, the epigenetic characteristics present in this generation should be passed over to the following F2 generation.

4.5 Consequences of the stable $\dot{M}O_2$

Lack of oxygen is associated with enforced effects of harmful PAH-exposures (Mu et al. 2017). Development in phenanthrene-enriched environment (100 µg/l) accompanied with hypoxic conditions (2 mg/l O_2) has shown to be significantly more harmful to the development of larval zebrafish, than the same phenanthrene exposure in oxygen-rich (6.0 mg/l O_2) concentrations (Cypher 2017). In research by Negreiros et al. (2011), cellular damage of gill lamellar epithelium, indicated by hyperplasia and hypertrophy, was found significantly more frequent in the slender seahorse (*Hippocampus reidi*) when exposed (8 h) to hypoxia (1.5 mg/l O_2) and crude oil (18 mg/l) simultaneously than if either of these factors occurred separately. Analysis of the immediately conserved 30%-V WSF revealed much of the same components which were present in the exposure by Negreiros et al. (2011) (naphthalene 12 mg/l, phenanthrene 0.41 mg/l, and fluorene 0.87 mg/l). The heavily exposed individuals in the F0 generation would, thus, supposedly have been very sensitive to reduced oxygen levels in the environment during the time when the reproduction success was monitored following the exposure. It would, thus, be interesting to study in the future whether this kind of sensitivity is present in the offspring of heavily exposed organisms.

The loss of variation in the $\dot{M}O_2$ seen in the F0 and F1 of the heavily exposed population could mean that the ability to adjust the metabolism to respond to changing environmental conditions, like the temperature, was reduced due to the parental exposure. This type of rigidness could be very harmful in natural environments where adapting to constantly

changing environmental conditions is necessary for the survival of daphnids (Carvalho 1987). In general, adaptation to e.g. changing temperatures can be considered as necessary for all living organisms especially in the Baltic Sea due to the presence of the four seasons. For example, the mean temperature of surface water varied from 1 °C to 16 °C yearly during 1990-2017 in Bothnia Bay (HELCOM 2018). Offspring originating from the heavily hydrocarbon-exposed parental organism could have difficulties tackling environmental changes and, thus, the survival of these individuals could decrease drastically in the Baltic Sea.

An acute decline in the population densities of planktonic species with short generation times often follows an oil spill and the increase is usually seen when a couple of generations have passed (Notini 1978, Linden 1979, Elmgren 1983). In light of the current results, it can be concluded that recovery from oil exposure can last for several generations and, since abnormal $\dot{M}O_2$ could be assumed to increase mortality in nature (Clarke & Fraser 2004), the recovery time of a couple of generations could explain the observed short lag-phase of decrease in planktonic population densities following oil spills. This would mean that also transgenerational effects lie behind the population oscillations following oil spills and that the effects documented are not merely caused by acute toxification of individuals that experience the oil spill.

4.6 Reasons for variation in $\dot{M}O_2$ in the control and the lightly exposed population

The laboratory conditions were only maintained between 18 and 22 ºC and the room temperature was not fixed. This could have influenced the $\dot{M}O_2$ of the model organism used, since small poikilotherm animals as *D. magna* have been observed to be especially sensitive to the variation of the environmental temperature (Clarke & Fraser 2004). Thus, higher temperatures are expected to stimulate increased metabolism and to cause higher $\dot{M}O_2$ (Khan & Khan, 2008). The effect of small temperature variations is indicated by the significant differences in the $\dot{M}O_2$ between the two identical 10%-V WSF exposures of the pilot experiments. The only difference between the first and the second set 10%-V WSF exposure was a 0.4 ºC temperature increase between the first (19.8 ºC) and second pilot (20.2ºC). This elevation in temperature could be assumed to lie behind the significantly higher $\dot{M}O_2$ seen in

the 10%-V WSF exposure of the second pilot. It needs to be noted, however, that measurements were made at the same temperature. Nevertheless, it seems that the cultivation temperature had a significant influence on the $\dot{M}O2$ in both the lightly exposed and the control population, but not in the heavily exposed population.

