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Faculty of Environmental Sciences and Natural Resource Management

The effect of tunnel construction particles on *Daphnia magna*

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Environment and Natural Resources

Preface

This master thesis represents the finalization of my master´s degree in Environment and Natural Resources at the Norwegian University of Life sciences (NMBU).

I want to thank my primary supervisor Hans-Christian Teien (NMBU), for the support during the laboratory work, analysis, and writing process. I would also like to thank my co-supervisors, Emelie Skogsberg (NIVA) and Lene Sørlie Heier (NPRA), for additional help during this journey. Additionally, I would like to thank Shane Scheibener for the guidance in daphnia handling and keeping an algae culture.

Abstract

Particles originating from drilling and blasting during tunnel construction have the potential to end up in the environment, however, there is a knowledge gap concerning the effect of these particles on aquatic biota. In this master thesis, acute toxicity tests (48h and 72h) were performed with the test species *Daphnia magna* to identify concentrations causing immobilization, particle uptake, and abnormal appearance. In a laboratory setup, the daphnids were exposed to starting concentration of total suspended solids (TSS) with a range between 200-3000 mg/L of tunnel particles and control. TSS and turbidity were measured to identify changes in suspended solids (SS) during test periods.

The particles concentration (EC_{50} values) that caused immobilizations were 761 (± 532) mg/L for the 48h test and 649 (± 82) mg/L for the 72h test. The EC_{90} values were 1641 (± 2361) mg/L for the 48h test and 941 (± 271) mg/L for the 72h test. For the higher estimated doses, the daphnids were more sensitive in the more extended test. The no observed effect concentration (NOEC) for the 48h and 72h tests were 492 mg/L and 562 mg/L, respectively. Exposed daphnids differed from the control in weight. Correlation tests showed a moderate negative correlation between the daphnids' weight and TSS for both 48h (R= -0.57) and 72h (R= -0.54). As the TSS increased, the daphnid's weight decreased. This can be explained by the particles being within the daphnid feeding size, which led to the daphnid ingesting the particles and getting less nutrition than the control. Therefore, the growth rate was lower. Particles in the filter apparatus of daphnids possibly leading to ingestion were also confirmed with an environmental scanning electron microscope (ESEM) of exposed daphnids. Additionally, element quantification of digested daphnids with ICP-MS showed elements like Yttrium (Y) and Thorium (Th) associated with the daphnids and positively correlated with TSS. These elements have low leaching capacity and are most likely associated with particles in or on the daphnids. The immobilization of daphnids occurred on concentrations over usually permitted values for projects. Furthermore, there were other effects like weight decrease and particles associated with the daphnids. Therefore, chronic effects would be interesting to study.

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1 Introduction

In Norway, tunnels are essential to modern transport infrastructure, as vast areas are dominated by mountains, valleys, and fjords. Tunnels are constructed to increase transport efficiency through mountains or as underwater passages (NFF, 2017; SSB, 2017; Statens vegvesen, 2015). The construction of tunnels involves blasting and drilling of rocks, which produces an enormous volume of particles that can potentially be emitted into the environment (Pabst et al., 2015). The input of pollution into surface waters because of human alterations poses a potential threat to ecosystems. Therefore, regulations, recommendations, and guidelines have been made to limit the supply of harmful substances (Bilotta et al., 2008). The Norwegian Water Regulation (*Vannforeskriften*) aims to set sustainable limit values, leading the waters to a very good or good chemical- and ecological state (Lovdata, 2019). However, there are no set limit values for the supply of suspended solids (SS). Following the Norwegian Pollution Control Act (*Foruresningsloven*) §11, projects like constructing of roads and tunnels that pollute the environment need a special permit from the authorities (Klima- og miljødepartementet, 2021). These emission permits for construction sites are typically based on naturally eroded particles, and it is assumed that the impact of construction particles on aquatic biota differs from the naturally eroded ones (Weideborg et al., 2009). The emission permit for Norwegian construction sites varies, but the value usually is 100 mg/L of SS for freshwater systems. For marine waters, the value is typically 400 mg/L of SS (Statens vegvesen, 2021).

1.1 Particle concentration

The concentration of particles plays a crucial role in the impact on aquatic biota (Bilotta et al., 2008). Bilotta et al. (2008) define SS as organic or inorganic matter dispersed in the water column by turbulence. SS occur naturally in the aquatic systems, but human alterations of the landscape enhance the concentration in the water bodies from their natural background levels and change the chemical, biological, and physical properties (Bilotta et al., 2008). The background levels of SS in Norwegian waterbodies are dependent on several factors, like geology, climate, vegetation, and land use (Hessen, 1992). But usually, the natural background levels of SS rarely surpass 100 mg/L (Sørensen, 1998). Anthropogenic activities can potentially increase the concentration of SS in waterbodies from the natural background levels and pose as a problem (Hessen, 1992). There are several ways humans alter the landscape; one of them is construction projects (e.g., roads, tunnels, rails, etc.) (Pabst et al.,

2015). Particles originating from drilling and blasting differ from naturally eroded particles in being more elongated (Skogsberg et al., 2022).

Water from Norwegian tunnel construction contains concentrations of SS ranging from 5000 to 10 000 mg/L. A purification process is necessary to decrease this to an acceptable level. Therefore, the water is transported through sedimentation basins, and occasionally, a coagulant and acid are added (Vikan et al., 2013). When tunnel construction material is left in water bodies, especially in shallow water, the dumping may cause sediment particles to come into suspension, which leads to an even higher concentration of total suspended solids (TSS) (Sørensen, 1998). Excess particle masses may also be deposited in nearby terrain and occasionally used in other construction projects. The smaller particles can relocate from the construction site or deposit site to the recipient via precipitation, ground- and surface water. The particle mobilization depends on the particle characteristic and the transport method (Pabst et al., 2015).

The volume of suspended particles in a water column can be analyzed directly by weighing dried TSS or indirectly as turbidity (Kjelland et al., 2015). TSS identifies the accurate particle weight for a given volume (mgL^{-1}) and is considered time-consuming. Turbidity contributes to information about TSS in the water column and is the lack of clarity in the water body. The method measures the light scattered in the water (Bilotta et al., 2008; Wetzel, 2001). TSS and turbidity do not convey any other information about the particles, such as what the particles consist of, size, or shape (Pabst et al., 2015)

1.2 Particle size and exposure time

The particle size is crucial in how long the particles stay suspended. Hence an essential factor determining the concentration of particles in the water column. Furthermore, the shape and size of the particles from tunnel construction blasting depends on the blasting method and the type of bedrock being blasted. The particle size influences the sedimentation rate and affects the exposure time for aquatic organisms (Table 1) (Pabst et al., 2015). The smaller and lighter ones stay suspended for longer, e.g., particles smaller than 1 µm have a longer suspension time than larger particles (Filella et al., 2008). Therefore, potentially having a higher risk of being collected by species feeding in the water column.

Table 1: The sinking rate of particles depends on the particle size (Bjerknes, 2001).

Description	Grain size (mm)	Sinking rate (w/hour)
Fine sand	0.063 – 0.125	18
Coarse silt	0.031 – 0.063	4
Medium silt	0.016 – 0.031	1.5
Fine silt	0.002 – 0.016	0.2
Clay	0.001 – 0.002	0.02
	< 0.001	0

The exposure period of given particles in the water column also depends on the concentration supplied and the duration of the project, e.g., the length of the tunnel constructed (Pabst et al., 2015)

1.3 Leaching of metals

The relocation of the rocks from the excavation can potentially change the concentration of elements in the recipient due to leaching. Elements, like metals and rare-earth elements (REE) that were previously non-available in the bedrock, can become available for uptake by organisms (Pabst et al., 2015; Tabelin et al., 2014; Walker et al., 2012). Some elements are harmful, and some are essential nutrients and only toxic when in excessive volumes (Nikinmaa, 2014). For instance, aquatic biota in water bodies that receive runoff from construction sites is negatively impacted, as the enhanced volumes of potentially toxic metals (e.g., As, Pb, Cd, Cu, Cr, Hg, Ni, Zn) in aquatic systems pose a critical factor for the organisms in the system since they can´t effectively metabolize (Smirnov, 2017). Depending on the physicochemical conditions in water bodies, soluble elements can transform from solid to dissolved phase (VanLoon et al., 2017). The dissolved metals are more bioavailable than non-dissolved metals and therefore considered more toxic for aquatic species (VanLoon et al., 2017).

The Norwegian Environment Agency has published a guideline (*Vannforeskriften*) with limit values and condition classes for metals that have been proved to be harmful in aquatic systems (Table 2). This policy aims to support work to achieve and obtain good water conditions in Norwegian waterways (Lovdata, 2019). The leaching of metals from tunnel construction particles can potentially increase the presence of these metals in the waterways.

Table 2: Limit values in µg/L and classification set by the Norwegian Environmental Agency for heavy metals in fresh surface water. Footnote 1: the cadmium value is dependent on the hardness of the water (Pettersen, 2016).

Substance	**Class 1**	**Class 2**	**Class 3**	**Class 4**	**Class 5**
	Background	Good	Moderate	Bad	Very bad
Metals					
Arsenic (As)	0 - 0.15	0.15 – 0.5	0.5 – 8.5	8.5 - 85	> 85
Lead (Pb)	0 - 0.02	0.02 – 1.2	1.2 - 14	14 - 57	> 57
Cadmium (Cd)	0 - 0.003	Footnote 1	Footnote 1	Footnote 1	Footnote 1
Copper (Cu)	0 - 0.3	0.3 – 7.8		7.8 – 15.6	> 15.6
Chromium (Cr)	0 - 0.1	0.1 – 3.4			> 3.4
Mercury (Hg)	0 - 0.001	0.001 – 0.047	0.047 – 0.07	0.07 – 0.14	> 0.14
Nickel (Ni)	0 - 0.5	0.5 - 4	4 - 34	34 - 67	> 67
Zink (Zn)	0 – 1.5	1.5 - 11		11 – 60	> 60

1.4 Ammonium nitrate from explosives

During tunnel construction in Norway, a slurry of explosives is usually used, mainly consisting of ammonium nitrate (NH_4NO_3) (Vikan, 2013). This is highly soluble in contact with water and, therefore, can be transported to the recipients (Bækken, 1998). Total ammonia nitrogen (TAN) includes both unionized ammonia (NH_3) and ionized ammonium (NH_4^+) (Francis-Floyd et al., 2009). The speciation between these depends on ionic strength, temperature, and pH. The higher the temperature and pH, the higher the percentage of ammonia (Emerson et al., 1975; Hach, 2021). Ammonium is recognized as a harmless ion. In contrast, ammonia is harmful to aquatic biota (Table 3) (Emerson et al., 1975; Weideborg et al., 2009).

$$NH_3 + H_2O \leftrightarrows NH_4^+ + OH^- \quad \text{(equation 1)}$$

Table 3: Values set by the authorities for ammonia levels in Norwegian waterways (Direktoratsguppen Vanndirektivet, 2018).

Parameter	**Class 1**	**Class 2**	**Class 3**	**Class 4**	**Class 5**
	Background	Good	Moderate	Bad	Very bad
Ammonia (µm/L, 90 percentile)	1	5	10	15	25

The main problem is the undetonated explosives (Vikan, 2013). Typically, in standard blasting, the percentage of undetonated explosives is 10-15% (Weideborg et al., 2009). To prevent the critical effects of ammonia in water bodies, a pH adjustment is usually performed during the treatment of tunneling water (Vikan, 2013).

1.5 Effect of particle exposure on aquatic organisms

The European Inland Fisheries Advisory Commission (EIFAC) has a set of criteria (Table 4) for the concentration of SS in water bodies. These criteria are based on natural SS and, therefore, most likely differ from particles from tunnel construction (DFO, 2000).

Table 4: Criteria for suspended solids by the European Inland Fisheries Advisory Commission. The values are based on naturally eroded particles (DFO, 2000).

Suspended solids	Effect
< 25 mg/L	No evidence of harmful effect
25 - 80 mg/L	Possible to maintain good fisheries
80 – 400 mg/L	Unlikely to support good fisheries
> 400 mg/L	Harmful. Only poor fisheries are likely to be found

The total effect of SS on aquatic organisms is dependent on several stressors and factors, including the particle concentration, duration of exposure, particle size distribution, and the chemical composition (Bilotta et al., 2008). The increased SS concentration in waterways impacts the aquatic systems directly and indirectly. The enhanced level of pollution does not only cause harm on an individual level but may cause changes in the population dynamics on different levels of the food chain (Hessen, 1992). Not only are the leaching of metals into the dissolved phase a threat, but also contaminations adsorbed or chemically bound to the particles. The contaminations (e.g., metals) in particles may impact the species and, in the worst case, lead to mortality (Weltens et al., 2000).

The effects of the particles depend on the type of organism exposed. Aquatic species collect food with the help of different methods, and the impact of particles differs between the methods. Filter feeding species, like daphnids, ingest all particles within a specific size range and are therefore affected by the particles that are not needed for survival. Organisms that selectively choose the particle ingested are not as affected. An experiment indicated that the presence of clay suspended in the water column significantly impacted the population level. Unselective filter-feeding species (e.g., *Daphnia ambigua* and *Daphnia pulex*) had a larger negative effect on the presence than selective feeders like rotifer species (Hessen, 1992).

