Master's Thesis

Application to detect toxicity of recycled plastics using boar spermatozoa motility

Milja Aalto

University of Jyväskylä

Department of Biological and Environmental Science

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Aalto, Milja

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Supervisors: Associate Professor Suvi Ruuskanen and Doctoral Researcher Benjami Laine and Professor Marja Tiirola
Tarkastajat: Doctor of Science Eeva-Riikka Vehniäinen ja Associate Professor Sami J. Taipale
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Plastic as waste decomposes slowly and the quantities are large. Therefore, plastic recycling is essential. However, a key problem with recycled plastic and plastic products is their additives, such as plastic softeners and inhibitors for mold and bacteria, which contain chemicals that may be toxic to mammalian cells. Harmful additives are difficult to avoid as the manufacturer is not obliged to inform about them. Therefore, in recycled plastic mixtures, their exact content and toxicity, is not known. The aim of this study was to apply an assay method to measure changes in boar spermatozoa motility using the automated sperm analysis program (ISAS v1) with a particular focus on recycled plastic. The applied assay method did not give particularly accurate results. The inaccuracy of the results was affected e.g. by a large natural variability, deviation between replicate samples, and the computer program which did not calculate motility completely reliably. Despite the use of the ISAS program, the person doing the work also had to use subjective selection. The assay method works most reliably when there is a substantial change in spermatozoa motility within three days due to the chemical, and the semen is of good quality, in which case the negative control motility reduces only slightly. As part of application process, a case study was carried out, where spermatozoa motility measurements were conducted after exposure to two types of recycled plastic, a grocery store's plastic bag (90% was recycled plastic) and for Barbie doll's leg (from the 70s). In the tests, the negative control was the semen as it came from the artificial insemination station, and in the positive control chemical triclosan was used, which is an antibacterial agent used also in plastics. With this assay method and equipment and using 2 µg/ml triclosan, 50% of rapid movement of boar spermatozoa motility was lost between 1 and 3 days of exposure. The assay method gave indicative results that the plastic bag and Barbie contain substances toxic to boar spermatozoa. Rapid movement of boar spermatozoa decreased 53% when exposed to a piece of plastic bag (2x3 cm) and 60% when exposed to pieces of Barbie's leg (2 pieces of 0,5x2 cm) for three days. Overall, the case study demonstrated that presented assay method can be used to study the toxicity of recycled plastics.

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Tarkastajat: Filosofian tohtori Eeva-Riikka Vehniäinen ja apulaisprofessori Sami J. Taipale
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Muovi jätteenä hajoaa hitaasti ja määrät ovat suuria. Siksi muovin kierrätys on välttämätöntä. Kuitenkin kierrätysmuovin ja yleensä muovituotteiden keskeinen ongelma on lisäaineet, kuten muovin pehmennysaineet ja homeen ja bakteerien estäjät, jotka saattavat sisältää nisäkässoluille myrkyllisiä kemikaaleja. Haitallisia lisäaineita on vaikea välttää, koska valmistajalla ei ole velvollisuutta ilmoittaa niistä ja siksi kierrätysmuoviseoksien tarkkaa sisältöä ja myrkyllisyyttä ei tunneta. Tämän tutkimuksen tavoitteena oli menetelmäsovellus, joka mittaa muutosta sian siittiöiden liikkuvuudessa automaattisella siittiöanalyysillä (ISAS v1 tietokoneohjelma), erityisesti kierrätysmuoveihin liittyen. Sovellettu menetelmä ei antanut erityisen tarkkoja tuloksia. Tulosten epätarkkuuteen vaikutti mm. suuri luonnollinen vaihtelu, hajonta rinnakkaisnäytteiden välillä ja tietokoneohjelma, joka ei laskenut liikkuvuutta täysin luotettavasti. ISAS-ohjelman käytöstä huolimatta työn tekijä joutui käyttämään myös subjektiivista valintaa. Menetelmä toimii luotettavimmin, kun siittiöiden liikkuvuudessa tapahtuu merkittävä muutos kolmen päivän aikana johtuen kemikaalista ja siemenneste on hyvälaatuista, jolloin negatiivisen kontrollin liikkuvuus vähenee vain hieman. Osa soveltamisprosessia oli tapaustutkimus, jossa siittiöiden liikkuvuuden muutosta mitattiin sen jälkeen, kun niitä oli altistettu kahdentyyppiselle kierrätetylle muoville, ruokakaupan muovipussille (90 % kierrätysmuovia) ja Barbie-nuken jalalle (70-luvulta). Testeissä negatiivisena kontrollina oli siemenneste sellaisenaan kuin se tuli keinosiemennysasemalta ja positiivisessa kontrollissa käytettiin triklosaania, joka on myös muoveissa käytettävä antibakteerinen aine. Tällä menetelmällä ja laitteistolla sekä altistuksella triklosaanille (2 µg/ml), sian siittiöiden nopea liike väheni 50 % yhden ja kolmen päivän altistuksen välisenä aikana. Menetelmällä saatiin suuntaa antavat tulokset, että muovipussi ja Barbie sisältävät sian siittiöille toksisia aineita. Siittiöiden nopea liike väheni 53 %, kun niitä altistettiin muovipussin palaselle (2x3 cm) ja 60 %, kun niitä altistettiin Barbien jalalle (2 palasta, 0,5x2 cm) kolmen päivän ajan. Kaiken kaikkiaan tapaustutkimus osoitti, että esitettyä menetelmää voidaan käyttää kierrätettyjen muovien myrkyllisyyden tutkimiseen.

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TERMS AND ABBREVIATIONS

Terms

CASA	Computer-Aided Semen Analysis: automated instruments that use cameras and software to analyze data obtained by microscopic evaluation in order to provide semen parameter results
EC_{50}	A 50% loss of rapid movement of spermatozoa
ISAS v1®	Integrated Semen Analysis System, a commercial program for examining sperm
Triclosan	Antibacterial and antifungal chemical, widely used in personal care and medical disinfection products

1 INTRODUCTION

Plastic waste is a key environmental problem. It is a ubiquitous material, decomposes slowly and thus efficient recycling is important to overcome the quantitative challenge. In addition to that, chemicals and additives are added to plastics and many of them are cytotoxic. Therefore, it should be obvious that plastics in production will be a major problem in future. A considerable amount of additives are used in plastics (Verma *et al.* 2016), which make them durable, flexible (plasticizers, phthalates), less flammable (fire retardants), grease-resistant (fluorinated chemicals, PFAS) or sterile (biocides) (Petrlik *et al.* 2021). Many of these additives are toxic and leak from the products during use, as they break down and become brittle, during recycling and from recycled products (Petrlik *et al.* 2021). Toxic chemicals associated with plastic waste have an impact on human and animal health (Verma *et al.* 2016). For the consumer plastic additives are problematic because the manufacturer is not obliged to inform about them. Especially, in recycled plastic mixtures, their exact content and toxicity, is not known.

The toxicity of plastics has been minimally tested so far. I think it is useful to test plastic materials as well, not just pure raw materials in them. Plastic mixtures can behave in different ways than raw materials by themselves, they can strengthen or weaken each other's effect. This is a viable option, especially in the recycled plastic mixture, where recycling process can even generate new toxic chemicals (Brosché *et al.* 2021). Boar (*Sus domesticus*) spermatozoa have been shown to be sensitive to toxic compounds in plastics, as fertility problems have occurred due to toxic compounds in multi-layered plastic bags of semen (Nerín *et al.* 2014; Schulze *et al.* 2020).

Boar spermatozoa are widely used as indicator cells in various toxicity studies (Andersson *et al.* 2010; Vicente-Carrillo *et al.* 2015; Castagnoli *et al.* 2018; Vicente-Carrillo 2018). Boar semen is easily commercially available, collecting is painless for the animal and the operation is ethically acceptable (Vicente-Carrillo 2018). Metabolism of spermatozoa is simple compared to somatic cells, i.e. non-germ cells (Andersson 1999). The functions and metabolism of the spermatozoa are greatly influenced by the function of its cell membrane (Harrison 1997; Andersson 1999). Boar spermatozoa are particularly suitable as test cells because the steroid content of their cell membrane is low, which enables chemicals to affect the cell (Paulenz 1993; Andersson 1999). Among the functions of semen, spermatozoa motility is the one most likely to be affected by the external environment, e.g. toxins (Long *et al.* 2018), because spermatozoa are highly dependent on mitochondrial production and consumption of ATP for their metabolism (Vicente-Carrillo *et al.* 2015). Compared to drug-induced mitochondrial toxicity testing, which has traditionally been assessed in isolated mitochondria from killed animals, and in cell-based *in vitro* models such as

human hepatocytes, sperm testing is fast, easy and cost-efficient (Vicente-Carrillo 2018). The main challenge in sperm testing is the standardization and optimization of the equipment and procedures related to CASA systems to ensure accurate scientific and clinical results (Bompart *et al.* 2018).

The goal of this thesis was to establish an easy, reliable assay method to analyze cytotoxicity of plastics using boar sperm. The research questions were: What factors should be considered when applying this assay method and can the cytotoxicity of plastics be detected using this assay method? The assay included exposing commercial boar sperm to test materials in tubes and analyzing the effects on spermatozoa motility on slides using automated sperm analysis (ISAS v1 program). First, the equipment and processing methods were tested, and the final phase involved testing with two types of recycled plastic. In the tests, the storage and handling of the semen bag were tested and minimum requirements for spermatozoa liveliness were searched. Negative control, positive control and EC_{50} (lost of rapid movement) were investigated. Exposure conditions, correct mixing of the samples, the choice of objective slides, heating, and optimal pipetting methods were further tested. During the semen analysis different kinematic parameters and microscope adjustments were tested, and repeatability and randomization were examined. Solutions were sought for problems such as achieving uniform quality samples and increasing the accuracy of the automated measurements. As a case study were tested the plastic bag (90% recycled plastic) which represented a product made of recycled plastic, and Barbie doll (from the 70s) which represented a recycled plastic toy that today's children also play with. The hypothesis was that exposure to recycled plastic reduces the motility of boar spermatozoa.

2 MATERIALS AND METHODS

2.1 Practical set up

2.1.1 Plastics in laboratory materials

Since an assay method was applied for testing plastics, it was important to consider whether plastic laboratory materials might affect the test results. According to Andersson et al. (2010), plastic laboratory disposables can dissolve substances into ethanol and methanol, that are toxic to boar spermatozoa and there may be differences between brands. Plastic disposables were still chosen to use because of their usefulness (single use, cleanliness) and because of their price compared to glass disposables. Plastic tubes and pipette tips were chosen based on the knowledge that was available from previous studies (Andersson *et al.* 2010). In the present work polypropene tubes (5 ml, blue caps, lot: 210179, Mekalasi Ltd., Helsinki, Finland) from Nuova Aptaca were used. Pipette tips (Optifit Tips, non-sterile, 10 µl lot: 501637187, 200 µl lot: 501668703, 1000 µl lot:

501507590) were ordered from Sartorius AG (Goettingen, Germany, former name was Biohit Ltd.). Eppendorf tubes (1,5 ml, lot: 211125001, Mekalasi Oy, Helsinki, Finland) from Nuova Aptaca were used with the slide-coverslip.

Non-sterile was considered clean enough although some kind of plastic contamination is possible. Sterility does not prevent chemicals from dissolving and boar semen itself is not sterile. Chosen plastic disposables were not pre-tested nor compared to other plastic or glass disposables. The possibility of chemicals dissolving into plastic disposables should be considered in future uses. Plastic cloves were not used, because it is known that cloves can affect spermatozoa (Andersson *et al.* 2010).