Changing $\dot{M}O_2$ throughout the generations in the control and the lightly exposed daphnid-population could also be associated with the fluctuating temperatures of the laboratory during the experimental period. The temperature of the stock aquarium had a slightly decreasing trend during the experiment period. This decreasing trend in temperature could provide an explanation for the lower $\dot{M}O_2$ observed in the F1 & F2 of the control and the lightly exposed populations compared with the F0. Of course, new experiments where the temperature would be stable between generations would need to be conducted to evaluate these assumptions.

An additional factor that might have caused differences in $\dot{M}O_2$ between the F0 generation and the two filial generations seen in the lightly exposed and the control population is that the parental generation was subjected to more relocation than the filial generations and,_ thus,_ the parental generations supposedly experienced more transfer-related stress than the filial generations. The attempt was to equalize the treatments of each generation by creating a sham exposure to the filial generations by letting them mature in a container to reach the corresponding 96 hours of age when the $\dot{M}O_2$ of the parental organism was measured, but an error was done in this sham exposure since the parental generation was let to mature in a container until only 48 hours of age, where after they were transferred to the exposure tanks. The filial generations were mistakenly not relocated at 48 hours of age but, instead, the filial generations were let to mature in a single container until they reached the age of 96 hours. This mistake might explain some of the differences seen between the different generations in the lightly exposed and the control population since it was creating differences between the treatments of generations and the amount of stress they were subjected to.

4.7 Evaporation of Water-Soluble Fraction of crude oil

High evaporation rates of PAHs from the WSF were indicated by the current results. Especially low-molecular-weight hydrocarbons have been documented to evaporate quickly

from the WSF (Wakeham et al. 1983). These low-molecular-weight PAHs, which are also found in the current WSF, are known to be highly volatile (Hong et al. 2017). When considering the PAH reduction during the experiment, the trend of the total hydrocarbon (C5-C40) levels seen in Table 4 is rather confusing. The concentration of the hydrocarbons was increasing from 3.6 mg/l to 4.7 mg/l from the sample representing the starting point to the end of the evaporation period. This pattern can, however, be explained by the fact that the analysis was conducted from separate samples and, thus, the initial concentrations of hydrocarbons may have been different.

The samples were collected into the bottles from the faucet at the bottom of the container and so the sample to be collected last presented the surface layer of the WSF. One possibility is that the surface layer contained dispersed oil droplets invisible to the eye that enriched the last sample with petroleum hydrocarbons. The last sample collected was indeed the bottle that was used to analyze the hydrocarbons following the 48-hour evaporation period. The genesis of oil droplets has been documented when the behavior of oil slicks has been observed following weathering processes, such as mixing oil with water by the waves (Wernersson 2004). The droplets possess the physical characteristics of oil and can flow in the most surface layer of water (Almeda 2014) and, thus, it is natural that they ended up in the sample filled up last.

5 Conclusions

The study aimed to investigate possible transgenerational effects following a crude oil-derived hydrocarbon exposure. The results of the study showed such effects in the heavily exposed population (30%-V WSF) where parental exposure was reflected in the filial generations by reduced variation. Stable $\dot{M}O_2$ and equal variances between the generations within the population could be recognized. In contrast, the $\dot{M}O_2$ varied and the variances were unequal between the different generations of the two additional populations, the control and the lightly exposed one.

The thesis in hand disputes the traditional methods to study crude oil hydrocarbon exposure-caused effects since these effects have mainly been investigated by tracking direct changes of function such as vitality, reproduction capacity, and other physiological changes of the exposed animals. The transgenerational effects of the current exposure were not however observed as direct changes in function, but rather as reduced variation: standard deviation of the $\dot{M}O_2$ was much lower in the heavily exposed population than it was in the two additional populations, the control and the lightly exposed.

In the future, it would be engaging to research the molecular mechanism behind the observed phenomenon and, also to investigate the impact of it on the affected individuals. A deeper understanding of the results in hand would allow better utilization of them in risk assessments considering oil spills in the Baltic Sea.

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Links:
Link 1. http://ilmatieteenlaitos.fi/saa/turku
Link 2. https://loligosystems.com/faq#faq-4

Appendix 1

Preparation of the cultivation medium M7

Daphnia magna was cultivated in a medium called "M7". This medium was prepared following the instructions in ´´OECD Guideline for Testing of Chemicals´´ (2014).