1.6 *Daphnia magna (D. magna)*

Planktonic *D. magna* is a member of the order *Cladocera* and the family *Daphniidae*. They mainly reproduce asexually through parthenogenesis and have the potential to produce many offspring rapidly (Smirnov, 2017). The specie is a popular test organism: this, combined with the fact that they are unselective filter feeders and, therefore, is sensitive to TSS, makes them a good option for these toxicity tests (Hessen, 1992). In their natural environment, *D. magna* feeds on bacteria, algae, and organic detrital fragments, with a diameter of 1 µm to 50 µm. In some of the largest individuals, particles up to 70 µm in diameter have been found in their gut. Even though their midgut (~100 µm) is more extensive, their esophagus is narrower (Smirnov, 2017).

1.6.1 Impact of suspended solids on *D. magna*

Preceding studies have investigated the effect of diverse materials of SS on the zooplankton, *D. magna*. The filter-feeding species can potentially ingest large volumes of solids (Weltens et al., 2000). In addition to nutritious particles in the daphnid's diet, they ingest particles like natural suspended solids, microplastic and inorganic particles from construction (Frydkjær et al., 2017; Hessen, 1992; Weltens et al., 2000). Depending on the particle type and other factors, the effects can positively and negatively impact the specie (Hessen, 1992).

There exists little knowledge on particle toxicity on primary consumers such as *D. magna*, particularly particles from tunnel construction. In a study by Hessen (1992), inorganic tunnel particles resulted in several effects on the zooplankton. Adverse effects included mechanical, toxic, and reduction in food availability (Hessen, 1992).

1.7 Research objectives and hypothesis

The primary goal of this master thesis was to gain more knowledge about which concentrations of tunnel construction particles contributed to the effects on *D. magna.* Acute toxicity tests were performed at the laboratory to identify immobilization, particle uptake, and abnormal appearance and behavior. Dose-response analyses were performed to identify critical values of tunnel construction particle exposure.

The following hypothesis was tested:

> H0: Exposure of particles from tunnel construction does not negatively influence *D. magna*.

H1: Tunnel construction particles have a toxic effect and immobilize *D. magna.*

2 Materials and methods

The test procedures were based on the Organization for Economic Cooperation and Development (OECD) Guidelines for testing of chemicals *Daphnia* sp. Acute Immobilization Test, with some explained deviations from the guideline (OECD, 2004). Preliminary tests were conducted to identify the optimal experimental design regarding daphnia well-being and the concentration range of tunnel construction particles used in the test solution.

2.1 Stressor: particle sludge from tunnel construction

Emelie Skogsberg already collected the particle sludge sample used in this master thesis as a part of her Ph.D. study. The sludge was collected with a spade into 11L buckets from a trench inside the tunnel Verket (59.4430, 10.6698), constructed between Moss and Sandbukta (Norway). The sampling location was 70 meters from the blasting area, where a slurry of explosives was used. The samples were stored dark and cool (4°C) until further use. A subsample of particles for the experiment was retrieved from the sludge while the suspensions were stirred. The subsample was wet sieved using a norm-complaint laboratory test sieve with a mesh size of 63 µm. The particle fraction was put in a new bucket and stored cool (4°C) until use in the exposure tests.

2.2 Study species: *D. magna*

The first batch of the *D. magna* was obtained from Norwegian Institute for Water Research (NIVA). The daphnids were maintained at The Isotope Laboratory at the Norwegian University of Life Sciences (NMBU). Daphnids (n= 40/beaker) were cultured in an incubator (Sanyo MIR-253) (20 ± 2°C, 16:8h light/dark cycle) in glass beakers with 800 ml cultivation media (Elendt M7 medium, Appendix 1). The medium was changed 3 times a week to assure good health and behavior. When changing the cultivation medium, the daphnids were collected with a Pasteur pipette with an expanded opening and released underwater to avoid air bubbles (Canada; OECD, 2004). During this procedure, daphnids in different life stages were separated. Excess daphnids were removed. The *D. magna* culture was fed daily according to the Daphnia Feeding Guide (appendix 3), except Saturdays and Sundays, with the green algae *Raphidocelis subcapitata*.

2.2.1 Diet: *Raphidocelis subcapitata*

The first batch of the green algae *Raphidocelis subcapitata* was obtained from NIVA. The algae culture was thereafter maintained at The Isotope Laboratory in 4- or 5-liter glass flasks in 20% Z8 cultivation medium under sterilized conditions. Once a week, 4- or 5-liter of deionized water, stock solutions Z-I, Z-II, Z-III, IV, and 40 or 50 ml of algae were added to the flask (Appendix 2). The algae culture from NIVA was the basis of the cultivation for the first week, for each of the following weeks, one subsample of algae for cultivation was collected after one week of growth when the cultivation flask was moved from the incubator to the fridge. The algae culture was added to the 4- or 5-liter cultivation media and put in the incubator (Sanyo MIR-253) (20 ± 2°C, 16:8h light/dark cycle) for one week with the supply of an air pump. After cultivation, the culture was put in the fridge (4°C) to settle. After settling, a subsample of 50 ml of algae was centrifuged (Beckman Coulter Allegra 64R Centrifuge) in 50 ml sterile tubes (7000 RPM, 3 minutes, 17°C, 9 ACC/DEC). After this process, the water was removed from the tube, and pellets were collected, and the process was repeated until the entire flask with algae culture was emptied of water. The whole procedure with cultivation and collection of pellets with algae was repeated weekly until the final week of exposure tests. A photometric analyzer (Shimadzu UV-1800 UV) using wavelength 560 nm was used to measure algae density to ensure the correct volume of algae was supplied to the daphnids. Water was used to autozero the instrument, and afterward, 30 µL of algae and 2970 µL of water were put in the tube in the front. The average of three measurements was used multiplied by 100 was used since the sample was watered down due to high density.

2.2.2 *D. magna* test organism

9 days old gravid *D. magna* females were used in the test. The gravid female was cultured from *D. magna* neonates that were separated from the primary culture. The neonates used were not the first progeny and were retrieved from healthy stock. Neonates were collected and cultured every week for 14 weeks to ensure access to gravid females during pilot experiments and exposure tests. The test organisms were maintained in several beakers under identical conditions as cultivation with similar medium, light, temperature, and algae supply.

2.3 Toxicity test set-up and sampling

In 1992, Hessen performed a similar toxicity test where *D. magna* was exposed to tunnel construction particles. The experimental test setup was inspired by Hessen´s method (Hessen, 1992).

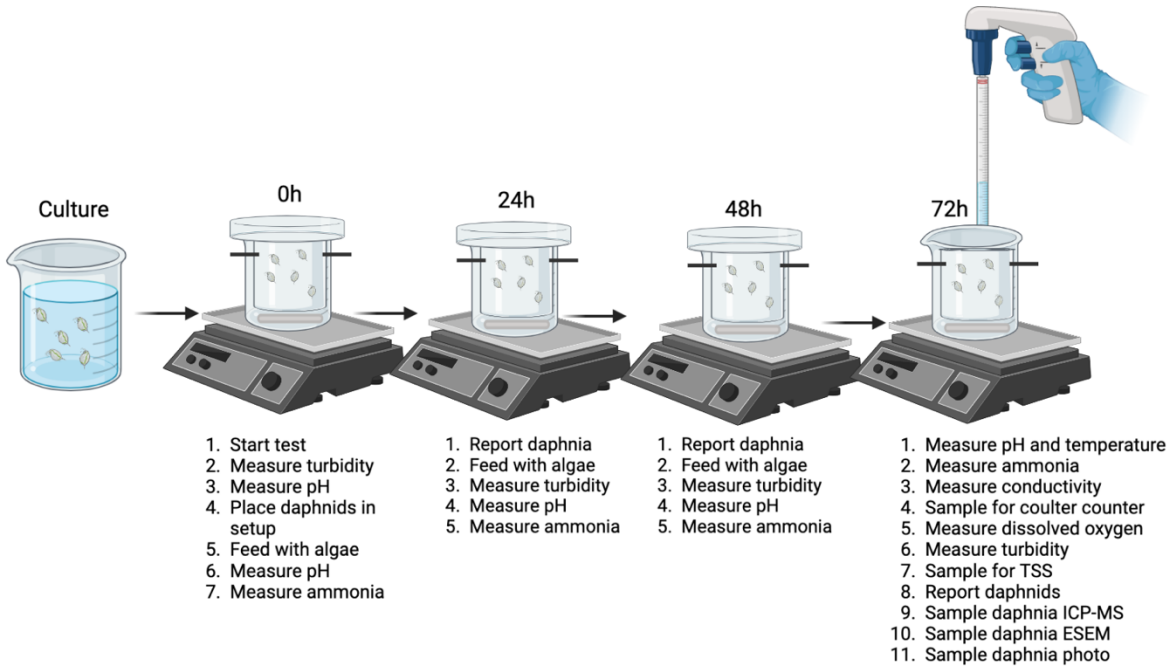
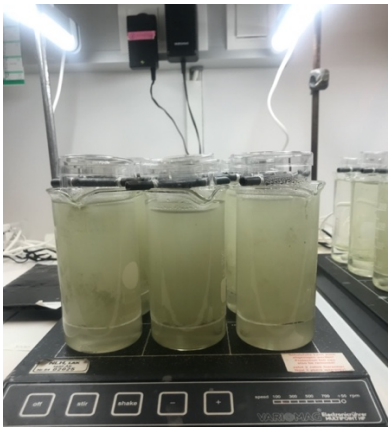
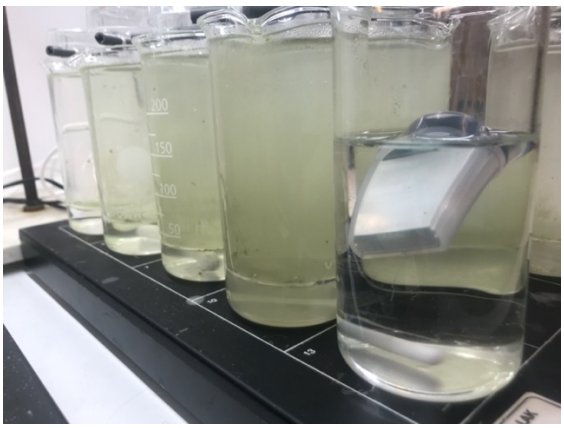


Figure 1: Timeline of the sampling during and at the end of the experiment. This timeline was from the 72 hours extended test. The sampling at the end of the 48 hours test was the same as at the end of the 72 hours test. The only difference was 24 hours less exposure time.

2.3.1 Laboratory set-up

One exposure unit consisted of 5 daphnids in 200 ml of exposure water. The daphnids in each replicate were exposed to particles in a plastic tube with a diameter of 4 cm with a 200 µm mesh glued on at the bottom with aquarium Casco silicone sealant. This setup was put in a 250 ml glass beaker (Figure 2 and figure 3). A plastic stick was placed through the top of the plastic tube, which lay on top of the glass beaker to ensure 1.5 cm clearing from the mesh to the bottom of the beaker. A 4 cm magnetic stir bar was put on the bottom of the beaker to keep the particles suspended. On top of this setup, a Petri dish was used as a cover to reduce water loss due to evaporation and reduce the risk of airborne contamination. The dish had some open space to allow oxygen (O_2) exchange.

Figures 2 & 3: The photo on the left shows the test setup from the front. The picture on the right shows the other magnetic stirrer board with setup. The HOBO Pendant temperature and light logger is shown in the front on the board. Image: Thea Oma.

Before starting the exposure, pilot experiments (Table 6) were performed to identify the optimal rotation of the magnet to ensure healthy daphnia (no immobilization in control) and as many particles in suspension as possible. During the final test, the set-ups were placed on two magnetic stirring boards (Variomag MULTIPOINT Magnetic Stirrer HP 15). M7 medium was added to each replicate, and the boards were turned on using the "stir" function and 200 RPM. When the magnets spun in a similar pattern, the test solution of particles was added as a one-time supply (Table 5). The total volume in each replicate was 200 ml, and the test solution was mixed the day prior in a 2-liter plastic bottle and pH adjusted, as later mentioned. The volume of M7 media and particle mix was calculated with the stock bottle's precise TSS concentration. Before the experiment started, turbidity and pH were measured. After this procedure (30 min.) *D. magna* (n=5/replicate, 15/concentration) was placed in the test setup.

Table 5: Overview of stressor, treatment concentrations at test start, and replicates for both tests performed.