2.1.2 Boar sperm

Fresh boar semen was acquired from artificial insemination station (Figen Ltd., Kauhava, Finland). Mix of five boar semen was ordered. No special permits were needed for the test. Boar semen doses (approx. 90 g) were shipped by courier service in a styrofoam box and delivery time was one day after extraction. During courier transport, the target temperature was +15–18 C, but there seemed to be challenges. Temperature was measured in one batch of semen after transportation in December. The temperature was 14 °C, but the liveliness of the spermatozoa was still good.

Promised best before time for semen was seven days. Figen diluted the semen before sending with commercial extender (MR-A®, Kubus S.A, Spain), (Figen unpublished data) and the function of the extender is to maintain spermatozoa motility. It was not possible to extend boar spermatozoa viability more, for example by freezing (Castagnoli *et al.* 2018; Maside *et al.* 2023). The target number of spermatozoa was 2.5–2.8x10^9 per 90 g (Figen unpublished data). The semen was not diluted, but it was used for the tests as it came from Figen. The dilution worked well as such for testing, the number of spermatozoa was sufficient. In general, a range between 2x10^6 and 50x10^6 sperm/ml is recommended particularly with a CASA system in breeding (Maside *et al.* 2023). Figen checks all their batches and promises that the minimum spermatozoa motility is 60% live spermatozoa (Figen Oy), which should be enough for fertilization (Jung *et al.* 2015). However, the motility of semen in each batch ordered did not always reach the 60% motility limit when compared to ISAS result parameter, 40% static % (Table 1).

Two different semen mix was used during the tests. In pre-tests 'Muskeli' mix was used and in the case study 'Supermuskeli' mix was used. The mix was changed in December because Muskeli mix had run out and Figen sent Supermuskeli mix instead. The difference between Muskeli and Supermuskeli is the muscle index, i.e. semen from the same boar can be present in Muskeli mix or Supermuskeli mix in different weeks (Figen Oy). Surprisingly, the spermatozoa of the Supermuskeli mix were livelier than of the Muskeli mix.

In the case study two different batches were used. The decision to use two batches from different weeks was based on to ensure the reliability of the results. According to Barquero et al. (2021a), there are variations in the motility and shape

of boar spermatozoa. Variation occurs between different breeds, lines and crosses, within a population and within the same animal (Barquero *et al.* 2021a). According to Harrison (1997) the quality and response of boar semen to treatments varies greatly.

2.1.3 The storage and handling of the semen bag

In the tests, one aim was to find out, how the semen bag should be stored and handled to keep the spermatozoa alive. The semen container can impact the stored semen: according to Nerín's et al. (2014) research, the chemicals leaching from the storage bags into the semen during the storage period of 1–7 days, were identified as the cause of decreased litter production among the swine. According to artificial insemination station Figen (unpublished data) the semen bag is the best storage for the semen and the bag does not pass oxygen when the container's 'straw' (for pouring the semen out) is closed. As a container for semen, I considered the storage bag, a glass bottle and a beaker covered with foil. The semen bag, where the semen was transported, was the best container for storage. The bag was easy to rock and the semen in the bag did not dry out. Based on the experience during the tests, the bag did not have any major effect on the vitality, spermatozoa stayed alive 5–18 days. In turn, proper mixing in the beaker was difficult and the vitality was worse, perhaps due to drying out. A glass bottle was not chosen to avoid excess plastic, as the glass bottle had a plastic cap which the semen would have had contact during mixing.

Storage temperature of the semen bag and light have an effect on spermatozoa viability (Vázquez and Navarro n.d.). If semen is exposed to too low or high temperatures motility diminishes rapidly (Schulze *et al.* 2013). Most used storage temperatures for boar semen are +15–18 °C (Castagnoli *et al.* 2018; Maside *et al.* 2023) and also, room temperature (+21–23 °C) has been used (Andersson *et al.* 2010; Vicente-Carrillo *et al.* 2015). It was decided to store the semen bags in a dark, +18 °C in compressor-cooled incubator (Memmert Gmbh) so that the storage temperature remained constant.

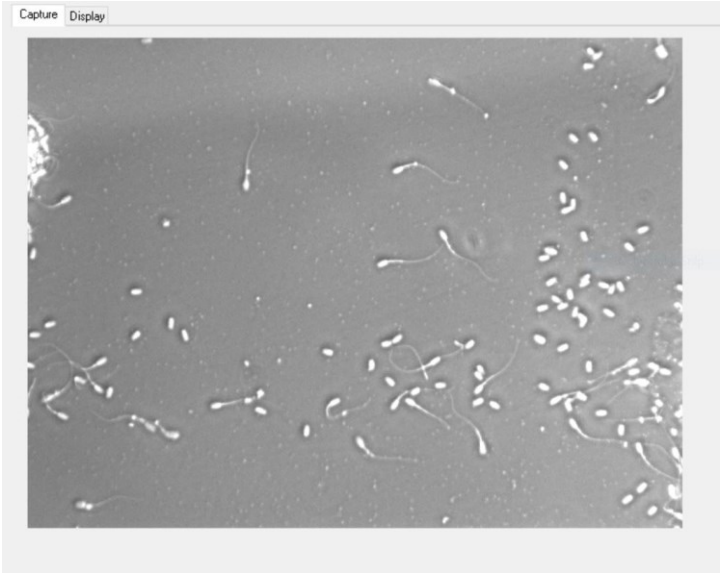
The semen bags were stored in the incubator both horizontally on the shelf and vertically in a Styrofoam box. The advantage of storing the bag on its side was that then the spermatozoa landed on the long side of the bag and the semen mixed faster. When the opened bag was stored upright, the opened straw did not need to be closed. If the bag was stored on its side, a stick and tape around it were used to close the straw.

Optimal handling and mixing the semen was also tested. In +18 °C most of the spermatozoa were in static state, and without mixing the spermatozoa sank to the bottom of the bag. Especially the older or poorer the quality of the semen, the faster the subsidence occurred. Therefore, the semen would need to be mixed before use, while extensive mixing could lead to tiring of the spermatozoa. Depending on species, they resistance to shaking varies (Vázquez and Navarro n.d.). It was found that the best way to mix the semen bag was to rock it gently about 50 times in all different directions. Gently shaking and slight rocking of the semen bag was not enough to get enough spermatozoa to the test tube and

therefore onto the slide. Rocking was better than shaking, because shaking the bag caused the semen to foam, and air bubbles are a nuisance when reading the slide with ISAS program (Picture 3). In practice, when mixing the bag, I kept the opened semen bag closed with my fingers by folding the bag under the straw and pinched tightly under the straw. I usually rocked the bag once a day when taking part of the semen into a beaker or test tube. During the tests, the semen bag was rocked for several days in a row and semen vitality did not suddenly collapse. When collapse happened, reason was poor quality at baseline (chapter 2.1.4) or because of long storage time.

2.1.4 Minimum requirements for spermatozoa liveliness

A total of five different batches of semen were used in the tests, over four months. There was great variability in the liveliness of semen batches: spermatozoa stayed motile from 5 to 18 days depending on the batch. In Picture 1 is a very poor-quality batch and other four batches did not look the same at any point. Because of the variation, it was decided that the tests should be started as soon as the semen arrived and not to make sequential tests with the same batch.



Picture 1. Picture of the spermatozoa batch with the poorest quality. Spermatozoa had lost their tails, and the solution was cloudy. Sample treatment: positive control and three-day exposure.

The estimation of minimum requirements of spermatozoa liveliness was done with poor batches. Out of five batches, two was very poor quality, and one good enough quality and could be used in the case study. Quality was estimated with ISAS parameters, Mobile Progressive % (rapid moving spermatozoa) and Static % (immotile spermatozoa). Table 1 shows the differences in vitality of two batches of which one was poor and another good enough quality. After three-day exposure, negative control dropped to 6% and to 29% depending on the batch. Same thing can be seen comparing negative controls in Figures 1 and 4. As

a minimum requirements of spermatozoa liveliness in a baseline were set to ≥35% of Mobile Progressive % and ≤50% of Static %. Of these results Mobile Progressive % was more reliable to monitor.

TABLE 1. Boar spermatozoa liveliness showed with result parameters, Static % and Mobile Progressive %. One-day refers to measurement after one-day exposure. Two batches of boar spermatozoa, one batch of poor quality and another of good enough quality. Sample processing: negative control (untreated) in the test tube.

batch of semen	Parameters (%)		
	Static % one-day	Mobile Progressive % one-day	Mobile Progressive % three-day
batch of poor quality	64	24	6
batch of good enough quality	48	34	29

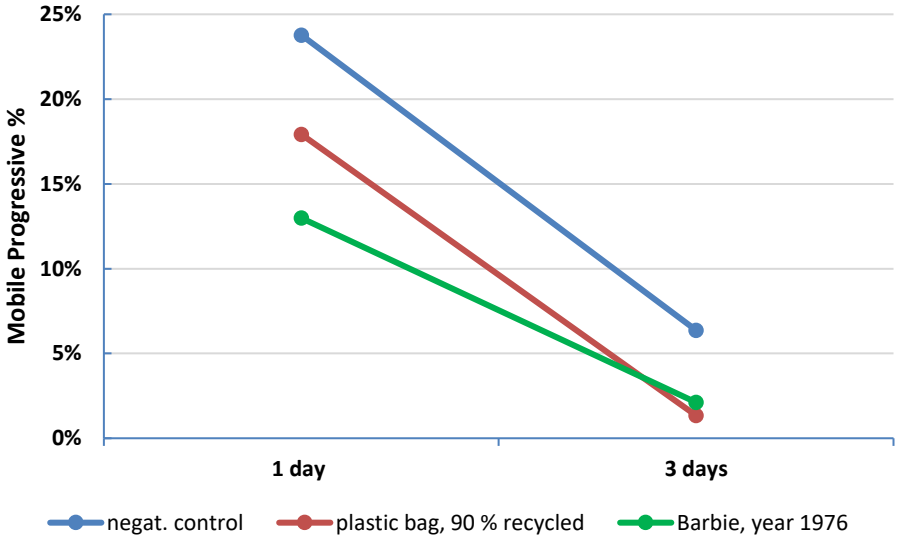


Figure 1. Effect of poor semen quality on samples over time. Sample processing: Technical replicates (n=18), three replicates from the same test tube after one-day exposure and three replicates from another test tube after three-day exposure.

2.2 Applying assay

2.2.1 Negative control, positive control and EC_{50}

The idea in this applied method was to detect the change in spermatozoa motility between the negative control and the sample exposed to the chemical or plastic material. The positive control, where all cells were immotile, was to confirm that the equipment and measurement method were working properly. Furthermore, a chemical triclosan with dose 2 µg/ml was used in the case study as a model alongside with the plastics, and to detect the time of EC_{50}. In this study, EC_{50} meant a 50% loss of rapid movement of spermatozoa. Exposure to chemicals was

done in test tubes. The amount of semen in a test tube was 2 ml according to Andersson et al. (2010) research, and to have some point of comparison.

The negative control was the extended semen as it came from the artificial insemination station Figen. Even without chemicals, boar spermatozoa motility varies in each batch, during storage days and according to temperature (see chapters 2.1.2 and 2.1.4 above). Therefore, negative control was read each time with other samples. Treated samples were compared to negative control and comparison was made only between the results of the reading day, not between different reading days. In the case study, two negative controls from different weeks, were quite close to each other (chapter 3.3). If the liveliness of two batches were very different from each other at the starting point, the results could be scaled to avoid a big deviation. Negative control would then be 100% at the starting point.