The preparations were made in two steps by first weighing and solving all the individual components of "Stock 1" and "Stock 2" into separate stock solutions. The stock solutions were stored at room temperature throughout the experiment period. The M7 was prepared by combining desired volumes of the stock solutions from Stock 1 (Table 5) and Stock 2 (Table 6) together and lastly adding 1 mL batch of melted vitamin solution (Table 7). Whenever components were dissolved into individual stock solutions or when a mixture of the stock solution was done these solutions were always stirred with a magnetic stirrer to assure the homogeneity of the solution. The pH of the ready M7 was set to 7.0-7.5 using 3M HCl (Fisher Scientific, Leicestershire, UK) and controlling the pH with Beckman Phi 32 pH meter (Beckman Coulter Inc. Brea, California, U.S.). M7 was stored in a cold room for a maximum of 1 week.

Table 5 The molecular components of Stock 1 are listed in the left-most column. Each component was weighed and dissolved individually into a stock solution with a concentration given on the second rightest column of the table. When a batch of M7 was prepared the individual solutions were combined in volumes presented in the rightest column to create an outset-solution where components from Stock 2 were added.

M7 Stock I

Molecular name	Molecular formula	Manufacturer, city, state, country	Concentration (mg/l)	Volume (ml/l of stock 2)
Boric acid	H_3BO_3	Sigma-Aldrich, Saint Louis, Missouri, USA	57 190	0.25
Manganese (III) chloride tetrahydrate	$MnCl_2$*4H2O	J.T. Baker Chemicals, Deventer, Holland	7 210	0.25
Lithium chloride	LiCl	VWR International, Radnor, Pennsylvania, USA	6 120	0.25
Rubium chloride	RbCl	Sigma-Aldrich, Saint Louis, Missouri, USA	1 420	0.25
Strontium Chloride Hexahydrate	$SrCl_2$*$6H_20$	Merck KGaA, Darmstand, Germany	3 040	0.25
Sodium bromide	NaBr	Sigma-Aldrich, Saint Louis, Missouri, USA	320	0.25
Sodium molybdate dihydrate	Na_2MoO_4*$2H_2O$	VWR International, Radnor, Pennsylvania, USA	1 230	0.25
Copper(II) chloride dihydrate	$CuCl_2$*$2H_2O$	Merck KGaA, Darmstand, Germany	335	0.25

Zinc chloride	$ZnCl_2$	J.T. Baker Chemicals, Deventer, Holland	260	1.0
Cobalt(II) chloride hexahydrate	$CoCl_2*6H_2O$	Sigma-Aldrich, Saint Louis, Missouri, USA	200	1.0
Potassium iodide	KI	Merck KGaA, Darmstand, Germany	65	1.0
Sodium selenite	Na_2SeO_3	Sigma-Aldrich, Saint Louis, Missouri, USA	43.8	1.0
Ammonium metavanadate	NH_4VO_3	Fluka Chemie Ag, Buchs, Switzerland	11.5	1.0
Na_2EDTA Dihydrate	$Na_2EDTA*2H_2O$	Merck KGaA, Darmstand, Germany	5 000	1.0
Iron(II) sulfate heptahydrate	$FeSO_4*7H_20$	Merck KGaA, Darmstand, Germany	1 991	1.0
Ferric EDTA	21 Fe-EDTA			5.0

Table 6 The molecular components of Stock 2 are listed in the left-most column. Each component was weighed and dissolved individually into a stock solution with a concentration given on the second rightest column of the Table. $Na_2EDTA*2H_2O$ and $FeSO_4*7H_20$ were prepared separately, poured together and autoclaved straight away. This results in the 21 Fe-EDTA needed. When a batch of M7 was prepared the individual solutions were combined in volumes presented in the rightest column with the solution that was previously created from the Stock 1 components.