Test duration	Stressor	Concentrations (Test start)	Replicates (Each treatment)	Total units
48-hours test	Particles from tunnel construction sludge	200, 400, 800, 1200, 1600, 2400, 3000 mg/L + control	3 replicates with 5 daphnids each	27
72-hours test	Particles from tunnel construction sludge	200, 400, 800, 1200, 1600, 2400, 3000 mg/L + control	3 replicates with 5 daphnids each	27

At test start (0h), the daphnids were fed 1.05e7 cells daphnia^{-1}, for the rest of the test, the daphnids were fed 5.25e6 cells daphnia^{-1} day^{-1}. The test setup was placed on the bench at

NMBU in room temperature. The light was supplied with 3 fluorescent lamps with a constant cycle (16h light: 8h dark). In addition, the setup was exposed to natural daylight through a window (December, Norway).

Table 6: Several preliminary tests were performed before the main experiments to optimize the best setup for both particles in suspension and daphnia well-being.

What	How
D. magna age and well-being	The OECD guideline prefers neonates (less than 24h); several tests indicated that these did not thrive in the test setup. Different mesh sizes (100 µm and 200 µm) and RPMs (150, 200, and 250) were tested. For all neonates, there was either high or total immobilization. The same setup was performed with adults to identify which RPMs that did not have a negative impact on the daphnia. Adults did thrive with 200 mesh size and 200 RPMs.
Particles in suspension	Tests were performed with 100 µm and 200 µm mesh sizes. Additionally, both 150 and 200 RPMs were tested to identify the best rotation regarding the suspension of particles.
Algae supply during the test period	Preliminary tests were conducted with the same supply as recommended by the feeding guide, ½ of recommended volume, ¼ of recommended volume, and no algae supply. The recommended category used was for adults.

2.4 Characterization of exposure solution

All water samples, except samples for ammonia and ammonium, were collected directly from each exposure unit with an automatic pipette (Integra Pipetboy with a Falcon® 25 ml Serological Pipet). A squirt bottle with distilled water was used to wash the probes used between sampling in each replicate.

2.4.1 Ammonia (NH_3) and ammonium (NH_4^+)

During tunnel construction, bulk emulsion explosives were used for blasting (report-fieldwork). The explosives consist of Centra Gold 100, Civec Control, Fortis Extra 100, and Fortis Advantage 100. These contain substances such as sodium nitrate ($NaNO_3$), distillates (petroleum), and around 60-80% ammonium nitrate (NH_4NO_3) (Orica Norway AS, 2021).

The test solution was collected with a 10 ml syringe (HENKE-JECT®) from a replicate with the highest exposure concentration to obtain information about ammonia in the test solutions. At the test start, the sample was collected from the 2-liter plastic bottle. The samples were filtered using a 25 mm syringe filter 0.45 µm before analyzing ammonia and ammonium

using a Portable Spectrometer (HACH® DR1900). Ammonia was analyzed using the Salicylate method. Two sample cells were prepared. One with 10 ml of deionized water as a control and one with the sample from the test. In both cells, the Ammonia Salicylate powder was added, shaken, and put aside for three minutes. After this, the Ammonia Cyanurate powder was added. After the cells were shocked, they were set aside for fifteen more minutes. After cleaning the cells outside, the absorbance was measured using 385 nm*, Ammonia, Salic.* First, the control sample was put in, and the button ZERO was pushed. After this, the cell with the test sample was put in, and the button READ was pushed. Equation 1 was calculated with a value depending on the pH and temperature. For this sample, this value was 31.40 (Hach, 2020).

$$\frac{{}^{mg}/_{L}NH3{-}N\ x\ value\ depedning\ on\ pH\ and\ temperature}{100}\ x\ 1.2 \qquad \text{(Equation 1)}$$

2.4.2 pH

According to the OECD guideline, the pH of the test solution should range between 6-9 (OECD, 2004). A preliminary test was performed, and the pH in the test solution did not remain within this range. Therefore, the pH was adjusted before tests started as a one-time adjustment. 0.1 M hydrochloric acid (HCl) was added to decrease the pH to 7.85, an acid preferred by the OECD guideline. The acid was added to the bottle the day before the test and exposed to air until test day. More HCl was added one hour before the test started if the pH was higher than 8. The pH was measured at test start and every 24 hours using a Multi 340i with SenTix® pH probe (Figure 1).

2.4.3 Dissolved oxygen (DO)

At the end of the tests, dissolved oxygen (DO) was measured in beakers for the controls and the replicates with the highest concentration of particles (Figure 1). The instrument Multi 340i was used with a WTW Digital IDS Dissolved Oxygen Sensor FDO® 925.

2.4.4 Conductivity

At the end of the test, conductivity (µS/cm) was measured at the end of the exposure in the beakers of all the replicates using the instrument Multi 340i with a WTW TetraCon® 325 (Figure 1).

2.4.5 Temperature and light

Temperature and light conditions were logged (HOBO Pendant Temperature/Light 64K Data Logger) every 15 minutes. The pendant was placed in a separate 200 ml glass beaker with M7 medium and a magnet on the magnetic stirrer board to resemble test replicates. The temperature was also measured manually in each replicate at the end of the tests (Multi 340i), indicating the differences between the replicates (Figure 1).

2.5 Characterization of particles in the test solution

2.5.1 Total suspended solids (TSS)

The TSS was identified following NIVA´s Method of analysis B2 Suspended solids and suspended embers (STS and SGR) (NIVA, 2020). The Whatman GF/C filters were prepared by dipping them in distilled water and then balancing them on the edge of beaker glasses to drip-dry them. The filters were wrapped in aluminum foil and covered in it. This was placed in the drying oven (VENTI-line® 56 Prime) at 105°C for 15 minutes. After this, they were combusted at 480°C in a chamber furnace (Carbolite CWF 1200) for 2 hours. Then they were cooled down in the drying oven for 30 minutes. After completely being cooled down, the filters were weighed (Mettler Toledo) and put in zip lock bags until use.

Prior to the test start, the stock bottle containing the test solutions was shaken, and 50 ml of samples were collected. This stock sample was filtered through the already prepared Whatman GF/C filter in a set-up with a vacuum pump. A squirt bottle with distilled water was used to “clean” the edge of the vacuum pump. The filter was dry sucked for 10-20 seconds before removing the vacuum hose. With a tweezer, the filter was removed, wrapped in aluminum foil, and dried in a VENTI-line® 56 Prime drying oven at 105°C for 2 hours. After this, the filter was weighed, and the TSS was calculated (equation 2).

$$TSS\ (mg/L) = \frac{1000\ (Weight\ after\ drying\ oven - weight\ filter)}{Sample\ volume\ (ml)} \qquad \text{(Equation 2)}$$

The same procedure was used to measure TSS at the end of the test in all the replicates.

2.5.2 Turbidity

Pre-, during, and at the end of the experiment, turbidity was measured (Figure 1) with a portable turbidity meter (Turbiquant® 1100 IR) controlled with reference solutions. The

instrument indicates the Formazin Nephelometric Units (FNU). A unit describing the quantitative decrease in the water transparency because of particles in the water column (Hongve et al., 1996). The turbidity was measured in 10 ml of sample collected with an automatic pipette (Integra Pipetboy, Falcon® 10 ml Serological Pipet). The sample collected pre and during the test were gently put back in the replicate before the sample cell was rinsed with distilled water between samplings.

2.5.3 Size fraction distribution

To obtain information on particle size distribution, 66.66 ml of sample were collected from each replicate at the end of the test. The 3 samples from each replicate at each concentration were pooled, totaling 200 ml of sample. To the pooled sample, 50 ml 0.05 M tetrasodium pyrophosphate ($Na_4P_2O_7$) was added, and the solution was ultrasonication (3 min) to avoid agglomeration before being stirred on a magnetic stirrer board. When the particles were suspended, 50-140 ml of sample was taken out by a syringe and analyzed. The analysis was performed with laser diffraction (Beckman Coulter LS 13320). Both background and loading were measured, sample information was noted, and each sample was analyzed 4 times. All tests were supposed to reach 7-9% obscuring when loading the instrument, which indicates the correct sample size. But some of them were lower. Between samples, the instrument was auto rinsed. A source of error was air bubbles in the instrument, as it does not differ between particles and bubbles. Usually, this was seen as a secondary peak at around 100 µm (Sabin, 2011).

2.6 Characterization of exposed daphnia

2.6.1 *D. magna immobilization, appearance,* and abnormal behavior

At the end of both tests, the daphnids were transferred to a Petri dish, where abnormal behavior and immobilization were reported (Figure 1). In the OECD guideline, immobilization is defined as: *"Those animals that are not able to swim within 15 seconds, after gentle agitation of the test vessel are considered to be immobilized (even if they can still move their antennae)" (OECD, 2004)*. In addition, observations of appearance and abnormal behavior were reported, including circling, floating, lethargy, swimming distance, and -pattern. At the end of the tests, the tube in the setup was carefully lifted, and the daphnids were placed in a Petri dish with M7 medium. If daphnids were stuck to the tube or mesh, a wash bottle with cultivation medium was used. The daphnids state was reported, and daphnids

that were not moving were slightly touched with the end of the pipette to check for immobilization. Daphnids (n=1/treatment) were collected and rinsed in Petri dishes with distilled water (x3) and transferred to a Petri dish with M7 medium with a white background. These daphnids were photographed with an iPhone.

2.6.2 Element quantification of daphnids

To obtain information of particles associated with daphnia after exposure, daphnia were quantified for element concentration, daphnids were collected (n=1/beaker, 3/treatment) with a pipette after exposure and rinsed in Petri dishes with deionized water (x3). The daphnids were gently pipetted into 2 ml VWR® cryotubes, vials made for low temperatures. The excess water droplets in the cryotubes were removed using a pipette with a Finntip Flex 200 µm. The samples were stored in a freezer (-21ºC). The samples were freeze-dried for 24 hours (Christ the Epsilon 2-4 LSC), programmed at 800 mbar and < -60ºC. After closing the side valve, the mbar slowly decreased to 0.1 mbar and < -80 ºC. After 24 the samples were taken out of the machine, the caps were put on the tubes and then put in dry storage. The freeze-dried daphnids were micro-balance weighed (Mettler Toledo MX5 microbalance) with the help of Neutralizing Electrostatic Charges to ensure accurate values presented in mg weight.

Exposed rinsed daphnids and certified reference material (CRM) were transferred to Teflon Autowave tubes before digestion. For the 3 blanks and the biological samples, 0.6 ultrapure nitric acid (HNO_3) were added to the tube, and 5 ml of ultrapure HNO_3 were added to the CRMs. The samples were digested with an Ultrawave (Milestone) (Program: 10 min 110°C, 20 minutes 260°C, 10 minutes 260°C). After digestion, samples were transferred to Sarstedt tubes, and the inside of the Ultrawave tubes was rinsed (x3 with deionized water) before dilution to 6 ml and 50 ml for samples and CRMs, respectively. Triple quadrupole ICP-MS (Agilent 8900) was used to quantify the concentration of elements accumulated in and on the daphnids, with an accuracy of 10%. The concentration of elements in each daphnid was calculated according to the weight of the daphnid and measured concentration in the digest.

2.6.3 Environmental scanning electron microscopy (ESEM)

An environmental scanning electron microscope (ESEM) was used to image daphnids from concentrations of 200, 1200, and 3000 mg/L to obtain information on the distribution of particles associated with the daphnia. Daphnids (n=1/treatment) were collected and rinsed in

Petri dishes with deionized water (x3) before gently pipetting into vials with 2 ml of fixative, completely covering the daphnia. The fixative, stored in the fridge (4ºC), was prepared by Emelie Skogsberg, and contained 50 ml 4% PFA Para-formalin, 25 ml 0.4 PIPES buffer, 5 ml 25 % Glutaraldehyde, and 20 ml dH_2O. The dehydration, critical point drier, and SEM processes were performed by Skogsberg (Appendix 7). Using an Environmental Scanning Electron microscope (ESEM; Zeiss EVO 50 variable pressure, 38-50 Pa, 30 kV accelerating voltage, 7-9 mm working distance, magnification 76-1500X), daphnids were imaged in backscattered electron imaging modes.

2.7 Deviations from guideline

There were several deviations from the OECD guideline for testing of chemicals. The parameters of test duration, temperature, age, feeding frequency, pH, and recording of immobilization differed from the set standard in the guideline (OECD, 2004). These deviations were supported by scientific literature. The first test duration of 48 hours was according to the OECD guideline. A second prolonged test of 72 hours was performed to support the result from the first test and identify if there was a different result from 24 hours more. Immobilization during the test should be reported according to the guideline. The high turbidity in the test solution made it difficult to observe some of the exposure concentrations. Therefore, not all were reported during the test. The control and the lowest concentrations were reported.

In the guideline, daphnids less than 24 hours were preferred (OECD, 2004). Knowledge from preliminary tests indicates that neonates did not thrive in the setup, and the turbulence caused by the spinning magnet impacted their swimming. In addition, offspring produced during the test period by adult individuals did not swim and, therefore, got stuck to the mesh.

