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**Front Matter**

Title

Microplastics are present in cow follicular fluid and can compromise gametes function *in vitro*: Is the Anthropocene throw-away society throwing away fertility?

* Short title can be a maximum of 50 characters.

Microplastics impaired gametes function *in vitro*

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**Abstract**

The abstract should be a single paragraph (**no more than 150 words**) written in plain language that a general reader can understand. Do not include citations. The abstract should provide:

* An opening sentence that states the question/problem addressed by the research
* Enough background content to give context to the study
* A brief statement of primary results
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**MAIN TEXT**

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**Introduction**

Decades of careless use and improper disposal have resulted in plastic pollution accumulating almost everywhere on earth (*22*, *23*) and microplastics – defined as plastic particles <5 mm in size that are insoluble in water – have now been documented in even the most remote environments. The exponentially increasing volume of plastic pollution making its way into rivers, lakes and oceans has drawn considerable scientific, public, and governmental attention (REFS). However, despite the tens of thousands of peer reviewed publications on plastic pollution, the overwhelming majority of these have focused on aquatic ecosystems, and very little is known about the effects of MPs in terrestrial systems. This knowledge gap is made all the more noteworthy by the fact that that 80% of the Earth’s species live on land18 and terrestrial systems may represent a larger environmental reservoir of MPs than oceans (6,7). With the plastic pollution crisis only expected to worsen over the coming decades, it is imperative that we improve our understanding of the potential health effects of the tens of billions of tons of plastic pollution that litter the globe.

While any health effects are undesirable, the potential impacts of MPs on reproductive systems are of particular concern. Reproduction is central to the capacity for species to maintain stable populations. Poor reproductive health not only reduces individual fecundity but, if widespread, species survival. With human activity around the globe having triggered the Earth's sixth major episode of mass extinction10–12, understanding the long-lasting effects of human activity on reproductive systems is essential for developing the tools and strategies needed to ensure the future of life on Earth. Over the past several decades there has been an alarming increase in the rates of reproductive dysfunctions and gamete abnormalities, reductions in gamete production, and altered embryo development in humans, animals and plants1–9 . While the overarching cause of these declines in fertility has yet to be identified, emerging studies are showing that MPs represent a potentially serious threat to the reproductive health of terrestrial species19–22. Data have been limited to studies on laboratory animals, however, and the extent to which they are representative of the conditions animals are actually experiencing in the real world is unknown. For example, Jin et al. studied the effects of MPs on fertility by feeding mice MPs at concentrations that were ca. 100,000 times greater than they would be exposed to in the wild. This means that it is unclear to what extent MPs can bio-accumulate in free-ranging terrestrial mammals and influence fertility. Here, we assessed the extent to which MPs might be bioaccumulating in the follicular fluid of domestic cows. We then used the concentrations of identified MPs to guide a biologically realistic investigation into the effects of MPs on bovine male and female gametes *in vitro*.

**Results**

**MPs, pigments, and plasticizers in cow follicular fluid**

MPs were found in all follicular fluid (FF) samples, with a mean concentration of 132.3 particles mL-1(Fig 1A; Table S1). The number of MP particles varied substantially between samples, with a total of 17 mL-1 in FF1, 21 mL-1 in FF2 and 359 mL-1 in FF3. ~~the total number of particles was adjusted to the particles detected in the water control~~. Non-plastic related particles were the majority of identified particles (56 to 74 %), while MP polymers accounted for 9.4 to 19.9 % of total identified particles. Other plastic-related particles that were identified included pigments (0.3 to 2.5 %), plasticizers (1.6 to 18.0 %), and coating, solubilizers and fillers (5.9 to 12.2 %). A total of 24 different MP polymers were identified, with the most abundant being cotton, followed by cellulose, polyvinyl chloride, polyethylene, polystyrene, polypropylene and silicon, respectively. The sizes of the MPs in bovine follicular fluid ranged from 3.2 to 56.9 μm in length (mean±SD = 9.8±4.5 μm) and 1.6 to 23.6 μm in width (mean±SD = 5.8±2.9 μm). Based on their sizes and densities, we determined that MPs summed to 0.002 μg mL-1 in FF1, 0.005 μg mL-1 in FF2 and 0.172 μg mL-1 in FF3. Of importance for the present study, polystyrene in FF3 equalled to 0.0126 μg mL-1 and to 0.00035 μg mL-1 in FF2, while it was not identified in FF1. Full details in the MP particles are shown in Table S2.