A positive control refers to a sample where all cells were immotile and this was achieved by using triclosan (20 µg per ml of semen, exposed in ethanol-solution). Triclosan was used as it is toxic to boar spermatozoa (Ajao *et al.* 2015). With dose of 10 µg/ml of triclosan, there were still some oscillating spermatozoa. In this research, 100% static was considered only those samples in which all spermatozoa were non-motile. This was important for evaluating the functionality of the ISAS program. Positive control had the same exposure time than other samples or it was made on reading day, when exposure time was about three hours. Because an error was found in tests (chapter 2.2.8), it was decided to read also positive control each time with other samples.

In the case study, EC_{50} time for loosing progressive motility was detected with 2 µg/ml of triclosan. The amount was based on articles, for example Andersson et al. (2010), found EC_{50} value for triclosan to be 1 µg/ml of triclosan after 3-4 days exposure using subjective estimation of rapid and progressive motility.

The spermatozoa were exposed to triclosan as solution. Stock solution was made of triclosan powder (403,2 mg) which was dissolved in ethanol (100 ml) thus concentration was 4 mg/ml. Triclosan (72779 Irgasan, CAS: 3380-34-5), analytical grade, ⩾97.0% (HPLC) was from Sigma–Aldrich and ethanol (Etax, CAS: 64-27-5), min 94,0 p-% was from Anora Group Ltd. Dilutions from the stock solution were made in ultrapure water (Milli-Q) to avoid extra chemicals. Dilutions were calculated using the formula

$$C_1 * V_1 = C_2 * V_2 \quad (1)$$

where C is concentration and V is volume. All water dilutions did not succeed. Succeeded one was 2 mg/ml (50 ml stock solution and 50 ml water) in a bottle. Water dilution which failed was 400 µg/ml (10 ml stock solution and 90 ml water) in a bottle which meant 2 µg/ml in a test tube. Dilution stayed as turbid solution and the next day the triclosan powder had settled to the bottom of the bottle. The solution was nevertheless used as turbidity solution in the case study. The

following week, a new batch of spermatozoa was subjected to a similar, turbidity solution.

2.2.2 Exposure conditions

In this test, exposure time refers to sample's time in a test tube with the chemical. The exposure time were one and three days following by Andersson et al. (2010). The three-day test was not successful each time, because of the variance in the viability of the semen batches (Figure 1), and therefore longer effective period was not reasonable. Also 30 min exposure was tested as done by Andersson et al. (2010) but this test did not bring any added value for this assay method, because differences between negative control and treated samples were clear only after one-day exposure.

Expose to chemicals in the test tubes was conducted at room temperature (21-23 °C) as done by Andersson et al. (2010) and Ajao et al. (2015). This study was made in autumn and wintertime and room temperature stayed constant. The test tubes were protected from light with foil during exposure.

The test tubes were stored in a tube rack in an upright position. During the exposure, the test tubes were turned upside down about five times by hand, not shaking, to make sure that spermatozoa got in contact with plastic pieces. In the case study, during the three-day exposure, the test tubes were rocked after one-day but not after two-day exposure. The test tubes could have been mixed on the 2nd day or not mixed after the 1st day, but the impact was not tested.

2.2.3 Mixing the test tubes before measurements

Samples were exposed to chemicals in test tubes. Mixing the test tube, before taking a sub-sample for motility measurements, affected the number of spermatozoa on the slide, and consequently influenced the repeatability of the measurements. In this point the spermatozoa was mostly in static state, before heating to 37 °C. Therefore, it was important to select a proper mixing method.

It was decided to use a shaker to mix the test tubes, to standardize the step. Mixing and rocking the test tubes by hand was not as good method, because the sample foamed easily, and the mixing was then not exactly the same for all test tubes. The level shaker (Heidolph DSG 9601282) worked the best for mixing the test tubes (Picture 2), since it made same kind of movement as by hand. Different levels of speed were tested and a speed of 178 rpm was chosen as the movement was suitably careful rocking, efficient enough, but semen did not foam much. Five minutes was considered to be sufficient time. The test tube was inverted once upside down before and after the shaker. During the shaking, the test tube was covered with a cloth to avoid the light.

It was tested if technical replicates could be used. The aim was to save semen. Samples were taken from the same test tube during the same day and rocked 2-6 times. There was no substantial collapse in motility due to the fact that technical replicates were used (Figure 1 and 2 and Table 2).

Picture 2. Mixing the covered test tube with the level shaker.

2.2.4 The choice of objective slides

Different kind of objective slides were tested to achieve the most repeatable spermatozoa motility measurements. First, I tested and compared ordinary slides (VWR, ref. 631-1553, ground edges frosted) and coverslips (different sizes), diagnostic microscope slides (10 wells, 6,7 mm) and manufacturer's own ISAS 4-chamber slide (D4C10, 10 microns height). Based on data from previous studies and price, ordinary slide-coverslip were chosen and most of the pre-tests were made with that. According to Gączarzewicz (2015), in the CASA system, in the microscopic examination of boar spermatozoa motility, chamber slide and ordinary slide-coverslip work almost equally well in examining motility. Manufacturer Proiser recommended to use ISAS disposable chambers in sperm analysis (Vázquez and Navarro n.d.). Because with slide-coverslip high deviation occurred in replicate samples, later I tested more expensive Leja 2-chamber slide (20 µm height, Standard Count, ref: SC200102BCE, Leja Products B.V.) and ISAS 4-chamber slide (D4C10, 10 microns height).

2-chamber slide was found to be the best (see also chapters 2.2.5, 2.2.9 and 2.2.10) and was used in the case study. The pipetting volume was chosen to be 5 µl based on previous studies were 2-chamber slide has been in use (Karjalainen *et al.* 2020). 5 µl did not fill the entire chamber, so the flow on the slide (see below concerning the effect of 'flow') decreased quickly. On the 2-chamber slide, the sample was of more uniform quality and the results of the replicates corresponded better compared to the slide-coverslip (Table 4 and 5). The spermatozoa motility remained better and longer on the chamber slide than on the slide-coverslip. Also, ISAS was able to count piles of spermatozoa from 2-chamber slide better than from slide-coverslip. With the 2-chamber slide the number of spermatozoa was 70–120 per image (Table 4) and the error rate

remained below 3% measured with immotile spermatozoa (Figure 4). With 2-chamber slide there were fewer intermediate steps and materials used than with the slide-coverslip, which meant faster sample processing and less chance of error.

Before start using the 2-chamber slide, many aspects of the slide-coverslip method were tested as this would be a more cost-efficient method. The slide-coverslip can be used to check the motility of spermatozoa before starting the test to save expensive 2-chamber slide. The ISAS manual (Vázquez and Navarro n.d.) advises that the cover slip must be placed over the sample in 5 seconds. Different sizes of coverslips were tested, 18x18 mm, 22x22 mm and 21x26 mm. The size of coverslip, 22x22 mm, was found the best for ordinary slide (VWR, ref. 631-1553, ground edges frosted). When the size of the coverslip was 22x22 mm, two samples could fit side by side on the slide. The size 21x26 mm was difficult to fit two pieces on the slide. With the size 18x18 mm the spermatozoa motility slowed down quickly. There were differences in the cleanliness of the coverslips. Coverslips (VWR, 22x22 mm, thickness nro. 1 cat. nro. 631-0124) were ordered from Avantor and they were a good quality. With slide-coverslip, different pipetting amounts onto the slide, 10, 7, 5, 4 µl were tested. The volume was chosen to be 5 µl when the size of the coverslip was 22x22 mm. 5 µl had less flowing than the larger amounts and retained spermatozoa motility better than the smaller amount.

Furthermore, when the slide-coverslip was in use, a key problem was that there was 'flow' i.e. passive movement of the semen, that disturbed motility counting. The ISAS program counts these passive moving sperm as motile spermatozoa. ISAS manual (Vázquez and Navarro n.d.) advises that one must wait for the movement of the liquid between the slides to stabilize before observing. The flow was reduced by using a sturdy stone table and avoiding the leaning on the table and microscope. In the slide-coverslip there was a lot of continuous flow and jerky flow. I tried tapping, pressing, massaging, and poking the edge of the cover glass against the flow. The flow leveled off more quickly when the coverslip was lightly rubbed (with a clean pipette tip) so that the coverslip moved in all directions. Also, the flow was reduced by standing the sample in the microscope for about 2 min to settle, before taking the image. With the slide-coverslip, the best result for the reading time was 1min 50 sec – 2 min 40 sec, reading of the result in 10 sec intervals, taking six images. The flow had time to decrease in 2 minutes and after 3 minutes the spermatozoa motility started to decrease.

A 4-chamber slide was also tested. The area to count the sperm was relatively small and the sample spread differently compared to the 2-chamber slide and the slide-coverslip. 4-chamber slide's chamber was narrow and shallower (10 µl) compared to the 2-chamber slide (20 µl) and pipetted amount (3 µl) was smaller than into the 2-chamber slide (5 µl). Gączarzewicz's (2015) research supported the decision to use the 2-chamber slide.

2.2.5 Heating before and during measurements

Boar spermatozoa motility is significantly increased by heat (Andersson 1999) and that feature is used in detection of movement. The most commonly used temperatures to activate the movement are 37–38 °C and preheating before reading varies 5 min – 30 min (Vicente-Carrillo *et al.* 2015; Ajao *et al.* 2015; Castagnoli *et al.* 2018; Barquero *et al.* 2021b). In this study, temperature 37 °C was used. A heater stage (Argumeia) suitable for the microscope was acquired to keep the sample and slide in 37 °C. The heating block (Grant) was used to preheat Eppendorfs and all slides and coverslips to 37 °C.

A five-minute heating for the spermatozoa to 37 °C was considered as a suitable time. The test showed that between 5 and 10 minutes heating, motility started to decrease (Figure 2). I estimate that heating should not be less than 5 min because I saw how the spermatozoa 'woke up' during the heating on the 2-champer slide. I also noticed that if spermatozoa were old or poor quality, they still could move nicely but moving collapsed between 0,5–2 min. When spermatozoa were good quality, there was no substantial collapse in spermatozoa motility in 15 min of heating (37 °C), even when exposed to plastics (Figure 2). So, in this assay method measuring points 10 ja 15 min did not bring any added value. Perhaps a 10 min measurement could add value if the exposure time was short (30 min) or if it is necessary to know how alive the spermatozoa are in general.