2.8 Quality assurance

To ensure that the values from the ICP-MS analysis were reliable CRMs (NCS DC73325-soil-China National Analysis Center and NCS ZC73007-soil-China National Analysis Center) were analyzed. The percentage error was calculated following equation 3. Values within 10% were accepted measurements.

$$\%\ error = \frac{Observed\ concentration - Expected\ concentration}{Expected\ concentration} x\ 100 \qquad \text{(Equation 3)}$$

The Multi 340i, to measure pH, was calibrated in two-point WTW technical buffer solutions of 4 and 7. The turbidimeter (Turbiquant® 1100 IR) was calibrated using 3 standard calibration points in the following order 1000, 10.0, and 0.02 FNU, in accordance with the instrument´s manual.

2.9 Data handling and statical treatment

Statistical treatment was necessary to determine the effect of the tunnel sludge particles on the *D. magna*. The data obtained were summarized in Microsoft Office Excel 2021 (version 16.56 (21121100)) in a tabular form for both tests. The software RStudio (version 1.4.1717) was used for statistical treatment with the α-level set at 0.05 for statistical significance.

2.9.1 Correlation tests

RStudio was used for correlation plots to identify similarities between data obtained during the test periods. The Pearson method was used for the normally distributed data, and the Kendall method was used for the datasets that were not normally distributed.
For the correlation plot exploring the similarities between turbidity and TSS, the mean value of measured turbidity pre, during, and at the end of the test periods was used (OECD, 2004, 2012). For all the plots with TSS, the measured concentration at the end of the tests was used.

2.9.2 Dose-response analysis

Dose-response studies investigate the estimated doses to affect the tested organism. Controlled doses of a substance are supplied, and the response was reported (VanLoon et al., 2017; Walker et al., 2012). In this study, particles from tunnel construction were used as a substance, and the response reported was immobilization of daphnids. Determination of the parameters no observed effect concentration (NOEC), lowest observed effect concentration (LOEC), effective doses (EC_x), and making dose-response curves were made in RStudio using the drc package (Ritz, Strebig, et al. 2016, Ritz, Jensen, et al. 2020). The identified LOEC value is the lowest concentration that differs from the control, and NOEC is the concentration below the LOEC value (Walker et al., 2012).

3 Results and discussion

The primary endpoint of both toxicity tests was immobilization of daphnids due to exposure to tunnel construction particles. These tunnel particles were environmental samples and, therefore, not a single toxicant but a complex mixture. Meaning several factors may influence the daphnids. Other parameters not related to the concentration of particle exposure were measured to exclude that these parameters contributed to the adverse effects. Other abnormal observations of the daphnids were reported following the OECD guideline (OECD, 2004). Additionally, several different procedures and analyses were used to identify particle uptake in the daphnids. Using an Environmental Scanning Electron Microscope (ESEM), particles can be seen in organs in the organism (Hessen, 1992). Another example of identification was the use of element quantification with ICP-MS.

3.1 Characterization of test solution

3.1.1 Dissolved oxygen (DO)

The dissolved oxygen (DO) varied between 8.95 - 9.75 mg/L for both tests (Appendix 4). Like many organisms, oxygen is essential for daphnids. Therefore, for the acute test to be valid by the OECD guideline, the DO levels should be over 3 mg/L (Cole et al., 2016; OECD, 2004; Wetzel, 2001). Dissolved oxygen enters the test replicate by either diffusion or as a by-product of photosynthesis (Cole et al., 2016; Fondriest Environmental Inc, 2013). The effect of test duration on DO depends on the concentration of particles. The 72h test had a higher dissolved oxygen concentration in the control replicates than the 48h test. For the replicates with the highest concentration of particles, the 48 hours test had the highest level of DO. Reduced light penetration and reduced algae photosynthesis activity can explain the differences (Fondriest Environmental Inc, 2013).

3.1.2 Temperature

For the 48 hours test, the temperature varied between 20.2-21.5 ºC, and for the 72 hours test, the temperature ranged between 20.5-22.2 ºC (Appendix 4). Several factors can be the reason for the variation, but variable heating and the room temperature was assumed to be the main reason since the experiment was conducted in December (December, Norway). For the 48 hours test, the temperature varied between 20.9-22.9ºC, and for the 72 hours test, 21-23ºC (Appendix 4). Higher temperatures were found in replicates with a higher TSS. The suspended matter increases temperatures in water bodies due to the absorbance of heat energy

(Kjelland et al., 2015). The observed temperatures were higher than the criteria in the OECD guideline, which pinpoint a preferred temperature between 18-22 ºC and should not vary by more than one degree throughout the test period (OECD, 2004).

3.1.3 Conductivity

The conductivity measurements for both tests showed an increase in conductivity with increasing TSS exposure for both tests (figure 4 and figure 5). For the 48 hours test, the conductivity varied between 590 to 897 µS/cm (Appendix 4), with a positive correlation (R= 0.82) between total suspended solids and conductivity. For the 72 hours test, the value varied between 645 and 974 µS/cm with a positive correlation (R= 0.89). The correlation between TSS and conductivity was significant for both tests.

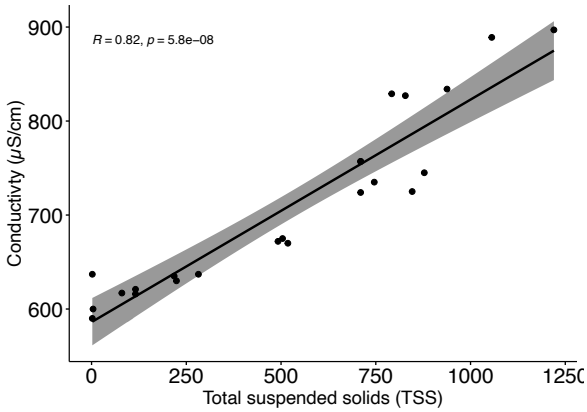


Figure 1: Correlation between conductivity (µS/cm) and total suspended solids (TSS) for the 48 hours toxicity test. (Kendall: R-value = 0.82, p-value = 5.8e-08).

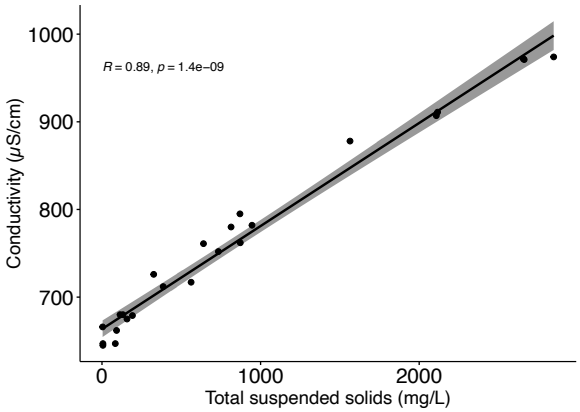


Figure 2: Correlation between conductivity (µS/cm) and total suspended solids (TSS) for the 72 hours toxicity test. (Kendall: R-value = 0.89, p-value = 1.4e-09).

Conductivity is defined as the capability to carry electrical flow, and the parameter is related to the number of free ions in the water column (Fondriest Environmental Inc, 2014a). According to an earlier study, the NOEC value identified for conductivity for *D. magna* is 7170 µS/cm (Frąk et al., 2021). Therefore, values under this were accepted.

The increase of conductivity with TSS in the current study was likely a result of two factors: the addition of acid and the particles themselves. HCl is a strong acid and, therefore, ionizes when in contact with the water (Equation 4) (Bauer, 2018).

$$HCl_{(g)} + H_2O_{(l)} \rightarrow H_3O^+_{(aq)} + Cl^-_{(aq)} \quad \text{(Equation 4)}$$

The enhanced volume of HCl in replicates with a higher concentration of TSS can enhance the free ion concentration in the test replicates. Furthermore, rocks release ions into the water column (CWT, 2004). Results from Hansen (2022) showed increased leaching of ions from these particles by increasing the time of suspension and increasing the particle concentration (Hansen, 2022). Therefore, the conductivity was heightened with a higher concentration of particles. The prolonged test had higher conductivity values than the test of 48 hours.

3.1.4 pH

pH in the treatments varied throughout the test periods. For the 48 hours test, 8.65 to 9.44, and for the 72 hours test, 8.5 to 9.4 (Appendix 4). According to the OECD guideline, the pH value of the test solution should range between 6-9 (OECD, 2004).

With the combination of algae and particles added to the Elendt M7 medium, the pH value surpassed 9. Therefore, several replicates have a higher pH than this during the test. A study from 2011 by Ghazy et al. reports that the daphnids need a pH lower than 10.13 (Ghazy et al., 2011; OECD, 2004). Therefore, the focus was to ensure the pH stayed under this level. The pH value in the aquatic system plays a crucial role in the physiological functions of the organisms in the system (Ghazy et al., 2011).

Table 7: pH pre and after algae in several of the replicates (48h test).

Concentration	pH pre algae	pH after algae
Control 1	8.32	8.73
Control 2	8.42	8.65
Control 3	8.41	8.80
2400 mg/L 1	8.49	8.85
2400 mg/L 2	8.50	8.86
2400 mg/L 3	8.54	8.81
3000 mg/L 1	8.53	8.85
3000 mg/L 2	8.52	8.86
3000 mg/L 3	8.55	8. 88

Several factors influence the pH in the mixture of particles and medium. The algae supply increases the pH in the test solution. The measurement of the test solution before and after algae supply showed an immediate pH increase. Table 7 shows the rise in pH right after algae supply for the 48 hours test.

Prior to the supply of the acid, HCl, the particles had a naturally higher pH than wanted during the main experiment. The use of grout and shotcrete during tunneling enhances the pH (Vikan et al., 2013). Therefore, the particle mixture had a high pH. The supply of HCl increases the H^+ concentration in the water solution, and therefore decreases the pH (Bauer, 2018).

3.1.5 Ammonium (NH_4^+) and Ammonia (NH_3)

0.15 mg/L was the highest measured concentration of total ammonia nitrogen (TAN) during the test periods. This gives the highest measured concentration of 56.5 µg/L ammonia (NH_3) at the specific pH and temperature (Hach, 2020). Studies indicate that the lowest observed effect concentration (LOEC) of ammonia for *D. magna* was 1.3 mg/L in a chronic toxicity test (Reinbold et al., 1982). The ammonia concentrations in these tests were about 23 times lower than LOEC. Thus, it's not expected that the ammonia originating from explosives has a negative effect on the daphnids during the test periods.

3.1.6 Total suspended solids and turbidity

Pilot tests were performed to identify the highest possible rotation of the magnet that did not immobilize the daphnids and the lowest possible rotation to keep particles in suspension. One pilot test determined that 200 RPM was the maximum to avoid immobilization of daphnids. Another pilot experiment showed that 200 rotations per minute (RPM) maintained a higher concentration of particles in suspension than 150 RPMs. Turbidity was measured in the replicates (n=3) for each concentration. From 0.5h to 24h, the replicates from the 200 RPMs for both the high and low concentrations of TSS had a percentage reduction varying from 21% to 53% FNU. For the 150 RPMs, the decline in the same period for both concentrations ranged between 35% and 73% FNU. Therefore, 200 RPMs were preferred during the main experiments as the FNU concentration had a smaller decrease. A higher frequency would negatively affect the daphnids as the spinning impacted them. A slower frequency would have a negative impact on the concentration of particles in suspension.

For both tests, TSS was measured to identify differences from supplied concentration to final concentration (Appendix 5). Complete suspension of particles was not expected due to sedimentation. The sedimentation rate of the particles depends on the particle size and the water flow (Pabst et al., 2015). For the 48h extended test, there was a decrease in the concentration of particles. The reduction of TSS varied from 30% to 67%. For the 72 hours test, the reduction of TSS from test start to test end varied from 5% to 72%.

Usually, there is a good correlation between TSS and turbidity (Kaste et al., 2020). The correlation analysis of the test data indicates a positive relationship between TSS and turbidity (Figure 6 and Figure 7). The correlation coefficient (R) was closer to 1 for both tests. The correlation coefficient for the 48h test was 0.97, and for the 72 hours test, the value was 0.98.

The p-value for both tests was <0.05 and, therefore, statistically significant. The data for turbidity was based on the average value over the sampling period.

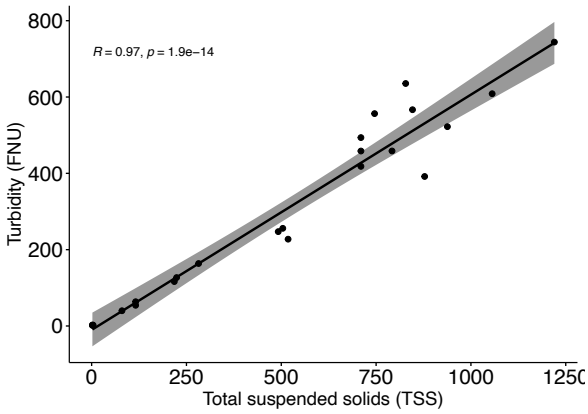


Figure 6: Correlation between total suspended solids (TSS) and turbidity (FNU) for the 48 hours test (Pearson: R-value = 0.97, p-value = 1.9e-14).