M7 Stock II

Molecular name	Molecular formula	Manufacturer, city, state, country	Amount added to water (mg/l)	Volume (ml/l of stock 2)
Stock solution 2	-	-	-	50.0
Calcium chloride	$CaCl_2*2H_2O$	VWR International, Radnor, Pennsylvania, USA	2903 800	1.0
Magnesium sulfate heptahydrate	$MgSO_4*7H_2O$	Merck KGaA, Darmstand, Germany	246 600	0.5
Potassium chloride	KCl	VWR International, Radnor, Pennsylvania, USA	58 000	0.1
Sodium bicarbonate	$NaHCO_3$	Sigma-Aldrich, Saint Louis, Missouri, USA	64 800	1.0
Sodium Metasilicate Nonahydrate	$Na_2SiO_3*9H_2O$	Sigma-Aldrich, Saint Louis, Missouri, USA	50 000	0.2
Sodium nitrate	$NaNO_3$	Honeywell, Seelze, Germany	2 740	0.1
Monopotassium phosphate	KH_2PO_4	Merck KGaA, Darmstand, Germany	1 430	0.1
Dibasic potassium phosphate	K_2HPO_4	Merck KGaA, Darmstand, Germany	1 840	0.1

Table 7 The molecular components of the used vitamin stock. The components are listed in the left-most column. Each component was weighed and dissolved individually to a solution with a concentration given on the second rightest column of the Table. After a 1 L of ready stock was prepared it was divided into batches of 1 mL and stored at freeze at -20 °C.

Vitamin Stock

Molecular name	Molecular formula	Manufacturer, city, state, country	Amount added to water (mg/l)
Thiamine hydrochloride (B_1)	$C_{12\ h17}N_4OS^+$	Merck KGaA, Darmstand, Germany	750
Cyanocobalamin (B_{12})	$C_{63}H_{88}CoN_{14}O_{14}P$	Sigma-Aldrich, Saint Louis, Missouri, USA	10
Biotin (B_7)	$C_{10}H_{16}N_2O_3S$	Sigma-Aldrich, Saint Louis, Missouri, USA	7.5

Appendix 2

Cultivation of the algae *Pseudokirchneriella subcapitata*

The received stock culture of *Pseudokirchneriella subcapitata* (24th May 2017) was divided into 6 x 1L Erlenmeyer flasks. A stock liquid for the *P. subcapitata* cultivation was prepared following a protocol obtained from the Laboratory Centre for Ecotoxicology and Risk Assessment of the Finnish Environment Institute (SYKE) (Jyväskylä, Finland). A stock liquid for the algae cultivation liquid was prepared by weighing and dissolving all the individual components listed in Table 8. This stock liquid was then divided into 10 ml falcon tubes and stored in a freeze at -20 °C. Actual cultivation liquid was prepared by melting and mixing a single 10 mL batch of the stock liquid with 1990 mL of boiled and again cooled tap water. This procedure gave a total of 2 l of cultivation liquid.

The growth flasks were re-filled with algae growth medium approximately twice a week to keep a stable 800 mL liquid volume within each cultivation flask. Oxygen was supplied to the flasks through MILLEX®-GP 0,22 µm sterile filters (Millipore, Bedford, Massachusetts, U.S.) with air-pumps. A photoperiod of 16 h light and 8 h dark was fixed for the algae using the OSRAM Relax 15W 2700 K light bulbs.

Table 8 The molecular components of the used stock for algae cultivation. The components are listed in the left-most column. Each component was weighed and dissolved individually to a solution with a concentration given on the second rightest column of the Table. After a 1 L of ready stock was prepared it was divided into batches of 10 mL and freezed.

Stock liquid for algae cultivation

Molecular name	Molecular formula	Manufacturer, city, state, country	(g/l)
Sodiumnitrate	$NaNO_3$	Honeywell, Seelze, Germany	137.4
Magnesium sulfate heptahydrate	$MgSO_4*7H_20$	Merck KGaA, Darmstand, Germany	25.0
Monopotassium phosphate	KH_2PO_4	Merck KGaA, Darmstand, Germany	10.0
Citrate monohydrate	$C_6H_6O_7*H_20$	Merck KGaA, Darmstand, Germany	2.5
Ammonium iron(III) citrate	$FeNH_4C_6H_4O_7$	Merck KGaA, Darmstand, Germany	2.5

Preparation of nutrition for *Daphnia magna*

Nutrition for daphnids was prepared from the living algae. For one dose of nutrition, 45 mL of the algae culture was filtered through a FALCON® 40 µm sterile cell strainer (Corning Incorporated, Corning, New York, U.S.) into 50 mL VWR® plastic High-Performance centrifuge tubes (VWR, Radnor, Pennsylvania, U.S.). The dosage in the tubes was centrifuged for 5 minutes at 4000 rpm with a Biofuge™ Stratos™-centrifuge (Thermo Fisher Scientific, Waltham Massachusetts, U.S.).