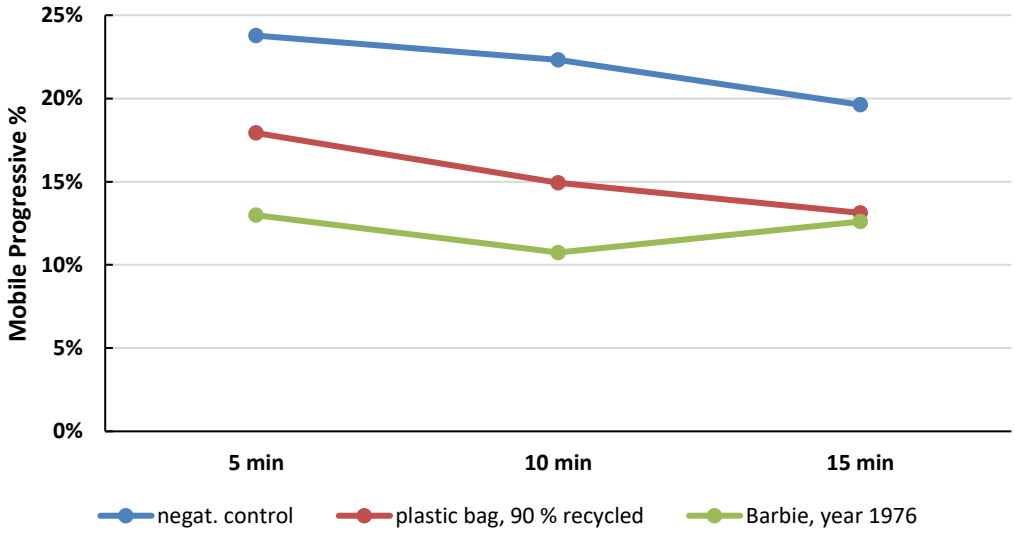


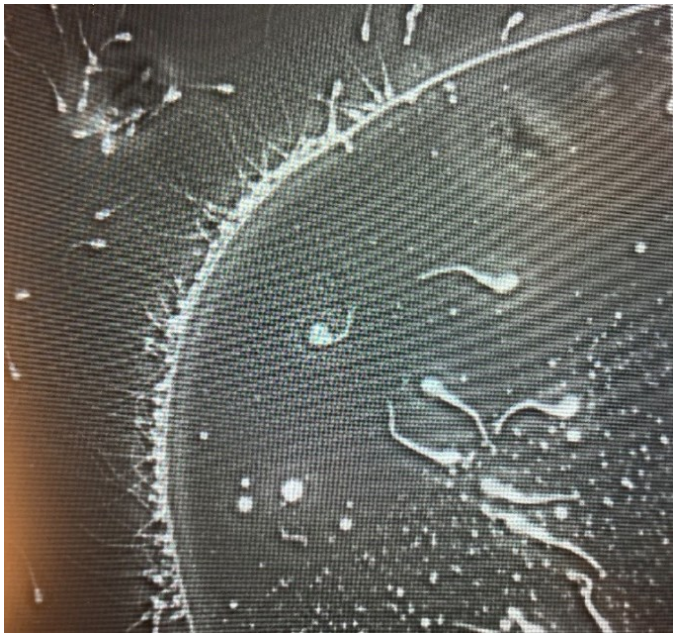
Figure 2. Rapid movement of the boar spermatozoa after 5, 10 and 15 min heating (37 °C) on the 2-chamber slide. The Barbie sample showed increase after 15 min of heating, but this indicates the inaccuracy of the assay method. Sample processing: Technical replicates (n=9), 3 of each after one-day exposure.

When the 2-chamber slide was in use, the sample was heated, and the movement stabilized at the same time (5 min, +37 °C) in microscope heater stage. When the slide-coverslip was in use, heating was different and heating time increased to eight minutes total. A sub-sample (200 µl) was heated in Eppendorf

on the heating block for five minutes before pipetting the sample on the slide following by Andersson et al. (2010). After preheating, Eppendorf was mixed with vortex mixer 10 sec at 800 rpm (less mixing was not enough to get evenly spermatozoa onto the slides), immediately pipetted onto the slide, and let the sample to stabilize about two minutes before reading with ISAS. According to my test, with the slide-coverslip, spermatozoa motility decreased after five min heating in Eppendorf and after three minutes in slide-coverslip. Heating in Eppendorf might have effect, if Eppendorf is poisonous to spermatozoa, but I got a similar result with the 2-chamber slide, the motility of the spermatozoa started to decrease after five minutes heating (Figure 2).

2.2.6 Pipetting

Pipetting influences the homogeneity of the sample on the slide. In practice, I noticed that it matters which point in the test tube the sub-sample is taken. I tested pipetting from the surface of the liquid, in the middle of the liquid, against the wall of the test tube and right next to the piece of the plastic bag which was in a liquid. The best place to pipet from the test tube was right in the middle. The immotile spermatozoa sank to the bottom and the surface had the least sperm. I noticed that the spermatozoa reacted to the plastic bag. I pushed a piece of plastic out of the way with the tip of the pipette and the pipette was against the plastic. As a result, only a few spermatozoa came on the slide. Spermatozoa tend to react with surfaces (Amann and Waberski 2014) as also seeing in Picture 3. When pipetting into a test tube and Eppendorf, air bubbles could be prevented by pipetting against the wall of the tube, not into the solution. Furthermore, reverse pipetting onto the slide was chosen to avoid air bubbles and sample flowing.



Picture 3. Boar spermatozoa and air bubble.

When the 2-chamber slide was in use, pipetting into the chamber was done at a steady speed, not too fast, directly towards the center of the chamber. It was easy to pipet into the chamber, even though the slide was already in read position in the microscope. When the slide-coverslip was in use, there was an extra pipetting compared to the chamber slide. 200 µl of spermatozoa were pipetted to preheated Eppendorf and after five minutes, 5 µl was pipetted onto the slide, in the middle of the place where the coverslip came. The slide was ready on the heated stage of the microscope, but not ready for reading, but in such a way that you could pipet onto it. The coverslip was immediately put on and the slide was placed in the reading position so that the sample began to settle.

2.2.7 ISAS and kinematic parameters

The CASA-system (Computer Assisted Semen Analysis) for sperm analysis was used to observe the change in spermatozoa motility. Microscope (UB203i, SN201000953), phase contrast objective (Plan PH N, 10x/0.30, ∞/0.17 SN:PHO100906052), phase condenser (multipurpose, JG40), heater stage with heater controller unit (Argumeia, ISAS CT-03), ISAS v1® -program (Proiser R+D, ISAS v1.2, integrated semen analysis system, serial number: 00221DDE) and more precisely ISAS Motil –module (2004). ISAS v1 is a multi-specie system that can work with many different sperm samples, including boar sperm. Each species had its own configuration and analysis algorithm. According to the manufacturer Proiser, the used program was old production (ISAS v1.2 and Motil–module, 2004) and updates were no longer available.

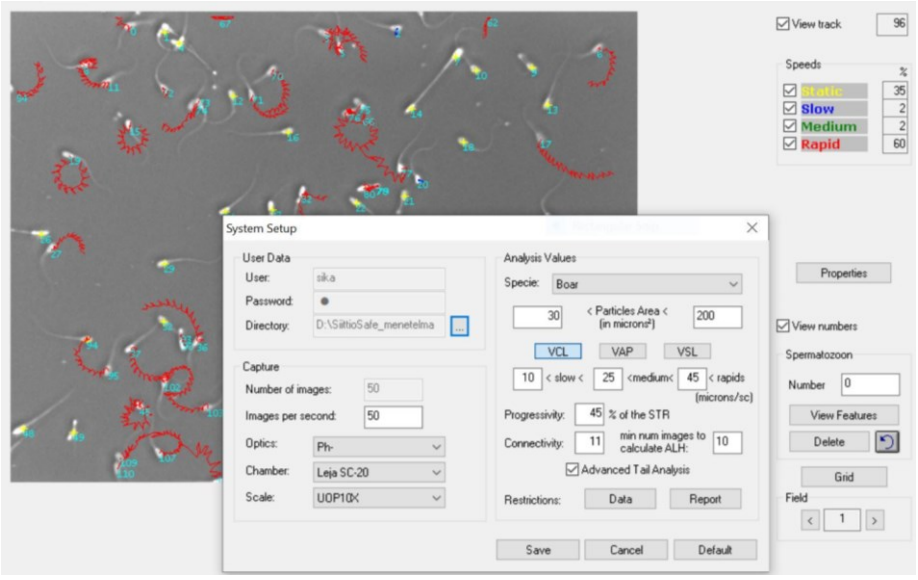
The ISAS program offered default values for boar spermatozoa and according to the manufacturer Proiser, the default values work the best, but some of them were changed (Picture 4). The default size for Particles Area parameter, i.e. the size of the spermatozoa heads, was changed from 10–80 µm² to 30–200 µm². Then, all easily counted spermatozoa heads were included and small debris was left out of the calculations. Optics parameter was selected to be Ph-. Chamber parameter was selected as Leja SC-20 for 2-champer slide. If slide-coverslip was in use, values coverslip (22x22) and 5 µl dose was selected. Since this study did not monitor concentration, the slide option did not matter as much.

In the ISAS program there were three parameters for measuring spermatozoa trajectories, VCL (Velocity Curved Line, µm/s), VAP (Velocity average path, µm/s) and VSL (Velocity straight line, µm/s), and one of which had to be selected. Parameter VAP was tested based on Soler et al. (2017) and Didion (2008). In Didion's (2008) study, the deviation in VAP was claimed to be small. Parameter VSL was tested based on Soler et al. (2017) and in Valverde's et al. (2019) research, VSL had the least difference when images are 25–200 fps. According to Soler et al. (2017), VCL is sensitive to gaps in image capture. The only way to estimate the accuracy on the result, was to compare it to the subjective observation, and parameter VCL was chosen based on that. Subjective evaluations were conducted from the live screen, and from the same video frames as the video is recorded, and the speed of the video can be slowed down.

The parameters used to record the semen traits, number of images and images per second were examined, and some modifications to the default were made. ISAS capture parameters, Number of Images was changed from 25 to 50 and Images per Second was changed from 25 to 50. Number of images influence how spermatozoa motility is calculated (Amann and Waberski 2014), likewise images per second (frame rate, frames per second, fps) has an effect on how the program calculates spermatozoa motility (Amann and Waberski 2014; Vicente-Carrillo 2018; Valverde *et al.* 2019; Barquero *et al.* 2021b). The manual recommended for this ISAS V1.2-program to use 25 Images per Second to obtain optimal use of the system, (Vázquez and Navarro n.d.). 25 frames per second is considered these days very slow and effects on calculations of spermatozoa motility (Valverde *et al.* 2019). 25, 50 75 ja 100 Images per Second were tested, and comparison showed that there was difference between 25 images per second and others (Table 2). 25 images stood out from the other images. No more than 50 images per second was chosen so that the program still worked. With these options the program worked quite well and made the change in spermatozoa motility visible.

TABLE 2. Parameter Images per Second expressed with the result parameter Static %. Sample processing: all samples taken from the same test tube.

Images per second	Result (%)
	Static %
25 images	55.6
50 images	28.9
75 images	28.4
100 images	25.6
again 25 images	50.5



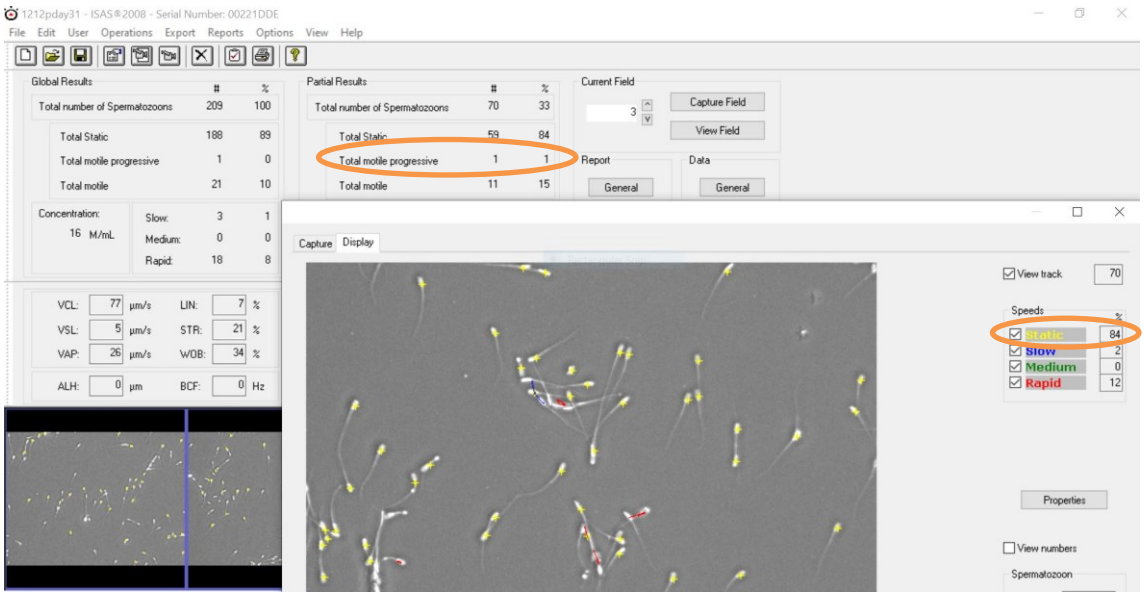
Picture 4. Chosen parameters in the ISAS program.