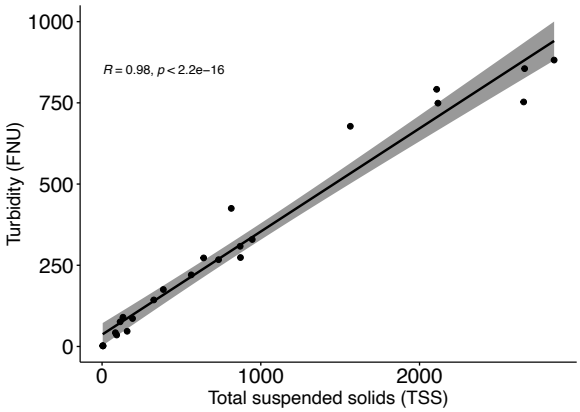


Figure 7: Correlation between total suspended solids (TSS) and turbidity (FNU) for the 72 hours test (Pearson: R-value = 0.98, p-value = <2.2e-16).

Several factors influence both the TSS and turbidity results. The sampling and measuring of other parameters can be a source of error. The movement in the test solution may lead to the resuspension of sedimented particles. The particles may be in the bottom of the beaker or stuck in the mesh. Also, the vertical and horizontal sampling location in the beakers differs from replicate to replicate. This was because the daphnids should not be drawn into the pipette. The Whatman Grade GF/C glass filters used to measure TSS have a 0.7 µm pore size. The particles under this size will not be weighted; therefore, the TSS was higher than measured. Measurements of turbidity were also affected by the algae added to the experiment (Fondriest Environmental Inc, 2014b).

3.1.7 Size distribution

Particle size distribution D_{50} was 6.94 µm for the 48 hours test and 7.5 µm for the 72 hours test. This means that 50% of the particles were smaller than this (Horiba Scientific, 2018). D_{90} of the particles in exposure concentration have a diameter smaller than 27.7 µm for the 48 hours test and 29.8 µm for the 72 hours test (Table 8). This indicates a dominance of medium silt or finer (Bjerknes, 2001). One example of the size distribution of the tunnel construction particles is illustrated in figure 8.

Table 8: Average particle size distribution for the two acute tests excluding 200 and 400 mg/L.

	48h test	**72h test**
D_{10}	1.3 µm	1.3 µm
Median/D_{50}	6.9 µm	7.5 µm
D_{90}	27.7 µm	29.8 µm
(D_{90} / D_{10})	21.1 µm	21.6 µm
(D_{90} - D_{10})	26.4 µm	28.4 µm
(D_{75} / D_{25})	4.6 µm	4.7 µm
(D_{75} - D_{25})	11.1 µm	12.1 µm

Pre-test the particles sludge was sieved through a 63 µm sieve. Therefore, the particles were expected to be under this size. There were some errors in some of the analyses from the laser diffraction, as the instrument cannot differ between particles and air bubbles (Sabin, 2011). Several datasets from the laser diffraction were affected by air bubbles. This was noticed with a secondary peak in the data, around 100 µm (Figure 9). The result from 200 mg/L and 400 mg/L were not used to calculate the average values from the test. This was because the sample size was very small, and the secondary peak, which demonstrated the presence of air bubbles, almost dominated.

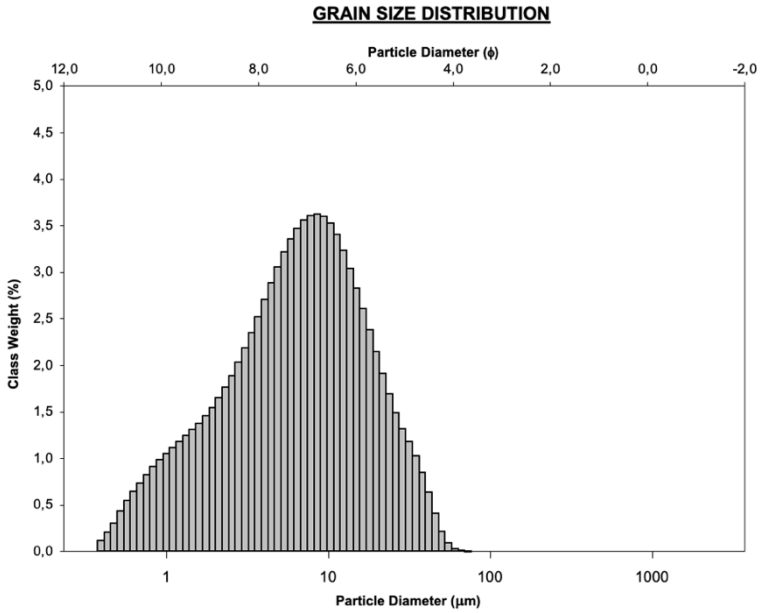


Figure 8: The grain size distribution for the starting concentration of 1600 mg/L for the 72 hours test. Y-axis: class weight in percent. Lower X-axis: particle diameter in micrometer.

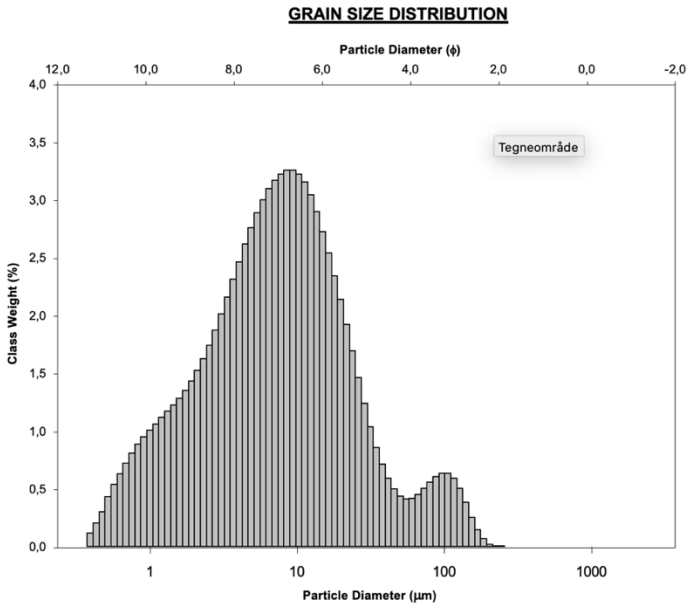


Figure 9: Grain size distribution for 800 mg/L for the 48 hours test. Y-axis: class weight in percent. Lower X-axis: particle diameter in micrometer.

3.1.8 Metal leaching

In the study by Hansen (2022), a leaching experiment was conducted on the same particles from Verket. The leaching of several metals (e.g., As, Pb, Cu, Cr, and Ni listed in the Norwegian Environmental Agency guideline) was quantified. As the values identified were from 100 g/L, they were divided by 33 to be adjusted to the highest concentration (3000 mg/L) used in these acute toxicity tests (Table 9) (Hansen, 2022). All values adjusted for 3000 mg/L indicated a good or better water quality when compared to the guideline.

Table 9: Element leaching from Verket particles in the study by Hansen (2022). The values from her research were divided by 33 to adjust it to 3000 mg/L. The values obtained from her study were from the highest values, from either 0-20 or 20-63 µm. The water was pH adjusted.

Metal	Values after 57 days	Adjusted for 3000 mg/L
Arsenic (As)	1.291 µg/L	0.039 µg/L
Lead (Pb)	0.039 µg/L	0.001 µg/L
Copper (Cu)	22.44 µg/L	0.68 µg/L
Chrome (Cr)	58.3 µg/L	1.76 µg/L
Nickel (Ni)	14.9 µg/L	0.45 µg/L

3.2 Effect on Daphnia magna

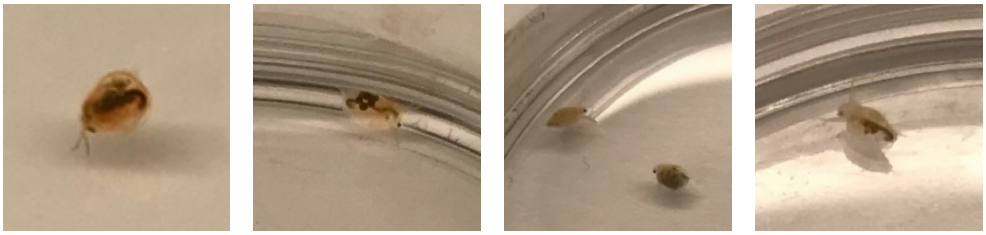
3.2.1 Effect with and without feeding

Before the main experiments, preliminary tests indicated that daphnids in all concentrations were negatively impacted by the laboratory set-up when algae were absent in replicates. The supply of green algae pre-and during the test duration deviates from the guideline. When not fed during the experiment, there was at least one immobile daphnid in each starting concentration. Earlier studies indicate that daphnids scrape the bottom when there is a lack of food in the water column (Gillis et al., 2005). Therefore, the individuals in the beaker without feeding were most likely to go down to the bottom and be affected by the rotation flow, which kept them at the bottom and near the mesh.

The daphnia in the controls and the lower treatments were observed to have a lower vertical swimming pattern and stay closer to the mesh at the bottom than individuals in the higher treatments. Daphnids are known to adapt their vertical presence in a water column according to present light (Serra et al., 2019).

3.2.2 Appearance and swimming pattern

When sampling at the end of the test, the particle exposed daphnia transferred to the petri dish differed in multiple ways compared to the controls (Figure 10, Figure 11, Figure 12, and Figure 13). They had different appearances and swimming patterns, and some were immobile. The daphnids in control had a transparent look, with a hint of green and yellow. This indicates healthy daphnids when fed on green algae (Kalff 2001, Ebert 2005). Even in the lowest exposure concentrations with particles, the daphnids' appearance was a bit paler, and as the particle concentrations got higher, the daphnids looked even paler. Some exposed individuals had a greyish color and looked bigger in size, which most likely was associated with the particle exposure (Figure 12).



Figures 10, 11, 12, and 13: Daphnids sampled at the end of the 72h test. A) One daphnid from the control. B) One daphnid from starting concentration of 200 mg/L. C) Two daphnids from staring concertation 2400 mg/L of SS. The daphnid to the left was still mobile. The daphnid to the right was immobile and looked like it was full of particles. D) One daphnid from the 3000 mg/LL of SS start concentration.

Observations also indicated an altered swimming behavior of the exposed individuals compared to control individuals. This behavior was mainly seen in the higher concentrations of particle exposure. One possible explanation for this was that the daphnids, earlier kept in greater turbidity, were not as adapted to the light intensity, and therefore were affected when suddenly exposed to a lighter environment when transferred to the Petri dish. Therefore, this sudden change may impact the swimming pattern (Bownik, 2017). The swimming patterns observed differed between "spinning" and slower movements.

3.2.3 Effect on weight

Analyses of freeze-dried daphnia's weight (n=1/daphnid, 1/replicate) showed a moderate negative correlation coefficient with TSS for both tests, the 48h test (Kendall, R= -0.57) (Figure 14) and the 72h test (Kendall, R= -0.54) (Figure 15). As the TSS concentration increased, the daphnid's weight decreased. This decrease was already seen in the lowest exposure concentrations.

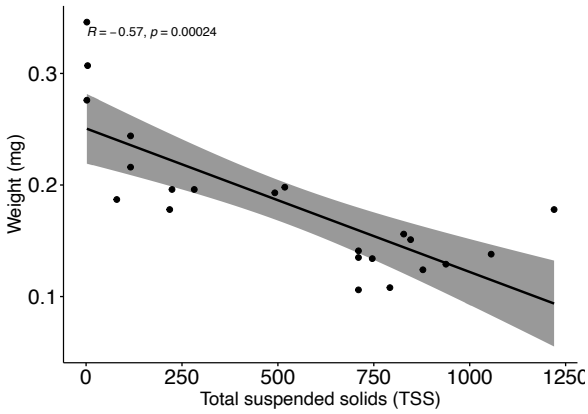


Figure 14: Correlation between total suspended solids (TSS) and weight (mg) of exposed daphnids for the 48 hours test. (Kendall: R-value = -0.57, p-value = 0.00024)

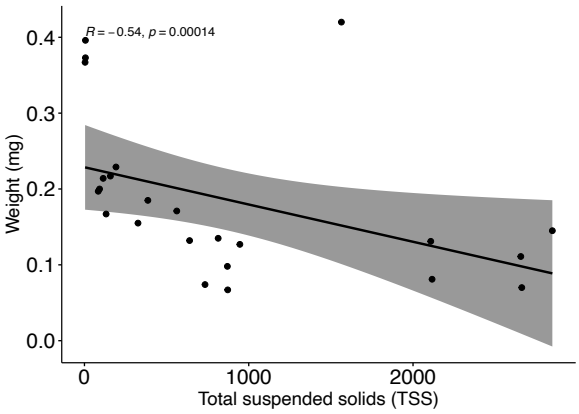


Figure 15: Correlation between total suspended solids (TSS) and weight (mg) of exposed daphnids for the 72 hours test. (Kendall: R-value = -0.54, p-value = 0.00014)

The particle-exposed animals did not thrive in their environment and most likely did not grow as much as the ones in control. Because of their age at the test start, the daphnids were expected to continue to grow during the test period. The particles in the tests (Table 8) were within the daphnids feeding range (less than 45 µm) (Gillis et al., 2005), and since they feed unselectively, they ingested inorganic particles, which were not nutritious and can, therefore, negatively impact the specie growth rate (Hessen, 1992; Kirk, 1991).