The supernatant was discharged, and the remaining algae pellets were suspended with 15 mL of fresh M7-media by shaking the centrifuge tube with a closed cap. The generated suspension of M7 and *P. subcapitata* was added to the cultivation aquarium of daphnids to maintain a constant light green in the cultivation medium.

Appendix 3

Preparation of one batch of a 100%-V WSF liquid

The procedure was performed adaptively following a description of WSF preparation by Martinez-Jeronimo et al. (2005). A stock liquid of 100%-V WSF was created by weighing 100 g of crude oil (Russian Export Blend medium crude oil, Neste Oil, Raisio, Finland) into a 2.5L clean plastic bottle in a fume hood. Nine hundred mL of fresh M7 growthM7 growth medium was then poured into the bottle and the bottle cap was closed. The mixture of oil and M7 was stirred with a magnetic stirrer ~200 rpm for 24 hours. The mixture was then left to separate from each other for one hour in a custom-made separation funnel. After the separation, the M7 fraction was collected into a clean 1L glass bottle. The collected liquid represented the 100%-V WSF.

WSF samples chemical analyses

Four samples of 30%-V WSF solution were prepared for chemical analysis. Four clean 1L glass bottles were filled with 300 mL of the 100%-V WSF and 700 mL of fresh M7. The bottle caps were sealed, and the liquids stirred by shaking the bottles by hand.

After the shaking two of the bottles were reopened and placed in a fume hood for a 48-hour period. The evaporated samples were created to see how time affected the hydrocarbon composition of the test-liquid. The liquids in the two remaining bottles were conserved immediately by adding 2 mL of 6M HCl (Fisher Scientific, Leicestershire, UK) to each and then shaking the bottles with the cap sealed. The conserved bottles were stored in a cold room under 5°C.

When the 48-hour period was reached, the bottles in the fume hood were conserved as previous. The conserved samples where sent for further analysis to Novalab Oy, Karkkila, Finland were the total hydrocarbon content (C5-C40) and concentrations of 16 different PAH compounds (US-EPA PAHs) were determined.

The total hydrocarbon content was determined with a gas chromatography-flame ionization detector (GC-FID) on heptane dissolved samples that had been cleaned, dried and consecrated before the analysis. Based on the results, the hydrocarbons were divided into middle heavy (>C10-C21) and heavy hydrocarbons (<C21-C40). External samples and blank control samples were analyzed simultaneously with the test-liquid samples as a control. The

16 PAH-compounds were extracted with Toluene and analyzed by gas chromatography-mass spectrometry (GC-MS) with selected ion monitoring (SIM) technique. Also, these sample extracts were cleaned, dried and consecrated before the analysis. The results were calculated by using two inner standards for each compound. The detection limits of the method were <0.05 mg /l for C10-C40 and 0.1 μg/l for PAH compounds.

Appendix 4

Temperature and $\dot{M}O_2$

The temperature in the laboratory was followed by measuring the temperature from the stock aquarium of *D. magna* on a daily basis. The monitoring was done with a handled meter pHenomenal® OX4100H (VWR, Radnor, Pennsylvania, U.S.).

The measurements were performed to control that the temperature of the M7 was held between 18 and22 °C. The measurements were not documented every day due to that all of the measure values were found to lie within the fixed range. The documented values of temperature during the experimental period are presented in figure 4. The vertical lines in Figure 4 are also indicating the points for the $\dot{M}O_2$-measurements conducted. The numeric values of the $\dot{M}O_2$-rates are given in table 9.