**were bovinenegatively influenced maturation** In the are shownThe boxplots in show the e 24 h exposure to0.3 and 1.1 μm beads*.*Ianel oocytes incubated for 24 h in the presence of μm μm polystyrene beadscompared to a control are also shown.

**MPs reduce oocyte maturation and induce damage of the zona pellucidae**

For evaluating the effects of MPs on *in vitro* oocyte maturation (IVM, 24 h), we have classified the oocytes as broken zona pellucida (BZP), degenerating (DG) and mature (MT; Fig 1B). The presence of a polar body and a clear chromosome alignment without degradation was considered for oocytes that were mature (MT). The BZP oocytes were all those that independently of the nuclear stage, had a disruption of the zona pellucidae (Fig 1B). For the degenerating oocytes, the shrinking of the cytoplasm, and the absence or degradation of genetic material was considered. Independent of the size, IVM performed in the presence of polystyrene beads resulted in a lower number of mature oocytes in comparison to the control group (68.1±10.3%, 42.0±14.8 and 41.0±17.6%, for CT, B0.3 and B1.1, p < 0.00001 for both B0.3 and B1.1; Fig 1B). No difference on the number of mature oocytes was observed between B0.3 and B1.1 groups (p = 0.963). The number of DG oocytes varied between 27% and 37% and was not significantly different between all groups. The CT group had a significant lower number of BZP when compared to the groups incubated with beads (4.4±5.2%, 21.4±7.6 and 26.2 ±10.9%, for CT, B0.3 and B1.1, respectively; p < 0.0001 for all groups). It is important to highlight that 80, 40 and 35 % of the BZP oocytes were mature in CT, B0.3 and B1.1, respectively, but these oocytes were counted as BZP, not as MT.

**Oocytes proteomics**

**MPs attach to the sperm surface but are not internalized**

Only the bigger beads (B0.3 and B1.1) were analysed for attachment, due to a limitation of the microscope, from which smaller beads could not be seen. We observed that B0.3 incubated sperm had a higher number of beads attached to the surface than B1.1 (for example at 30 min: 10.0±9.9% vs 3.0±2.9%, respectively; p < 0.001) and there was no effect of time on beads attachment (p = 0.3). Nevertheless, we did not observe beads being internalized by the sperm cells (Fig 2A).

**MPs induce acrosome damage and oxidative stress but has no influence on motility**

Independent of size, PS beads did not induce significant changes on motility when compared to the control group. Nevertheless, an effect of time was observed on total motile sperm for all groups (p < 0.001; Fig 2B). Regarding acrosome status, it was observed that the presence of MPs, independent of size, negatively impacted acrosome integrity when compared to the negative control (Fig 2C, p < 0.001). Finally, we also observed that PS beads increased ROS production significantly in all time points compared to the negative control (Fig 2D). The presence of smaller beads (B0.05, and B0.1) have induced ROS to levels comparable to the positive control (CT+), which was stimulated with hydrogen peroxide, a known cellular ROS inducer. The bigger beads groups (B0.3, and B1.1) also induced higher ROS compared to the negative control (p = 0.02, and p = 0.036 respectively), although not to the same level as the CT+, as seen in B0.05 and B0.1.