The lower algae ingest rate by daphnia when exposed to SS has been identified in multiple studies. Arruda et al. (1983) investigated the decreased algae intake on *Daphnia parvula* and *Daphnia pulex* when exposed to suspended sediments. The decrease was ~95% when exposed to 0.0 to 2451 mg/L of SS in a laboratory experiment (Arruda et al., 1983). Another experiment by Hessen (1992) showed an impact in the growth of *D. magna* from 10 mg/L (Hessen, 1992). As earlier mentioned, at the end of the test, the exposed daphnids looked paler than the control, indicating less ingestion of algae. The study by Kirk on *Daphnia ambigua* confirms that suspended clay reduces the algae intake by 87% when exposed to 200 mg/L of suspended clay (Kirk, 1991). This also supports that the exposed daphnids had a slower growth curve than the control daphnids. The starvation of individuals in an aquatic system can impact the future generation of daphnids and affect the population level in the long term (Walker et al., 2012).

3.2.4 Element quantification daphnids

Analyses of phosphorus (P) concentration in daphnia samples with ICP-MS show a negative correlation (Pearson, R= - 0.65) with TSS (Figure 16). This indicates a decrease in an essential element in the daphnids with increased exposure. However, a strong positive correlation (Pearson, R= 0.81) between P concentration and the daphnid weight (mg) was seen (Figure 17). Earlier analysis performed by Skogsberg of the particles identified that the particles contain 0.12% of P (Skogsberg, 2022). But since P and TSS have a negative correlation, P was used as an estimate of daphnia.

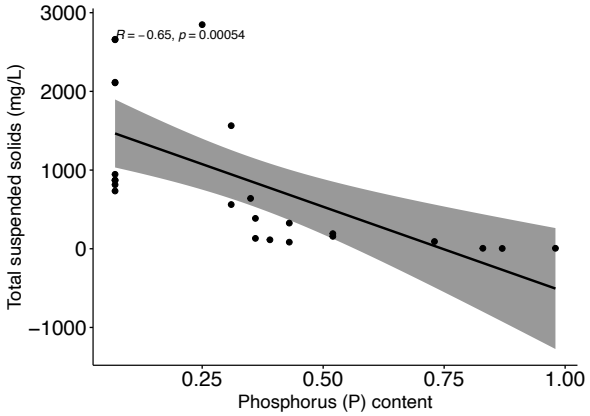


Figure 16: Correlation between total suspended solids (TSS) and phosphorus (P) content in exposed daphnids for the 72 hours test. (Pearson: R-value = -0.65, p-value = 0.00054)

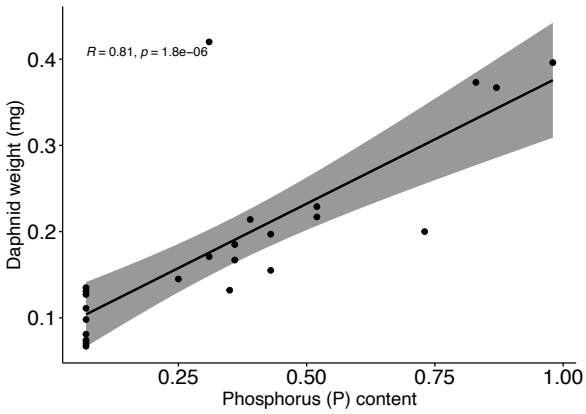


Figure 17: Correlation between the daphnid's weight (mg) and phosphorus (P) content for the 72 hours test. (Pearson: R-value = 0.81, p-value = 1.8e-06)

Results indicate that several metals (e.g., Al, Fe, Mn) and rare-earth elements (REE) (e.g., Y, Tm, La, Gd) were in close relationship with high particle exposure. Since the elements quantified in the daphnids can potentially be from the dissolved phase as well as particle uptake. Two elements, Yttrium (Y) and Thorium (Th) we selected for correlation tests with TSS and *D. magna* to indicate particle concentration associated with daphnia at different TSS exposures. These two elements have a low leaching capacity. This was confirmed with the leaching test performed by Hansen (2022). Additionally, a pilot leaching experiment performed by Skogsberg of Verket particles (pH: 6.5-7.5, 96h, 100 to 5000 mg/L) showed that 99.9% of Th was in the particles rather than being dissolved (Skogsberg, 2022). Cardon et al. (2019) also confirm the low leaching capacity of Y (Cardon et al., 2019). Therefore, these elements were more likely associated with being taken up via particles associated with the daphnids rather than elements from the dissolved phase.

3.2.4.1 Thorium (Th)

Thorium (Th), a radioactive metal (Ma et al., 2016), has a positive correlation with TSS (Kendall, R= 0.47) (Figure 18) and a negative correlation with P (Kendall, R= -0.34) (Figure 19). This indicates the content of the element in the daphnids increased with the increasing concentration of TSS.

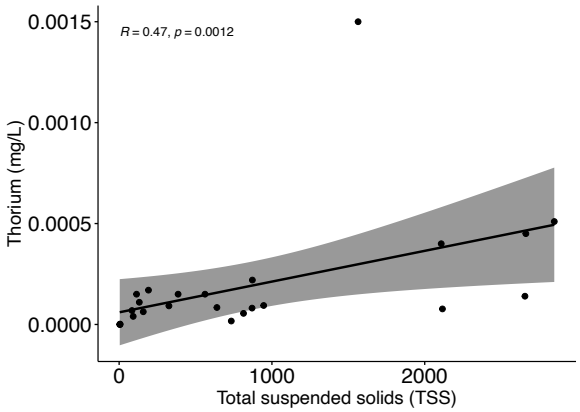


Figure 18: Correlation between total suspended solids (TSS) and thorium (Th) content in exposed daphnids for the 72 hours test (Kendall: R-value = 0.47, p-value = 0.0012).

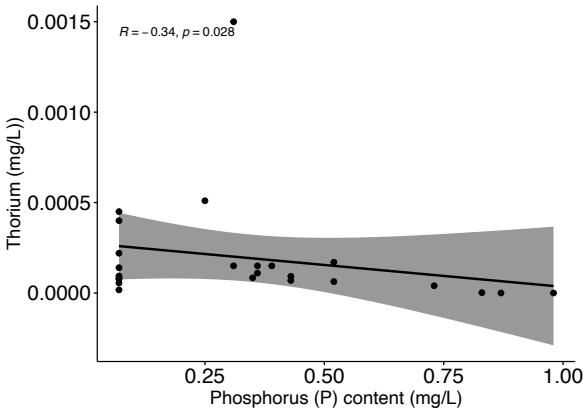


Figure 19: Correlation between thorium (Th) and phosphorus (P) content in exposed daphnids for the 72 hours test (Kendall: R-value = -0.34, p-value = 0.028

3.2.4.2 Yttrium (Y)

Yttrium (Y), a rare earth element (Cardon et al., 2019), had a strong positive correlation with TSS (Kendall, R=0.79) (Figure 20). The element had a negative correlation with phosphorus (Kendall, R= -0.68) (Figure 21). This indicates that there was an increase in the concentration of Y associated with daphnids when the concentration of TSS increased in test exposure.

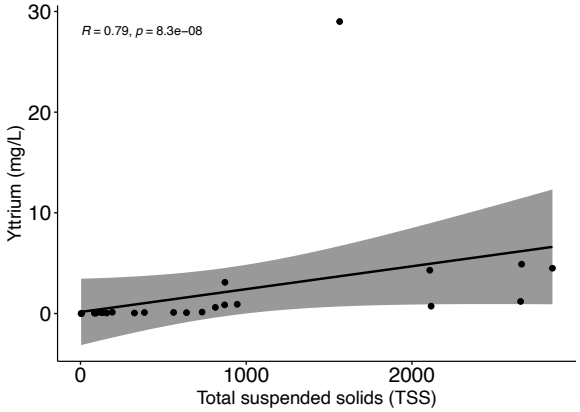


Figure 20: Correlation between total suspended solids (TSS) and yttrium (Y) content in exposed daphnids for the 72 hours test (Kendall: R-value = 0.79, p-value =8.3e-08).

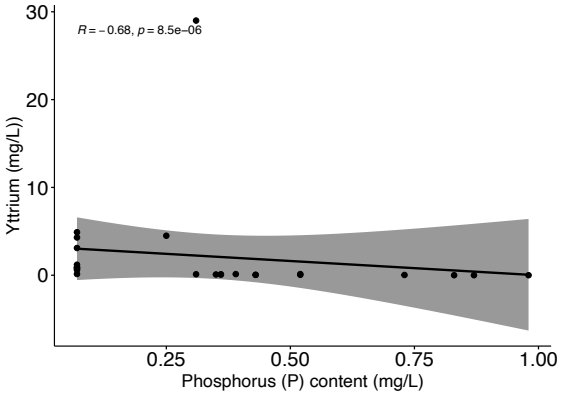


Figure 21: Correlation between yttrium (Y) and phosphorus (P) content in exposed daphnids for the 72 hours test (Kendall: R-value = -0.68, p-value = 8.5e-06).

3.2.5 Environmental Scanning Electron Microscopy (ESEM)

The images from the Environmental Scanning Electron Microscopy (ESEM) (Figure 22-27) show the filter apparatus of daphnids exposed to tunnel particles. The filter-feeding species has an apparatus consisting of a mesh screen. The screens were like combs of setae, setules, and setular bosses (Riisgård et al., 2015). The daphnid in the images (Figure 22, 23, 24, and 25) was exposed to 1200 mg/L TSS, and the photos show some particles in the filter comb.

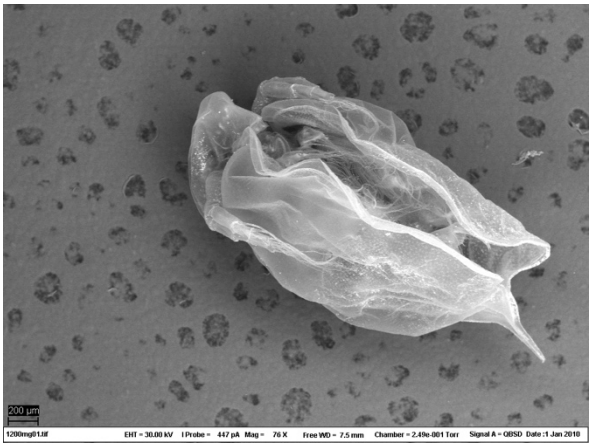


Figure 22: A SEM image of one D. magna exposed for tunnel particles from a 1200 mg/L starting concentration. Magnification: 76 X.

Figure 23: A SEM image of one D. magna exposed for tunnel particles from a 1200 mg/L starting concentration. Magnification: 1.39K X.

Figure 24: A SEM image of one D. magna exposed for tunnel particles from a 1200 mg/L starting concentration. Magnification: 350 X.

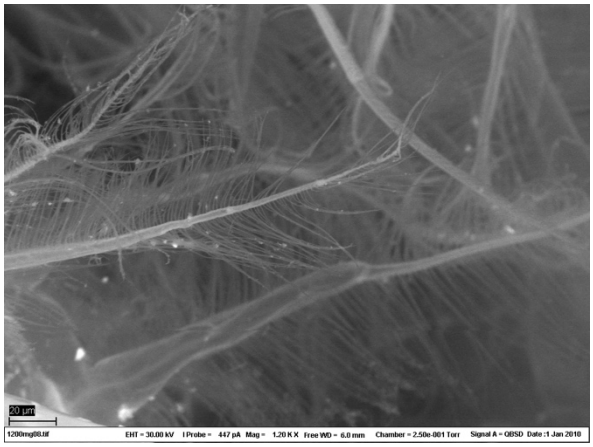


Figure 25: A SEM image of one D. magna exposed for tunnel particles from a 1200 mg/L starting concentration. Magnification: 1.20K X.

The ESEM images show the daphnid sampled from the 3000 mg/L treatment. The daphnia contained many particles; therefore, the closer images mostly show particles. Figure 26 shows the daphnid's outline consisting of many particles.

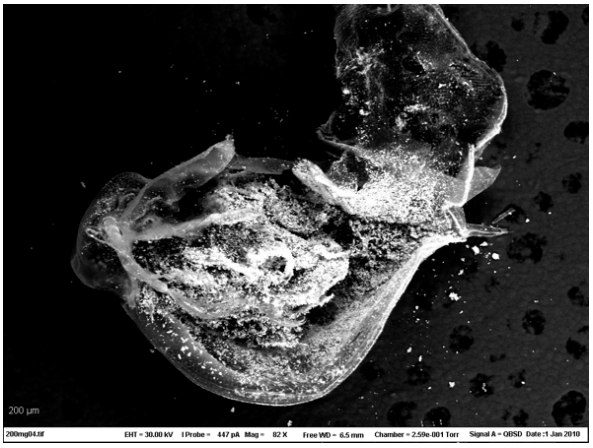


Figure 26: A SEM image of one D. magna exposed for tunnel particles from a 3000 mg/L starting concentration. Magnification: 82 X.