2.2.8 ISAS and result parameters

The ISAS program gave as a result about 15 different result parameters. Different options were considered: (1) Speed % (Static/Slow/Medium/Rapid), (2) Beat Cross Frequency Hz (BCF) and (3) Mobile Progressive %. Based on previous literature Speed % is not as used parameter as BCF and Mobile Progressive % (Gacem *et al.* 2020; Maside *et al.* 2023). During the tests, the averages given by ISAS were for Mobile Progressive % about 0–40%, Static % about 30–100% and BCF about 0–13 Hz.

First, I tested the functionality of ISAS program with the immotile samples (positive control) for the three above-mentioned parameters. I tried to get results either 100% or 0% depending on the result parameter (Table 3). The result of Static % (immotile spermatozoa) was usually 75–90%, rarely near 100% as it should. With the 2-chamber slide, static % was higher than with the slide-coverslip, suggesting better ability to count the immotile cells. However, ISAS program also included motile spermatozoa to Static % when compared to visual observation. Also, BCF (beat Cross Frequency, Hz) was not considered reliable to monitor because it was not consistently 0 Hz in immotile samples. It there were falsely claimed one rapid moving spermatozoon, BCF could be even 6 Hz.

From the result parameters given by the ISAS program, Mobile Progressive % was chosen to be monitored. Mobile Progressive (another name used by ISAS Total Motile Progressive), described rapid movement of spermatozoa and it could be verified from the live screen by visual observation as "two-tailed spermatozoa". Picture 5 shows an example of an immotile sample, when comparing result parameters, Static % and Mobile Progressive %.



Picture 5. Example of the result parameters when all cells were immotile. Static % is 84% and total motile progressive % is 1%. Sample processing: Positive control on the 2-chamber slide.

TABLE 3. Motility of boar spermatozoa described with result parameters, Mobile Progressive (%), Static (%) and Beat Cross Frequency (Hz). Sample processing: Positive control (all cells immotile) on 2-chamber slide, batches one and two. Number is average of three images.

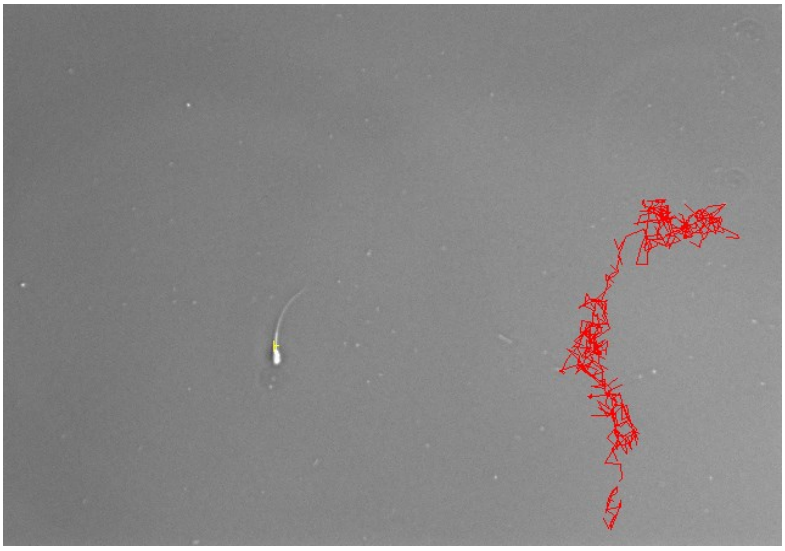
Sample	Results		
	Mobile Progressive %	Static %	BCF (Hz)
positive control 1, batch 1	1.4	93.1	2.6
positive control 2, batch 1	1.0	93.3	1.3
positive control 3, batch 1	1.1	93.9	0.3
positive control 1, batch 2	1.3	88.5	0.0
positive control 2, batch 2	2.1	92.9	3.6
positive control 3, batch 2	0.8	91.4	0.7

2.2.9 Microscope adjustments and repeatability

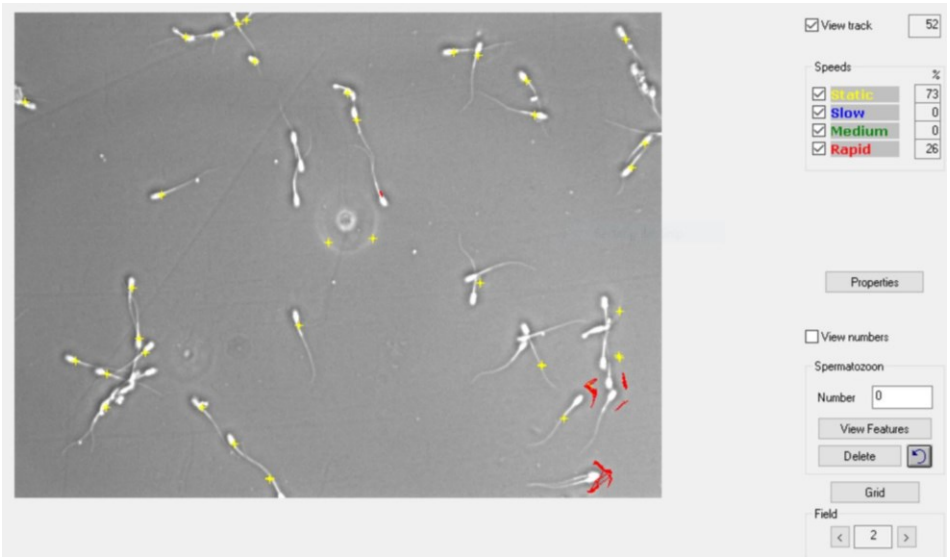
Several microscope adjustments affected the images taken by the ISAS program and it was possible to refine the image in many ways. By adjusting the focus of the image, the spermatozoa could be seen as black, bright white or dull white. Those colors were compared with phase contrasts, Ph- and Ph+. Negative phase contrast (Ph-) worked best when the boar spermatozoa were visible as bright white. With each new slide the image was zoomed in separately. The image focused on one layer, but ISAS program recognized also the slightly imprecise spermatozoa heads. To subtract the background from the image, the function Background Capture was used.

The darkness of the background influenced how the ISAS program counted spermatozoa heads. With a darker background, positive control improved. I looked for a point where both positive and negative control were reasonably good. I chose the background darkness by testing and the best was darkish background, not the darkest. The screw did not have scale of any kind.

During the tests, a "red rapid stripe" randomly appeared on the lower right edge (Picture 6 and 7). When the stripe appeared, it skewed the results, claiming the spermatozoa to be too motile. “The red rapid stripe” no longer occurred when the height from to the light source of the microscope was adjusted bigger.



Picture 6. "The red rapid stripe" in the image. The sample was not mixed, and pipetting was from the surface of the test tube to get semen without spermatozoa. Sample processing: All cells immotile on the slide-coverslip.



Picture 7. In the image occurs "red rapid stripes". Also, debris is included to counting and some spermatozoa in piles are not counted. Sample processing: All cells immotile on the slide-coverslip.

The number of spermatozoa in the image should be as constant as possible. If there are large differences in the numbers of spermatozoa, differences between results can affect the reliability and comparability. Also, the fewer spermatozoa are included in the calculations, the more deviations affect the result. According to the ISAS manual (Vázquez and Navarro n.d.), images should be taken so that the counts include at least 200 spermatozoa, in practice two images so that the number of spermatozoa in the image is 90–110.

In the tests, the number of spermatozoa in positive samples (cells immotile) was significantly lower than in negative samples (untreated) (Table 4 and

chapter 3.3). When using the 2-chamber slide, the number of the spermatozoa was near 70 per image in immotile sample and near 120 per image in untreated sample. When using the slide-coverslip, the number of spermatozoa was lower than with 2-chamber slide (Table 4).

The other reason for the difference was that ISAS program had difficulties to count piled spermatozoa. Especially when slide-coverslip was in use, piles were problem (Picture 7). Also, when the quality of the semen was poor, the spermatozoa made a considerable number of piles already in the storage bag on their own.

Because there was a lot of deviation between replicate samples (Table 4 and 5), one decision to improve the precision was to take multiple images from one slide and remove a deviating image. When using the 2-chamber slide, four images from the same slide were taken and one image was removed. When using slide-coverslip, six images were taken and one image removed, but I still could not get consistently as uniform results as with the 2-chamber slide. Three images were estimated to be the minimum amount that is sufficient and removing the fourth, deviating, image increased the precision (Table 6).

TABLE 4. The number of spermatozoa on 2-chamber slide and slide-coverslip. Comparison samples to both slides have been taken from the same test tube (n=12). Number of captured images was the same to all samples, four and one taken off. Sample processing: Positive control and negative control, three-day exposure in the test tube.

Sample	Number of spermatozoa	
	2 chamber slide	slide and coverslip
positive control 1	209	231
positive control 2	249	186
positive control 3	257	169
negative control 1	379	232
negative control 2	313	174
negative control 3	327	133

TABLE 5. Boar spermatozoa motility on 2-chamber slide and slide-coverslip expressed with result parameter Mobile Progressive %. Comparison samples have been taken from the same test tube (n=12). Number of captured images was the same to all samples, four and one taken off. Sample processing: Positive control and negative control, three-day exposure in the test tube.

Sample,	Mobile Progressive (%)	
3 days exposure	2 chamber slide	slide and coverslip
positive control 1	0.5	1.3
positive control 2	0.4	7.0
positive control 3	0.8	2.4
negative control 1	30.6	54.7
negative control 2	29.1	9.2
negative control 3	27.2	28.6

2.2.10 Subjectivity and randomization

In this study, the ISAS computer program was used to observe spermatozoa motility. Observation can also be done manually by subjective observation (Andersson 1999). However, visual inspection always involves a subjective perception, which may differ compared to the result obtained by the equipment and another person (Vyt *et al*. 2004; Amann and Waberski 2014). Using a computer program aims for a more uniform and efficient calculation.

There are benefits to use subjective observation compared to computer program. Person can ignore the flow, count all spermatozoa that are in piles, count as many spermatozoa as needed and count also imprecise spermatozoa heads. The program is not capable of such evaluation and that places demands to the sample on the slide.

During the tests, I found that I can influence the results, because the sample was not uniform quality on the slide. The 2-chamber slide worked quite well but when using the slide-coverslip this was a problem. I favored the moving spermatozoa because the immotile spermatozoa were in piles and especially on the slide-coverslip ISAS counted the piles poorly. Also, I noticed that very actively moving spermatozoa cleared space for themselves, so I rejected the image place because the number of spermatozoa in the image was less. These were important findings because choices affected the outcome.