**Discussion**

The ubiquitous and long-lived nature of MPs has made them synonymous with the seemingly irreversible mark of mankind on our planet. The growing volume of plastic pollution making its way into rivers, lakes and oceans has drawn considerable scientific, public, and governmental attention to the plight of aquatic ecosystems. Yet, despite the tens of thousands of peer reviewed publications on MPs, few of these have focused on terrestrial systems, and very little is known about the occurrence and fate of MPs in mammals. While evidence is still extremely limited, emerging studies are showing that MPs represent a potentially serious threat to the reproductive health of rodent species (*39*, *42*–*47*). However, there are currently no studies that link MP contamination to changes in fertility in other terrestrial mammals. Importantly, the lack of a reliable method to isolate MPs from tissues and biological fluids hinders our ability to precisely investigate the presence of MPs in complex biological samples. In the present study, we optimized a protocol to isolate small MPs from follicular fluid and have shown, for the first time, that MPs are present in bovine follicular fluid. Moreover, we also have shown that PS MPs exert a negative effect on both male and female bovine gametes *in vitro,* demonstrating that MPs should be treated as concerning environment reproductive toxicant. It is important to note that, in this study, the MPs concentration used for the *in vitro* investigations were based on a study carried out in mice that was fed PS microplastics *in vivo* (*41*), which is not necessarily representative of the number of plastics animals encounter in real life.

To perform a realistic experiment investigating the impact of MPs on fertility (and health in general) *in vitro,* it is necessary to have baseline information about what type, size, and amount of MPs can plausibly bio-accumulate in mammals. In reality, no such information is available for terrestrial mammals. This can be attributed to two main reasons: 1) the vast majority of MPs research has focused on aquatic ecosystems, and 2) due to cost, time, and technological limitations there are no reliable methods for isolating, characterizing and quantifying small MPs (<10 μm) from complex biological samples. In an attempt to overcome the latter limitation, we have tested different published protocols (*38*, *48*–*52*) for isolating spiked small MPs (< 3 μm) from bovine biological samples (ovary, oviduct, endometrium, follicular fluid, seminal plasma and faeces; *data not shown*). Unfortunately, none of the published protocols were optimal because they all resulted in large amounts of undigested biological matter, which in turn hindered our ability to analyse the samples by automated Raman spectroscopy. Therefore, we have developed a protocol that allowed the analysis of MPs in follicular fluid samples. Nevertheless, due to the still high number of undigested substances in the samples, the protocol required 3-7 days (per sample) to acquire the Raman spectra of all the detected particles, plus 4-18 hours to match it to the database, which is clearly a protocol that is not a viable option for large scale applications.

* Talk about Type of particles encountered in FF and other plastic related particles… compare size and kind with other published data
* Oocyte exposure – 24h only – in vivo would be for longer, during folliculogenesis…. Talk about the effects form rat/mice work and what we saw here + correlate proteomics data
* Sperm exposure –
* Impact and future
* Conclusions

**Materials and Methods**

**Contamination prevention**

To prevent sample contamination with airborne/materials MPs, all procedures were performed in a laminar flow, and flasks and other apparatus, whenever possible, were replaced by glass materials. Moreover, all materials and equipment used were rinsed three times with filtered (0.1 μm filter - brand) ultra-pure water prior to use. All reagents and water used were filtered using a 0.1 μm filter before use.

**Microplastic polystyrene beads**

Polystyrene beads (SURF-CAL™ particle size standards) having sizes 0.047 (B0.05), 0.100 (B0.1), 0.304 (B0.3) and 1.112 (B1.1) μm were purchased from Thermo Fisher Scientific. The beads were present in deionized filtered water in a concentration of 3 x 108 particles mL-1 and were diluted according to the concentration required for each experiment.

**Bovine oocyte and follicular fluid isolation**

Bovine ovaries were obtained from a local slaughterhouse and immediately transported to the laboratory at room temperature. Immature cumulus oocytes complexes (COCs) were aspirated from follicles with a size between 2 and 8 mm, together with follicular fluid, by using a vacuum pump and a 19G needle. Collected fluids with COCs from ~20-30 ovaries (N = 3 pools) were let to pellet for a maximum of 10 min. Pellet was then transferred to a petri dish with the equivalent amount of washing media (IVF Bioscience, UK) for oocyte selection, and remaining follicular fluid frozen at -20ºC for MPs isolation as described below.