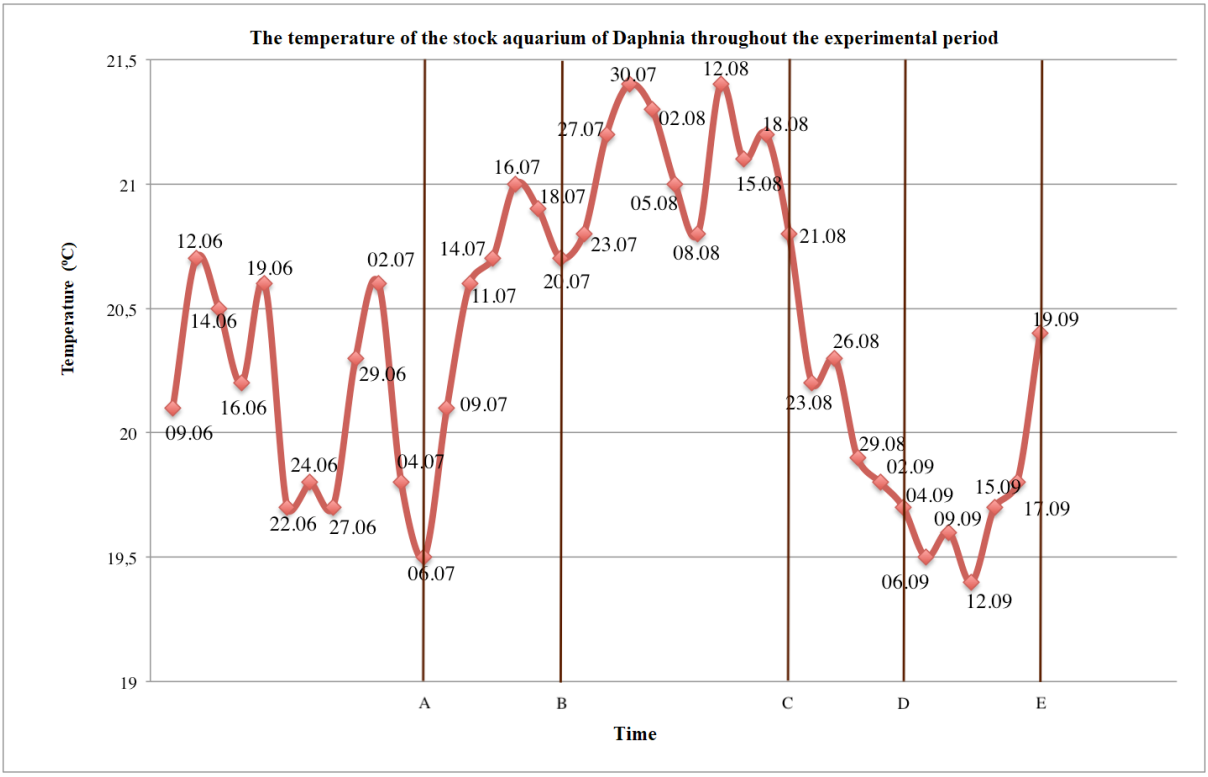

Figure 4 The temperature of the daphnid stock aquarium cultivation medium throughout the experimental period. Y-axis represents the temperature in Celsius degrees and X-axis the time proceeding. The dots on the red line are presenting temperature measure points and specific dates for the measurements given at each dot. The vertical lines titled A, B, C, D and E are points of $\dot{M}O_2$-measurement days. A is the measurement day of the first pilot experiment. B is the measurement day of the second pilot experiment. C is the measurement day of the F0 generation $\dot{M}O_2$ in the main experiment. D is the measurement day of the F1 generation $\dot{M}O_2$ in the main experiment. E is the measurement day of the F2 generation $\dot{M}O_2$ in the main experiment.

Table 9 The exact values of standard deviation and means of $\dot{M}O_2$ for every generation within the daphnid-stems studied in the experiment searching for Transgenerational effects.

Stem	Generation	Mean $\dot{M}O_2$ in Pmol/anim/min	Standard deviation (SD)	Sample size
30%-V WSF exposure	PO	436.7	±107.6	20
30%-V WSF exposure	F1	508.7	±98.1	20
30%-V WSF exposure	F2	466.9	±129.5	21
10%-V WSF exposure	PO	454.6	±162.5	18
10%-V WSF exposure	F1	315.4	±42.6	20
10%-V WSF exposure	F2	296.8	±83.4	21
Control	PO	465.0	±200.7	20
Control	F1	343.5	±135.1	20
Control	F2	383.8	±87.1	21