Subjective selection was used when choosing the image locations for video capture and when removing one image. Image locations for video capture were chosen randomly near the center of the drop. If there were air bubbles, large debris or if the number of spermatozoa was too little, another place were chosen. If there were only few piles of immotile spermatozoa, they were left out of the images, and if there were a lot of immotile piles, they were included. Similarly, Gączarzewicz (2015) had also read the samples on the central part of the chamber and did not use images with air bubbles.

Overview inspection through the oculars was relevant, as subjective observation could confirm the uniform quality of the sample and the reliability of the images. The 2-chamber slide and slide-coverslip gave a wide overview, on them the sample spread in every direction.

The expectations of person doing the work may influence the removal of image. However, compared to taking just one photo, the result is more reliable. The selection of the image was not always clear (Table 6), and then also result Static % was used to figure out which three images were closer to each other. If the results did not clearly differ from each other, then the choice did not matter so much. The images were deleted immediately instead making the selection with a statistical method. The applied assay method was not that accurate anyway and the use was smoother.

TABLE 6. Examples of image selection situations. Four images were captured from the same 2-chamber slide and the most deviating image was deleted (pic out). CV% is calculated of three images, after removing fourth image.

Sample	Mobile Progressive (%)					
	pic 1	pic 2	pic 3	pic 4	pic out	CV%
positive control	0	5	1	1	5	87
negative control	30	30	24	30	24	0
plastic bag	11	23	13	10	23	13
Barbie doll	13	9	17	12	17	18
triclosan 2 µg/ml	8	4	10	3	10	53
triclosan 2 µg/ml	7	4	7	4	7	29

Test randomization can be considered when selecting the reading order of the test tubes. In the case study, the sample reading was conducted in the same order as their preparation. The sample reading took about 45 min per three replicates, therefore the last samples had approx. three hours delay which might have some relevance, at least after one-day exposure. I suggest that the positive control should be done first. If for some reason, the sample was not immotile, there was time to do it again. Also, if problems appeared with the ISAS-program, this was the best moment to notice them.

2.2.11 Type and form of plastic to be tested in the case study

In this work, alternatives of the form of plastic in the exposure tests, were considered. Grit form, extract form and piece form were considered. Several factors affect the transfer of the chemical to the spermatozoa, such as contact time, temperature and the initial concentration of the compounds in the plastic material (Nerín *et al.* 2014).

Plastic in extract form would perhaps offer the best opportunities for the chemicals to come into contact with the spermatozoa because the extract is evenly distributed throughout the sample. However, extraction requires chemicals that can also be toxic to spermatozoa. Also, extra chemicals can act as solvents and dissolve chemicals from different materials into the liquid (Andersson *et al.* 2010), in which case the substance affected to spermatozoa motility can be completely different than assumed. Extraction would also be a new step in the assay method, which would make the assay more difficult and expensive. A uniform grit form would have required equipment to produce uniform grit, which was not available. Furthermore, a small grit (30–200 µm) would probably affect the spermatozoa count, because that size was defined as the size of the spermatozoon head and similar-sized grit could confound the measurements. The problem could have been circumvented by adding non-toxic grit to the negative control or by leaving the pieces of plastic large enough so that ISAS program would not count them. In piece form, the effect time of the chemical, can become a significant factor. If the toxic chemicals needed a longer time than three days (maximum time set for this assay method) for toxicity to manifest, then the toxic effect would not be seen. However, piece form would be easy to produce, and

does not have the confounding effects of extract in chemicals, and therefore was selected for this study.

For the case study, a plastic bag was chosen to be tested (chapter 2.3) and different sizes of pieces were tested. Initially 1x1 cm size was tested as it didn't stick to the wall of the test tube but floated in the liquid. However, 1x1 cm piece from the plastic bag showed no clear difference in motility, and in the test, the semen batch was poor quality. Two pieces 1x1 cm stuck to each other and 2x1 cm didn't float in the liquid but stuck to the edge of the test tube. So, for the plastic bag was chosen the largest possible piece, 2x3 cm, which went around the circumference of the test tube and stuck to the wall of the test tube. The piece circled the inside of the test tube to a height of 2 cm, so that 2 ml of semen covered the piece (Picture 10). Another plastic to be tested was Barbie. From Barbie's leg were cut pieces of the size as could get (about 0,5x2 cm) and two pieces in 2 ml of semen was enough to make a significant difference in boar spermatozoa motility.

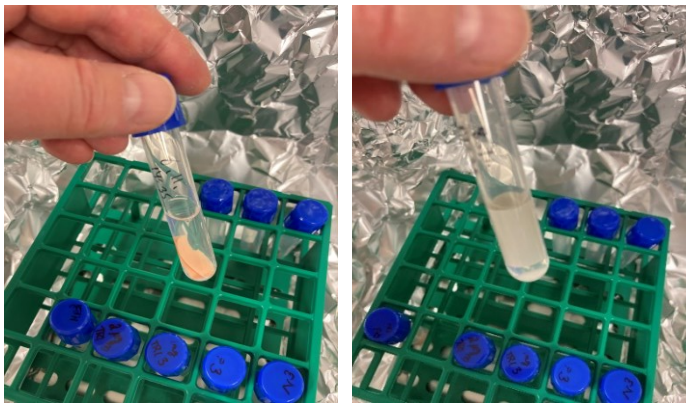
2.3 Case study

The plastics to be tested were (1) grocery store's plastic bag, which contained 90% recycled plastic and (2) Barbie doll from 70s (head Mattel INC. 1976, torso Mattel INC. 1966, China) from second hand store (Picture 8). The plastic bag represented a product made of recycled plastic. Bags are easily available to everyone in everyday use and may be in contact with food. Barbie from 70s represented recycled toy, which also today's children play with. The material of the new Barbies is not the same. (Miller and Harris 2015) tested vintage plastic toys from 1970s and 1980s, including Barbie doll's legs, and for example cadmium and lead were found frequently at concentrations exceeding current U.S. and European limits. According to (Miller and Harris 2015), the material of Barbie's legs from 1970s–1980s is mostly polyvinyl chloride (PVC) and according to Raman test at University of Jyväskylä (pers comm. Noora Risku), that was true. The material of the plastic bag was mostly polyethylene (PE) measured with Raman test (pers comm. Noora Risku).

Barbie doll was washed with dishwashing liquid and a towel. About 0.5 x 2 cm pieces were cut from the doll's leg with a potato peeler (Picture 9). Weights were between 0,0785–0,0899 g. Two pieces were placed in one test tube and the weight of the two pieces varied between 0,1568–0,2110 g. From the plastic bag, pieces of 2 x 3 cm were cut with the scissors (Picture 9). Colored areas were avoided in order not to introduce additional chemicals. Weights of pieces were between 0,0196–0,0238 g and one piece was placed to each test tube. Before putting the pieces into the test tube, all pieces were disinfected with 70% ethanol to avoid microbial growth. Pieces were placed first into the test tubes and later the semen was pipetted on top (Picture 10).

Picture 8. Tested plastics, Barbie from 70s and the plastic bag (90 % recycled).

Picture 9. Tested materials and tools. On the left are Barbie's legs and the peeling knife. On the right are the plastic bag and the scissors.



Picture 10. Tested materials in the test tubes. On the left are two pieces of Barbie's legs and on the right is the piece of the plastic bag.

Negative control was semen as it came from artificial insemination station Figen. As positive control triclosan (20 µg/ml) was used. Triclosan (2 µg/ml) were utilized to investigate the EC_{50} time and as a model alongside with the plastics. Total of 12 replicates per each material were processed, half of them for

day one and half for day three (Figure 3). Two batches of semen were used from two different weeks.

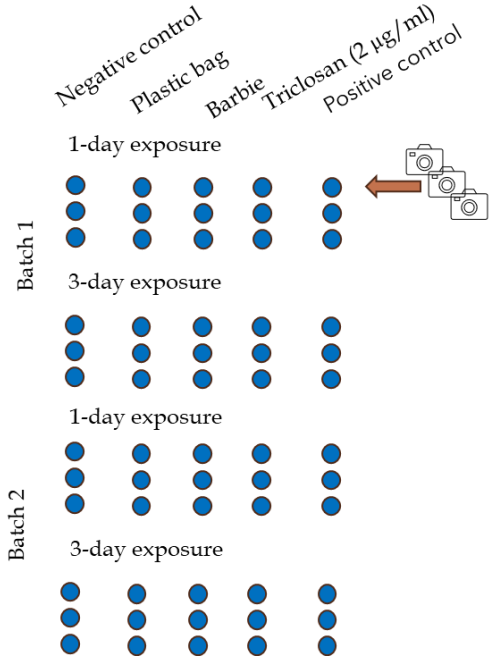


Figure 3. Test arrangement of the study. The blue spots represent samples in test tubes. Each test tube was one replicate (n=60). From each replicate was taken three images and average of them was used.

The motility of spermatozoa was checked before starting the test with slide-coverslip. The semen bag was rocked different directions about 50 times trying to avoid foaming. Immediately about 75 ml of semen was poured into a beaker. Semen was immediately pipetted from the beaker into the test tubes (2ml/tube, 30 tubes). The test tubes with plastic pieces and negative control were closed with caps. Triclosan (10 µl) was pipetted to the positive control tubes (20 µg/ml) and EC_{50} tubes (2 µg/ml). Rest of the caps were closed.

All test tubes, including negative control, were rocked upside down about five times. It was checked that all the pieces were submerged in the liquid. The test tubes were covered with foil to keep the light out. Exposure time for the test tubes were one and three days in room temperature. The test tubes of three-day exposure were rocked second time after one day.

After exposure the test tubes were rocked for 5 min with the shaker (covered with a cloth). The test tubes were turned once upside down before and after rocking. Immediately after rocking the sample, 5 µl was pipetted with diverse pipetting into the 2-chamber slide, which waited on the microscope. The sample was heated (37 °C) and settled for five minutes. While waiting, the image was focused and placed in a middle of the drop, overview of sample was checked trough oculars, and a test image was taken.

The samples were read using ISAS program. The sample reading was conducted every time in the same order as their preparation, positive control,

negative control, plastic bag, Barbie and triclosan 2 µl/ml. Four images of each slide were taken. Image locations for video capture were chosen as randomly as possible near the center of the drop. Bubbles and big debris were avoided, and roughly equal number of spermatozoa were captured in each image (mostly 70–120 cells / image). Taking four images took time less than 1 minute. The slide was moved gently, and leaning on the stone table or microscope was avoided, thus reducing flowing of liquids on the slide. One, the most divergent image was removed, by following Mobile Progressive %. If it was difficult to decide which one to remove, also Static % was used to make decision. Data was saved as Video file (.mot), General Data (.xls) and Data Report (.xls).

2.3.1 Statistical analysis

Statistical analyzes were used to evaluate dependencies and their statistical significance. The data were analyzed using IBM SPSS (Version 28.0.1.1). The ISAS program measured sperm motility as percentages. Although the data already met the assumptions of normality and homogeneity of variance, an arcsin-transformation was applied, prior to analysis, to ensure that these assumptions were strictly adhered to. For ease of interpretation, results were back-transformed from arcsin scale to percentage scale.

To test if there was a difference between number of spermatozoa in negative control and positive control, 12 negative and 12 positive controls (n=24), were tested with t-test for equality of means (Independent Samples t-test). Equality of variances were tested with Levene's Test.