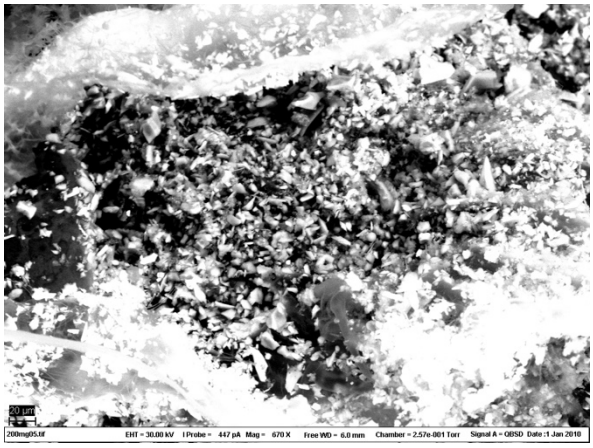


Figure 27: A SEM image of one D. magna exposed for tunnel particles from a 3000 mg/L starting concentration. Magnification: 670 X.

The ESEM images from the daphnid exposed to 1200 mg/L TSS show few particles in the daphnid´s filter comb. Also, the study by Hessen (1992) showed particles in the filter apparatus for the daphnids exposed to particles. In the same studies by Hessen, images done with the microscope did not show any negative impact on the filter apparatus (Hessen, 1992).

3.2.6 Dose-response curves - immobilization

Both tests (48h and 72h), showed an increase in immobile daphnids as the TSS concentration increased. Dose-response curves (Figure 28 and Figure 29) showed the response of increasing concentration of tunnel construction particles on *D. magna*. The 48-hours test results in an EC_5 value of 271.73 (± 180.9) mg/L. EC_5 is defined as the concentration which immobilizes 5% of the daphnids according to the OECD guideline (OECD, 2004). The EC_{50} value was 761.17 (± 532.91) mg/L and the EC_{90} value was 1641.76 (± 2361.33) mg/L. None of the starting nominal concentrations resulted in complete immobilization. Meanwhile, the 72 hours test resulted in a EC_5 value of 395.1 ± 136.52 mg/L, EC_{50} value was 649.77 ± 82.92 mg/L, and the EC_{90} value was 941.87 ± 271.59 mg/L. For NOEC, LOEC, EC_5, and EC_{10} the values for the 48h test were lower than the 72h test (Table 10). In contrast, the EC_{50} and EC_{90} were lower for the 72h test. This indicates that the higher concentrations of tunnel construction particles were more toxic for the daphnids when they were exposed for a longer duration.

Table 10: Estimated concentrations of the effect of tunnel construction particles on D. magna.

	48h exposure	72h exposure
NOEC	492 mg/L	562 mg/L
LOEC	504 mg/L	640 mg/L
EC_5	271.73 ± 180.9 mg/L	395.1 ± 136.52 mg/L
EC_{10}	352.9 ± 169.55 mg/L	448.26 ± 120.56 mg/L
EC_{50}	761.17 ± 532.91) mg/L	649.77 ± 82.92 mg/L
EC_{90}	1641.76 ± 2361.33 mg/L	941.87 ± 271.59 mg/L

The EC_{50} value for immobilization for both tests was higher than normally used values for permits in Norway. Therefore, these are most likely more relevant for acute episodes where higher concentration is suspended for shorter periods.

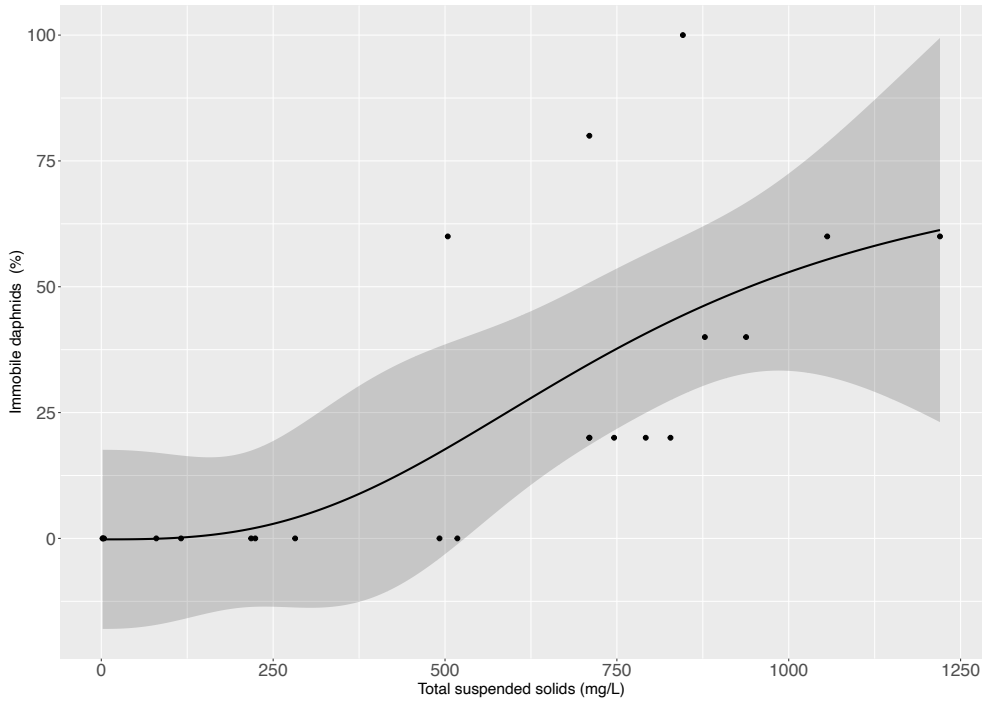


Figure 28: The dose-response curve for immobilization of D. magna exposed to tunnel particle particles in the laboratory for 48 hours. Y-axis: percentage of immobile daphnids. X-axis: TSS in mg measured at the end of the test.

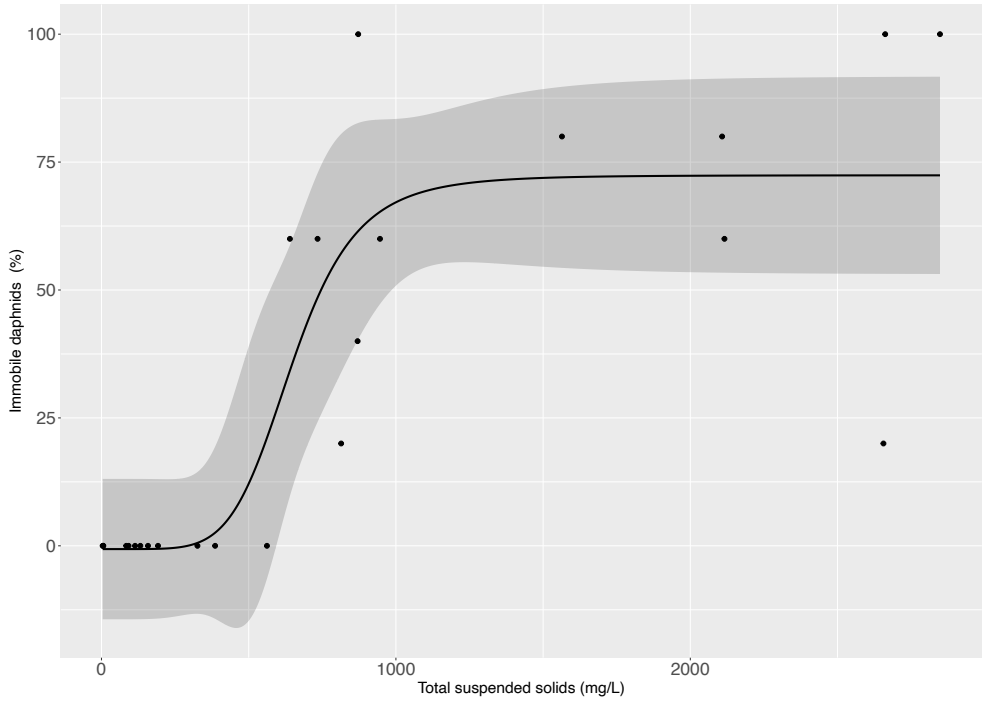


Figure 29: Dose-response curve for immobile daphnids exposed to tunnel construction particles (mg/L) for 72 hours. Y-axis: percentage of immobile daphnids. X-axis: TSS in mg measured at the end of the test.

During the first couple of hours of the 48 hours test, one daphnid in one of the 200 mg/L beakers was observed immobile at the bottom of the replicate on top of the mesh. The daphnid stayed there for the rest of the test duration and was identified as immobile at the end of the test. Reporting from the 72 hours extended test indicates that the immobilization was random. Therefore, this one daphnid was excluded from the statistic.

Earlier studies report that enhanced concentrations of inorganic particles in water bodies interfere with the filter apparatus of filter-feeding species. The particles clog the apparatus and the species starves to mortality (Langer, 1980). In a 72h exposure studying the survival and reproduction of *D. magna* harmful levels of kaolinite, montmorillonite and charcoal were identified as 392, 102, and 82 mg/L, respectively (Alabaster et al., 1982; Newcombe et al., 1991). The study by Hessen (1992) showed no effect on the survival of adult daphnids exposed to 100 mg/L of tunnel construction particles for 7 to 8 days (Hessen, 1992). Weltens et al. (2000) identified a difference in contaminated and not contaminated SS on daphnids. The study indicated that 500 mg/L of uncontaminated SS did not cause any mortality during the test period (48h). In contrast, particles, which were artificially contaminated by cadmium and zinc lead to mortality (Weltens et al., 2000).

Several studies have investigated the effect of microplastic (MP) on *D. magna*. MPs differs in material and shape and is often considered irregular compared to natural eroded particles (Pabst et al., 2015; Zimmermann et al., 2020). 9 days old daphnids were exposed to MPs for 120h, which gave an EC50 value of 52 mg/L (17.7-152.3). The experiment was performed on juveniles, and the result showed that they were around 50% more sensitive than the adults. In 2017, Frydkjær et al. investigated the differences in effect between regular and irregular-shaped microplastic on *D. magna.* The study resulted in a higher immobilization, longer egestion, and longer gut time for the daphnids exposed to the irregular-shaped particles (Figure 30) (Frydkjær et al., 2017).

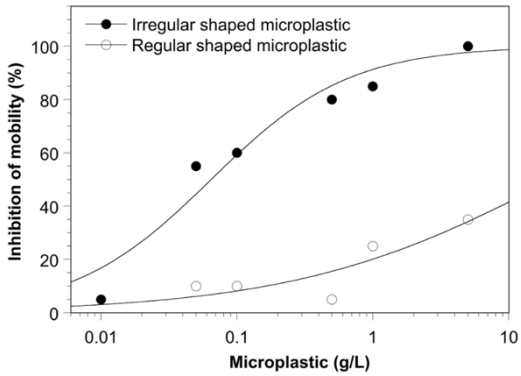


Figure 30: Dose-response of inhibition of mobility for daphnids exposed to microplastic for 48 hours. Specie exposed for both irregular- and regular-shaped microplastic (Frydkjær et al., 2017).

3.3 Cause of effects

The enhanced exposure concentrations of tunnel construction particles show a negative effect on *D. magna*. Earlier studies show tunnel particle exposure can cause lethal-, sublethal, and behavioral effects on the specie (Hessen, 1992; Newcombe et al., 1991). Although the daphnids were exposed to environmental samples, with potential for many stressors. Effects obtained were most likely to the effect of TSS and not the other stressors. This is supported by measurements of several potential stressors (such as conductivity, ammonia, temperature, DO, and pH) that were excluded as they were not over NOEC.

Elements from the dissolved phase have the potential to negatively impact the daphnids. Furthermore, the adjusted values of some metals from Hansen´s (2022) leaching experiment indicated good water quality according to the guideline by The Norwegian Environmental Agency. Therefore, these were not expected to be the reason for mortality. The specie, ingest

particles unselectively and therefore the tunnel construction particles have caused a decrease in nutrition uptake. Nutrition failure will most likely be a chronic effect of exposure. The presence of tunnel particles in the filter aperture was confirmed with the ESEM images and supported with element quantification, where particle elements (Y and Th) with low leaching capacity were identified in the exposed daphnids.

3.4 Further work

Further research should be conducted on unselective filter feeders (e.g., *D. magna*), focusing on the chronic effects, like reproduction and nutrition deficiency. Perhaps a different set-up, with bigger beakers would not negatively impact the neonates or the exposed adults could be transferred to a fresh medium, and differences between the control and exposed could be identified. Additionally, acute studies should investigate the differences between particles with different morphology and geochemical origin to obtain a broader knowledge of the effect of tunnel construction particles.