Potential differences in motility in four different treatments (negative control, plastic bag, Barbie, and 2 µg/ml triclosan) were analyzed using 2wayANOVA with repeated measures (4 treatments and 2 time points). To find out what kind of difference is between 1-day and 3-day exposure, test results were analyzed also using one-way analysis of variance (ANOVA), in two parts, separately for one-day exposure and three-day exposure. Pairwise comparisons were conducted with Tukey's test (Post Hoc). Data normality and homogeneity of variances were verified using Shapiro-Wilk normality tests and Levene's tests, days one and three were tested respectively. Data included 6 replicates per time point, which meant 12 of each sample (n=48). The limit of statistical significance was 0.05.

3 RESULTS

3.1 Assessment of optimal assay conditions

The applicability of the applied assay method to determine the cytotoxicity of recycled plastics was evaluated using experimental factors, accuracy, precision, sensitivity, and the ease of the test. Also, the costs and time spent on the work were considered. The presented protocol and the case study are explained in

chapters 3.2 and 2.3, and the results for the case study are showed in chapter 3.3. An application process showed that the assay being studied was suitable for determining recycled plastic toxicity.

The applied assay method was not very accurate based on precision and subjectivity. But despite of overall uncertainty of the assay, the case study provided satisfactory outcomes with modest sample sizes, and the possibility to use parametric test (ANOVA) increased the efficiency of the results (chapters 2.3.1 and 3.3).

To ensure that the overall method was functioning, a known motility-inhibiting chemical, triclosan, was used alongside the samples as an indicator (chapter 2.2.1). Also, two measurement points (1- and 3-day) were used to detect that there was a trend in decreasing mobility (chapter 2.2.2 and 3.3). For most accurate results the following ISAS parameters should be used: VCL of trajectory parameters, minimum 50 fps of Images per Second and 50 images of Number of Images and result parameter Mobile Progressive % (chapters 2.2.7, 2.2.8 and 3.3).

A few systematic errors were discovered. Positive control (cells immotile) was not constantly 0% as measured with result parameter Mobile Progressive % (chapter 2.2.8, 3.3). Despite the use of the computer program, the person doing the work had to use also subjective selection (chapter 2.2.10), and that could lead to errors.

The applied assay method was not particularly precise and therefore not particularly sensitive. During the tests, the variation between liveliness of batches was significant and it has a great impact on the reliability of the test. The case study showed that in order to obtain a significant difference between the negative sample and the treated samples, the semen had to be of good quality, in which case the negative control motility reduced only slightly (chapter 2.1.4), and the spermatozoa motile had to reduce significantly within three days due to the chemical (chapter 3.3). To reduce random variation, a mixture of semen from five boars was used, not a single boar, and two different batches were used for the same reason (chapter 2.1.2). Other reasons for low precision were heterogeneous quality on the slide, and inability to fully standardize the process of capturing the video image (chapter 2.2.10).

Precision was increased by taking several images of each objective slide (chapters 2.2.9 and 2.2.10). Homogeneous quality on the slide was improved many ways. As important steps were found mixing (chapter 2.1.4 and 2.2.3), pipetting (chapter 2.2.6) and use of the 2-chamber slide (chapter 2.2.4). Despite of all the effort done, there were difference in number of spermatozoa (70–120 pieces) on the slide and immotile samples had lower number (chapter 2.2.9). ISAS as a computer program could not separate moving of the spermatozoa from flowing with the liquid. As important steps to reduce the flowing were found a solid table (chapter 2.2.4), pipetting (chapter 2.2.6) and use of the 2-chamber slide (chapter 2.2.4 and 2.2.5).

3.2 Established protocol

The protocol is expressed with the case study objects.

HANDLING MATERIALS	**MORE INFORMATION**
PREPARATIONS	Chapter
1. Choose test materials: negative control (only semen) positive control (10 µl of triclosan 20 µg/ml) EC_{50} time (10 µl of triclosan 2 µg/ml) plastic materials	2.2.1, 2.2.11, 2.3
2. Plan test arrangement: three replicates per day, two reading days, two weeks = total 12 samples per each material	2.2.10, 2.3
3. Cut the plastics into pieces, weight, disinfect, and let to dry.	2.2.11, 2.3
4. Mark and arrange all test tubes. Put the plastic pieces into the test tubes with the tweezers.	2.3
BEFORE AND DURING EXPOSURE	
1. Check motility of spermatozoa before starting the test.	2.1.4
2. Remove all the caps from the test tubes.	
3. Rock the semen bag different directions about 50 times avoiding semen to foam.	2.1.3
4. Immediately pour semen into a beaker.	2.3
5. Immediately pipet semen into the test tubes (2ml/tube).	2.3
6. Close the caps of the tubes with negative control and pieces.	
7. Pipet 10 µl triclosan into test tubes with positive control and EC_{50}. Close rest of the caps.	2.2.1
8. Rock the test tubes upside down about five times, also negative control. Check that all the pieces remained in liquid.	2.2.2
9. Cover the test tubes with foil to keep the light out.	2.2.2
10. Expose semen to the chemicals in test tubes, for one and three days, at room temperature.	2.2.2
11. Mix three-day tubes once during exposure time by rocking the tubes five times upside down. Check that the pieces remained in liquid.	2.2.2

READING SAMPLES WITH ISAS	**MORE INFORMATION**
PREPARATIONS	Chapter
1. Select the reading order of the test tubes, positive control first.	2.2.10
2. Adjust the microscope. Check ISAS parameters.	2.2.7, 2.2.9

WITH 2-CHAMBER SLIDE

1. Preheat 2-chamber slides.	2.2.5
2. Place the 2-chamber slide into its reading place on the microscope, heating (37 °C) is the same time with the heater stage.	2.2.4, 2.2.5
3. Rock the test tube with the shaker 5 min. Use a stopwatch. Turn the test tube once upside down before and after rocking.	2.2.3
4. Immediately pipet 5 µl to the 2-chamber slide, use diverse pipetting.	2.2.4, 2.2.6
5. Wait for 5 min. Use a stopwatch. The sample heats (37° C) and settles.	2.2.5
While waiting: focus the image, place in the middle of the drop, check overview of sample trough oculars, and take a test image.	2.2.10
6. Select places to be photographed and take four images. Move the slide gently to avoid the flow.	2.2.4, 2.2.9, 2.2.10
7. Remove one, the most divergent image by following Mobile Progressive % - parameter. If decision is unclear, use static % for help.	2.2.8, 2.2.9, 2.2.10
8. Save data.	2.3

OR WITH SLIDE-COVERSSLIP

1. Preheat the slides, coverslips, and Eppendorfs on the heater stage of the microscope or on the heating block.	2.2.5
2. Place the slide on the heater stage so that you can pipet onto it. Coverslip next to it.	2.2.4, 2.2.5
3. Rock the test tube with the shaker 5 min. Use a stopwatch. Turn the test tube once upside down before and after rocking.	2.2.3
4. Immediately pipet 200 µl into Eppendorf. Let heat for 5 min (37 °C) on the heating block.	2.2.5, 2.2.6
5. Immediately mix 10 sec with the vortex (800 rpm).	2.2.5
6. Immediately pipet 5 µl onto the slide, use diverse pipetting. Immediately put the coverslip on. Move the slide to the reading place on the microscope to settle.	2.2.4, 2.2.5, 2.2.6
7. Wait 1 min 50 sec the sample to settle. Use a stopwatch.	2.2.4
While waiting: focus the image, check overview of sample trough oculars, place in the middle of the drop and take a test image.	2.2.10
8. Select places to be photographed and take six images between 1 min 50 sec and 2 min 40 sec in 10 sec intervals. Move the slide gently to avoid the flow.	2.2.4, 2.2.9, 2.2.10
9. Remove one, the most divergent image by following Mobile Progressive % - parameter. If decision is unclear, use static % for help.	2.2.8, 2.2.9, 2.2.10
10. Save data.	2.3

3.3 Case study

With this assay method and equipment, with dose 2 µg/ml of triclosan, 50% of rapid movement of spermatozoa were lost between one and three days of exposure (Figure 4). Negative controls (untreated) had a significantly higher number of spermatozoa cells than positive controls (immotile) (t-test, $p < 0.001$).

As part of applying the assay method, plastic testing was conducted, and the results are indicative. Effect of duration of the exposure was significant (2wayANOVA, $F = 195$; $df = 1,16$; $p < 0.001$, Figure 4). Also, both days separately showed significant effect (1wayANOVA, one-day exposure $F = 20$, $df = 3$, $p < 0.001$, three-day exposure, $F = 27$, $df = 3$, $p < 0.001$). Effect of treatments was significant (2wayANOVA, negative control, plastic bag, Barbie, and 2 µg/ml triclosan, $F = 120$; $df = 2,16$; $p < 0.001$, Figure 4). Interaction effect of time and treatment was significant (2wayANOVA, $F=8$, $df = 3$, $p = 0.001$).

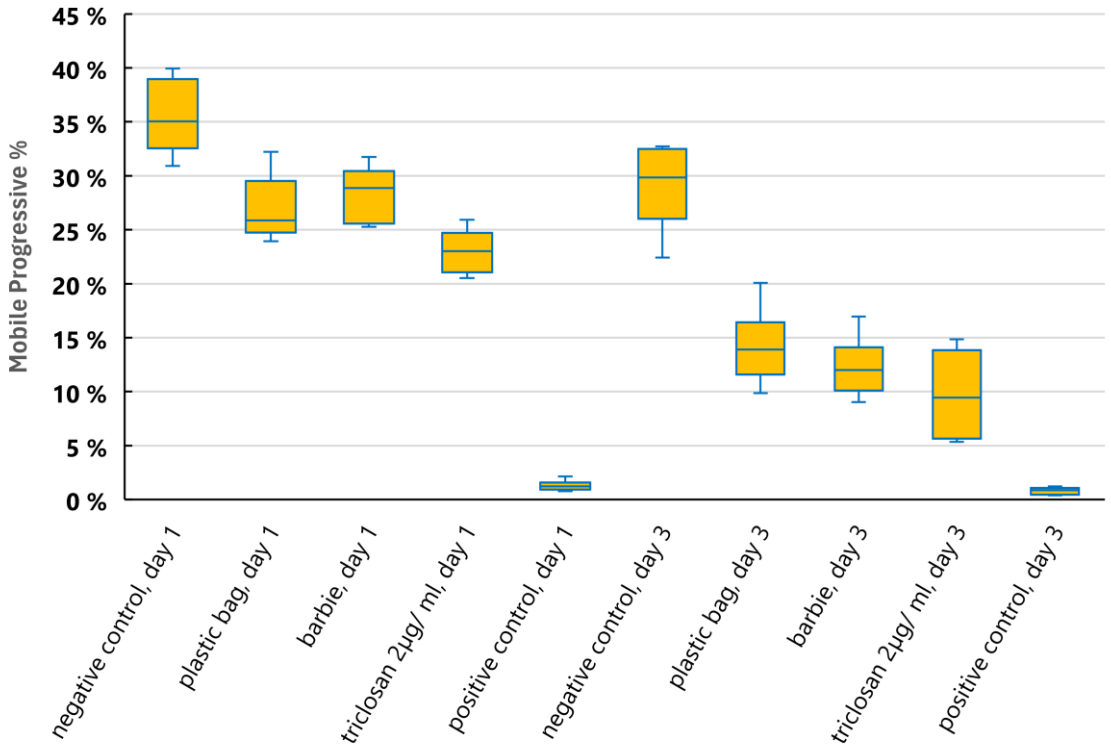


Figure 4. Effect of different treatments on rapid movement of boar spermatozoa at one and three days of exposure measured with progressive motility. The positive control is included to the figure, but it was not part of the analysis. Data from batches one and two have been pooled together. The sample size per group per time point was six i.e. each box corresponds to average of six replicates and each replicate is average of three images. The total sample size per group was 12, six replicates after one-day and six replicates after three-day exposure (n=48).