4 Conclusion

Hypothesis one is supported as the present study indicates that particles from tunnel construction suspended in the water column had a toxic effect and immobilize *D. magna.* In two tests (48h and 72h), there was an increase in immobilization when the concentration of particles increased. The EC_5 value was 271 (± 180) mg/L for the 48h test and 395 (± 136) mg/L for the 72h test. The EC_5 values for both tests were lower than the permit usually given for marine waters on Norwegian construction sites. The *D. magna* are a freshwater specie and the EC_5 values were over the normally permitted value of 100 mg/L. The EC_{50} values were 761 (± 532) mg/L for the 48h and 649 (± 82) mg/l for the 72h test.

The particles were within the daphnids' feeding size, and the use of element quantification of particles with low leaching elements (Y and Th) and imaging with ESEM indicates the ingestion of particles. An important finding was the decrease in the weight of the daphnids as the TSS increased. This also supports that those particles affected the growth of daphnids most likely due to the ingestion of the inorganic particles. Because the animals ingested these particles, they got a smaller proportion of nutrition and therefore, did not have the same growth rate as the control.

The potential decrease in the *D. magna* population can have high ecological relevance. Several other species in the aquatic system may be influenced by the decrease. Further research should be conducted on the chronic effects on daphnids and other aquatic biotas.

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6 Appendix

6.1 Appendix 1

Elendt M7 medium

The Elendt M7 medium (10x) was prepared in 10-liter cans with distilled water. An air pump supplied air and the medium was kept in the dark. The medium was prepared at least 24 hours before use.

Table 11: Elendt M7 medium stock solution 2.

M7 Stock II Solution Recipe				
Chemical	**mg/L**	**mL/0.5L**	**mL/L**	**10x (mL/L)**
H3BO3	57190	0,125	0,25	2,5
MnCl2	4584,585	0,125	0,25	2,5
LiCl	6120	0,125	0,25	2,5
RbCl	1420	0,125	0,25	2,5
SrCl2*6H2O	3040	0,125	0,25	2,5
NaBr	320	0,125	0,25	2,5
MoNa2O4*2H2O	1260	0,125	0,25	2,5
CuCl2*2H2O	335	0,125	0,25	2,5
ZnCl2	260	0,5	1	10
CoCl2*6H2O	200	0,5	1	10
KI	65	0,5	1	10
Na2SeO3	43,8	0,5	1	10
NH4VO3	11,5	0,5	1	10
Fe-EDTA solution		2,5	5	50

Table 12: Elendt M7 medium recipe.

M7 Media Recipe			
Stock	**mL/L**	**mL/10L**	**mL/20L**
Stock Solution II (10x)	5	50	100
CaCl2*6H2O	1	10	20
MgSO4*7H2O	0,5	5	10
KCl	0,1	1	2
NaHCO3	1	10	20
Na2SiO3*9H2O	0,2	2	4
NaNO3	0,1	1	2
KH2PO4	0,1	1	2
K2HPO4	0,1	1	2
Vitamin Stock	0,01	0,1	0,2

6.2 Appendix 2

20% Z8 medium

20% Z8 medium were prepared under sterilized conditions with deionized water. This was made with water under sterilized conditions and stocks Z-I, Z-II, Z-III, and trace element was added (Table 13). In addition, 40 or 50 mL of the algae was added to the mixture, depending on the size of flask.

Table 13:20% Z8 media for algae culturing.

20% Z8 Media		
Stock	**mL/4L**	**mL/5L**
Z-I	3,2	4
Z-II	0,8	1
Z-III	8	10
Trace Elements	0,8	1

6.3 Appendix 3

Feeding guide

Recommended feeding (cells/day/daphnia) during culturing of *D. magna*.

Table 14: Volume of recommended algae supply for daphnids depending on age from the daphnia feeding guide.

Age daphnids	**Recommended Feed (Cells/day/daphnia)**
Neonates (up to 4 days)	5.25E+06
4-8 days old	1.05E+07
For adults	2.10E+07

6.4 Appendix 4

Measured parameters test solution

Parameters that were measured in the test solutions. Dissolved oxygen was only measured in controls and the highest starting concentrations.

Table 15: Measured parameters in the test solution for the 48 hours test at the end.

Starting concentration	Replicate	Dissolved oxygen	Temperature	pH	Conductivity (µS/cm)
Control	1	8.95	21.5	8.91	637
Control	2	9.1	21.1	8.88	600
Control	3	9.18	20.7	9.04	590
200 mg/L	1		21.3	9.1	621
200 mg/L	2		21.1	9.07	617
200 mg/L	3		20.9	9.17	616
400 mg/L	1		21.3	9.17	630
400 mg/L	2		21.1	9.02	637
400 mg/L	3		20.9	9.13	635
800 mg/L	1		21	9.04	670
800 mg/L	2		20.9	9.04	672
800 mg/L	3		20.9	8.97	675
1200 mg/L	1		22.3	8.94	725
1200 mg/L	2		22.7	8.91	724
1200 mg/L	3		22.5	8.93	735
1600 mg/L	1		22.5	9.02	757
1600 mg/L	2		22.1	9.4	745
1600 mg/L	3		22.2	9.15	757
2400 mg/L	1		22.4	8.94	827
2400 mg/L	2		22.4	9.02	834
2400 mg/L	3		22.3	8.96	829
3000 mg/L	1	9.32	22.7	8.86	897
3000 mg/L	2	9.6	22.9	8.81	889
3000 mg/L	3	9.47	22.6	8.86	897

Table 16: Measured parameters in the test solution for the 72 hours test at the end.

Starting concentration	Replicate	Dissolved oxygen	Temperature	pH	Conductivity (µS/cm)
Control	1	9.24	21.7	9.04	666
Control	2	9.42	21.4	9.02	647
Control	3	9.12	21.2	8.66	645
200 mg/L	1		21.7	9.32	662
200 mg/L	2		21.3	9.25	647
200 mg/L	3		21	9.25	675
400 mg/L	1		21.4	9.11	680
400 mg/L	2		21.3	9.14	679
400 mg/L	3		21	9.08	680
800 mg/L	1		21.5	8.93	712
800 mg/L	2		21.3	8.92	717
800 mg/L	3		21.2	8.94	726
1200 mg/L	1		22.5	9.06	761
1200 mg/L	2		22.2	9.01	762

1200 mg/L	3		22.2	9.04	652
1600 mg/L	1		22.4	9.12	795
1600 mg/L	2		22.5	9.13	780
1600 mg/L	3		22	9.08	782
2400 mg/L	1		22.7	8.8	907
2400 mg/L	2		22.8	8.79	911
2400 mg/L	3		22.2	8.79	878
3000 mg/L	1	8.94	22.8	8.72	972
3000 mg/L	2	9.33	23	8.67	971
3000 mg/L	3	9.29	22.8	8.8	974

6.5 Appendix 5

Total suspended solids and turbidity

Table 17: TSS and turbidity measured for the 48 hours test at the start, during, and at the end. TSS for 3000 mg/L replicate 3 there were some errors when analyzing.

Particles supplied	Replicate	FNU 0h	FNU 24h	FNU 48h	TSS 48h
Control	1	1.08	4.22	2.48	2
Control	2	1.33	3.38	1.78	4
Control	3	0.42	2.23	2.25	2
200 mg/L	1	111.5	15.64	63.54	116
200 mg/L	2	95.95	11.49	12.45	80
200 mg/L	3	108.3	12.55	41.93	116
400 mg/L	1	224.0	53.35	103.2	224
400 mg/L	2	239.7	106.7	144.7	282
400 mg/L	3	250.2	51.45	47.55	218
800 mg/L	1	446.6	190.2	45.67	518
800 mg/L	2	388.2	135.4	218.1	492
800 mg/L	3	458.1	119.1	190.5	504
1200 mg/L	1	640.0	549.0	511.2	846
1200 mg/L	2	594.3	384.4	396.7	710
1200 mg/L	3	825.4	413.8	430.3	746
1600 mg/L	1	756.2	334.3	390.6	710
1600 mg/L	2	749.7	229.9	195.7	879
1600 mg/L	3	682.8	171.2	400.8	710
2400 mg/L	1	933.1	354.8	618.4	828
2400 mg/L	2	779.0	405.6	382.4	938
2400 mg/L	3	860.0	134.3	381.5	792
3000 mg/L	1	918.3	556.2	757.3	1220
3000 mg/L	2	894.4	265.3	666.2	1056
3000 mg/L	3	935.5	152.9	459.9	XXX

Table 18: TSS and turbidity measured for the 72 hours test at the start, during, and at the end. TSS for 3000 mg/L replicate 3 there were some errors when analyzing.

Particles supplied	Replicate	FNU 0h	FNU 24h	FNU 48h	FNU 72h	TSS 72h
Control	1	0.54	3.61	2.07	2.49	4
Control	2	0.3	2.19	2.17	3.2	6
Control	3	0.27	3	1.71	1.8	6
200 mg/L	1	106.6	4.65	3.83	26.03	92
200 mg/L	2	117.2	21.72	9.54	20.92	84
200 mg/L	3	115.1	15.38	12.02	44.9	158
400 mg/L	1	231.1	13.97	19.01	39.88	114
400 mg/L	2	221.2	21.18	33.44	68.67	192
400 mg/L	3	222.7	34.5	34.28	68.46	132
800 mg/L	1	417.9	57.22	52.27	173.9	386
800 mg/L	2	396.5	166.8	123.1	195.2	562
800 mg/L	3	413.8	41.71	72.82	44.54	326
1200 mg/L	1	585.5	60	230.7	214.1	640
1200 mg/L	2	635.5	113.7	173.4	172.4	872
1200 mg/L	3	631.6	71.03	222.4	143.6	734
1600 mg/L	1	759.9	202.8	178.5	92.67	870
1600 mg/L	2	820.4	132.5	199.8	547.8	814
1600 mg/L	3	716.2	230.8	254.8	115.8	946
2400 mg/L	1	947.9	619.5	687.3	912	2108
2400 mg/L	2	969.8	534.4	635.3	856.7	2116
2400 mg/L	3	980.3	630.3	867.6	233.2	1564
3000 mg/L	1	651.6	873.5	805	680.8	2656
3000 mg/L	2	784.3	809.1	938.3	889.6	2662
3000 mg/L	3	800.9	904.1	913.2	908.4	2848

6.6 Appendix 6

Table 19: Immobile daphnids in each replicate in percent for both tests.

Particles supplied	Replicate	Immobile daphnids (%) 48 hours test	Immobile daphnids (%) 72 hours test
Control	1	0	0
Control	2	0	0
Control	3	0	0
200 mg/L	1	0	0
200 mg/L	2	0	0
200 mg/L	3	20	0
400 mg/L	1	0	0
400 mg/L	2	0	0
400 mg/L	3	0	0
800 mg/L	1	0	0
800 mg/L	2	0	0
800 mg/L	3	60	0
1200 mg/L	1	100	60
1200 mg/L	2	20	100
1200 mg/L	3	20	60
1600 mg/L	1	20	40
1600 mg/L	2	40	20
1600 mg/L	3	80	60
2400 mg/L	1	20	80
2400 mg/L	2	40	60
2400 mg/L	3	20	80
3000 mg/L	1	60	20
3000 mg/L	2	60	100
3000 mg/L	3	60	100

6.7 Appendix 7

Environmental Scanning Electron Microscopy (ESEM):

Fixative

100ml of fixative to SEM samples

- 50ml 4% PFA Para-formalin (keep in fridge at 4°C)
- 25ml 0.4M PIPES buffer (keep in fridge at 4°C)
- 5ml 25% Glutaraldehyde (keep in fridge at 4°C)
- 20ml dH_20

Mixed the solutions a couple of days in advance of the experiment in a glass jar and kept in the fridge at 4°C until the end of the experiment.

Tissue fixation

1. Whole daphnids were put into glass vials with fixative (the fixative covered the tissue) how fast?
2. The daphnids were placed in a fridge at 4°C for about x weeks

Dehydration

The dehydration process followed the protocol below. In short, a pipet was used to take out most of the fixative from the glass vial without touching the tissue and discharged the fixative in a plastic flask. Next, 0.1M PIPES buffer was added, and the glass vials were put on a vibration board for 10 min. This procedure was done 3 times. Next, the buffer was replaced with increasing ethanol concentration (30-100%) as explained below. After reaching 100% ethanol, the ethanol with this concentration were replaced 4 times.

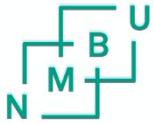
Critical point drier

The daphnids in ethanol were carefully but quickly transferred to small metal cassettes, still submerged in 100% ethanol with a pipette. The metal cassettes were transferred to the CPD.

Table 20: The procedure of SEM imaging.

Dehydration to CPD for SEM				
Date: Name Institute Sample ID	18/2-22 Emelie Skogsberg			
Step	Solution	Duration	Done	Comment
Fixation	2%PA+1.25%GA+0.125M PIPES buffer (pH 7)	About x weeks		
Washing	0.05M PIPES buffer, pH 7	5-15min	1. 2. 3.	
Dehydration	30% EtOH 50% EtOH 70% EtOH 90% EtOH 96% EtOH 100% EtOH (abs alcohol)	5-15min 5-15min 5-15min 5-15min 5-15min 5-15min	1. 1. 1. 1. 1. 1. 2. 3. 4.	

Critical Point Drying
Comment:



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