After the three-day exposure, there were significantly more rapid moving spermatozoa in the negative control compared to all the other treatments (Tukey

HSD, plastic bag, $p < 0.001$, Barbie, $p < 0.001$, and 2 µg/ml triclosan, $p < 0.001$, Figure 4). Already after one-day exposure, there was significant difference compared to negative control (Tukey HSD, plastic bag $p < 0.001$, Barbie $p = 0.002$, and 2 µg/ml triclosan $p < 0.001$, Figure 4). While Barbie and plastic bag exposure had more rapid moving spermatozoa compared to 2 µg/ml triclosan after one-day exposure (Tukey HSD, Barbie $p = 0.016$, plastic bag, $p = 0.106$, Figure 4), either Barbie or plastic bag exposure did not significantly differ from 2 µg/ml triclosan after three days exposure (Tukey HSD, Barbie, $p = 0.631$, plastic bag, $p = 0.181$, Figure 4).

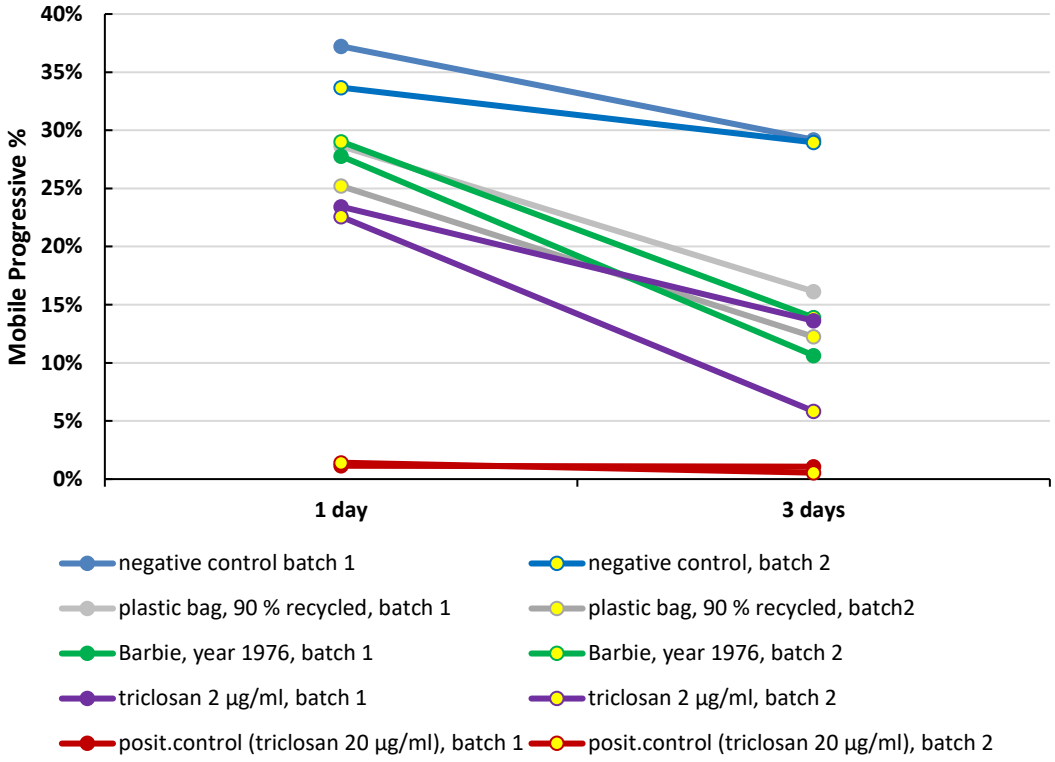


Figure 5. Effect of different treatments on rapid movement of boar spermatozoa at one and three days of exposure measured with progressive motility. The positive control is included to the figure, but it was not part of the analysis. Batches one and two are separately (two lines of the same color). Each spot corresponds to average of three replicates and each replicate is average of three images. The total sample size per group was 12, six replicates after one-day and six replicates after three-day exposure (n=48).

Without any treatment, rapid movement of spermatozoa in the negative control decreased by 6% between 1 and 3 days of exposure. Expressed with percentages, the rapid movement of boar spermatozoa decreased compared to the negative control (negative control, day 3) after three-day exposure: plastic bag by 53%, Barbie by 60% and 2 µg/ml triclosan by 68% (Figure 4). In the test, two different batches of sperm were used, and analysis shows similar effect in both batches (Figure 5). The used batches did not significantly differ from each

other, as interaction effect of time and batch was not significant (2wayANOVA, $F = 0{,}827$, $df = 1$, $p = 0{,}377$, Figure 5).

4 DISCUSSION

In this study, an assay method with boar spermatozoa motility and ISAS program was applied. The testing protocol was established, and the case study conducted with 90% recycled plastic bag and Barbie's leg from 70s.

The applied assay method did not give particularly accurate results. I came to same conclusions as Amann and Waberski (2014) and Valverde et al. (2020), that specimen chamber, imaging hardware and software, instrument settings and technician, affect the accuracy and precision of output values. For the results to be comparable, as many variables as possible should be controlled. For example, one problem I could not solve, was a significant difference in spermatozoa number between motile and immotile samples. I suspect the cause of difference was that immotile spermatozoa went with the flow and ended up to the edges, and when pipetting, immotile spermatozoa dropped near to the chambers mouth. Amann and Waberski (2014) explain the same kind of difference with the Segre-Silberberg effect, as motile spermatozoa redistribute themselves towards the center while immotile sperm do not relocate from the vicinity of the wall. Another issue was that although a computer program offers more detailed information, it did not completely eliminate subjectivity in this study. The homogeneous quality of the sample impacted the results, necessitating subjective observation. Consequently, the reliability of the results heavily depends on the user's expertise and training, as highlighted also by Maside et al. (2023), Valverde et al. (2020) and Bompart et al. (2018).

The inaccuracy of the results was also affected by a large natural variability. The sperm test is *ex vivo* test, taken directly from the animal and each spermatozoon is genetically unique. The sperm test can never be repeated with the same cells in a different lab or even with same batches, but the test can repeatedly show that there are substances that reduce the movement of spermatozoa, although the concentrations may vary. For example, boars can differ in their sensitivity to methanol (Sutkeviciene *et al.* 2005). The applied assay method works most reliably when there is a substantial change in spermatozoa motility within three days due to the chemical, and the semen is of good quality, in which case the negative control motility reduces only slightly.

My estimation as a minimum requirements of boar spermatozoa liveliness in a baseline was ≥35% of progressive motility moving measured with ISAS v1. Value for total immotile spermatozoa was ≤50%, but this was not as reliable parameter as progressive motility. These limits for usefulness in toxicity testing are lower than what Maside et al. (2023) represent for breeding. They present that a widely accepted value of total motile is ≥70% for breeding (Maside *et al.* 2023). Artificial insemination station Figen uses total motility of 60% as a limit (Figen Oy). Based on my testing, I suggest that measuring progressive motility, which

means two-tailed spermatozoa with subjective observation, and among ISAS parameters Mobile Progressive, tells more about spermatozoa vitality than total alive.

Boar spermatozoa and CASA-Mot systems are widely used to toxicity testing, and standardization would be highly beneficial for ensuring the accuracy of results and facilitating meaningful comparisons. However, it is unwise to assume that two different CASA system would produce the same outcome when analyzing the same sample, as noted by Amann and Waberski (2014) and Bompart et al. (2018). When the reproducibility of this assay method and equipment is compared to other studies, 50% of progressive motility of boar spermatozoa was lost in exposure to triclosan 2 µg/ml between one and three days. The result is in line with Andersson et al. (2010) and Salin et al. (2021) with their result of 1 µg/ml in three to four days and three-day exposure.

As part of the application process, recycled plastics were successfully tested with it. The boar spermatozoa tests have been studied to reveal certain type of toxins. The boar spermatozoa assay is sensitive to lethal toxins depleting plasma membrane integrity (Hintikka *et al.* 2024). According to Hoornsta et al. (2003) boar spermatozoa motility is sensitive to detecting sublethal toxins, such as mitochondrial toxins cereulide and gramicidin and protein kinase inhibitor staurosporine, which are not easily detected by other types of cells. On the other hand, spermatozoa are insensitive to substances affecting the synthesis of proteins, nucleic acids or their regulation (Hoornstra *et al.* 2003), and to cytostatic toxins that inhibit macromolecule synthesis, i.e. cell proliferation in cell cultures (Hintikka *et al.* 2024). Therefore, sperm test provides additional information about the biological target in spermatozoon and works best in parallel with other tests.

The results are indicative, but both the plastic bag (90% recycled) and Barbie's leg (from 70s) decreased the rapid movement of boar spermatozoa significantly compared to the untreated sample, indicating cytotoxicity. The results are quite startling, as both products are in use today, and recycling should be preferred. Cytotoxicity of Barbie's leg supports the result of Miller and Harris (2015) about harmfulness of vintage toys and according to them, possible toxin in PVC is lead or cadmium. Toxins in recycled plastic bag could be brominated flame retardants, UV stabilizers or bisphenol A in polyethylene (Brosché *et al.* 2021).

There were few things that bring uncertainty to the case study results. Firstly, the surface material of Barbie's leg had changed over time and could contain impurities despite cleaning. The surface was included in the study because that is the area to be touched. In the study, the motility of the spermatozoa could have been affected not only by the material, but also by impurities left on the surface, or by the change of the plastic material over time. Secondly, uncertainty to the results bring inaccuracy in weighting the triclosan and in pipetting, turbid water solution of triclosan (2 µg/ml), and the plastic pieces used in the replicates were not exactly the same size. Thirdly, the results for positive control (immotile cells) should be 0% but results were between 1.2–

1.6% measured with Mobile Progressive % and therefore, motility is slightly overestimated. Finally, the experimental setup could have been replicated and randomized more. For example, I could have done reading of the samples in different order in different days, and the test tubes could have been in a different order in the tube rack in different days. The reading took about 45 min per three replicates, therefore the last samples had approx. three hours delay which might have some relevance at least after one-day exposure. I used only one Barbie and one plastic bag, although using several of both would have given more reliable results. Pseudoreplication has been considered by using two different semen batches, but three batches would have been even better. With ANOVA tests, all six replicates were treated as independent, which they may not be, because three replicates out of six were from the same batch.

The applied assay method succeeded in determining the cytotoxicity of two different types of recycled plastic. However, the sperm test does not reveal the identities of individual compounds or their origin (Hintikka *et al.* 2024). Recycled plastics are mixture of plastics that were once new. Therefore, not only recycled plastics, but also new plastics should be tested for cytotoxicity before they are put in production.

In this study, an assay method was applied to measure changes in boar spermatozoa motility using the automated sperm analysis program (ISAS v1) with a particular focus on recycled plastic. The case study with 90% recycled plastic bag and Barbie from 70s, revealed two key findings: The applied assay is suitable for detecting cytotoxins in plastics, and recycled plastics, including their additives, may be cytotoxic to mammalian cells.

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Milja Aalto

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