**Microplastic isolation from follicular fluid**

Pools (20-30 ovaries) of bovine follicular fluid left after removal of the COCs (N=3) were used for microplastic isolation. Water controls from follicular fluid aspiration (N=3) were also processed using the same protocol. On day one, 2 mL of each sample were added to an Erlenmeyer for digestion in KOH 10% in a proportion of 1:25 (sample:digestion solution). The samples were incubated in a shaker at 60ºC and 250 rpm for 24 h. After this period, NaClO was added to each digestion to reach a final concentration of 7.5%, and samples were incubated for another 24 h in the shaker at 60ºC and 250 rpm. On day three, all samples were filtered in a 47 mm polytetrafluoroethylene polymer (PTFE) membrane (0.45 μm pores, Merck Millipore, USA) and rinsed with filtered ultra-pure water at least three times in order to prevent any NaClO and KOH contamination. Next, the membranes were placed in a beaker containing 50 mL of HNO3 20% and ran into an ultrasonic bath (TI-H-5 MF2 230 V, Elma Schmidbauer GmbH, Germany) with 100% power, in sweep function and a frequency of 45 kHz for 15 min, to transfer plastics/undigested matters from the membrane into the solution which was then kept in the shaker at 40ºC and 250 rpm for another 24 h. At the end of the third digestion day, the samples were filtered using 13 mm PTFE membranes (0.45 μm pores) and again rinsed three times with ultra-pure filtered water. The membranes were then mounted on a slide for microscopy and spectroscopy analysis.

**Oocyte isolation, incubation with MPs and nuclear stage analysis**

After isolation as described above, the Petri dish was screened with a stereomicroscope and good quality oocytes (homogeneous cytoplasm and, at least, 3 layers of cumulus cells) were selected. COCs were then washed three times in washing media, and one time in BO-IVM (IVF Bioscience, UK), before being randomly assigned to one of three incubation groups: 1) control group containing only maturation media (N = 114); 2) media containing PS beads of size 0.304 μm (N = 93); and 3) media containing PS beads of size 1.112 μm (N = 103). The concentration of the beads in each group was 1.2 million beads mL-1, or XXX μg mL-1. The groups were cultured for 24 h in an incubator at 38.5ºC in a humidified atmosphere of 5% CO2 and 95% O2. After the 24 h period of incubation, the oocytes were denuded, washed to remove any unattached beads, and either fixed in paraformaldehyde 4% for nuclear staging or frozen at -80oC in pools of 9 oocytes for proteomics analysis (N = 4-5 pools per group).

For determining oocyte nuclear stage, fixed oocytes were washed in PBS, stained with Hoechst 33342 (5 μg/mL) for 45 min and imaged in an EVOS M7000 Microscope using a ×40 NA 1.25 objective. The oocytes were analyzed for nuclear stage (metaphase 1 or 2 – determined by the presence of an aligned metaphase plate with/without a polar body, respectively), degenerated (no visible nuclear material, or pyknotic nucleus), and broken zona pellucida (visible breaks in zona pellucida). Some of the cumulus cells were still present in few of the oocytes and the nuclear stage couldn’t be determined, such oocytes were excluded from the analysis.

**Oocyte proteomics (Jan/Thomas)**

**Sperm incubation with MPs**

Frozen bull sperm were thawed at 37ºC for 30 s. The content of the straws were added to 3 mL of Tyrode’s medium supplemented with 2 mM sodium bicarbonate, 10 mM lactate, 1 mM pyruvate, 6 mg mL-1 fatty acid–free BSA, 100 U mL-1 penicillin and 100 μg mL-1 streptomycin, centrifuged for 5 min at 700xg, and the pellet submitted to a swim-up separation using Tyrode’s medium supplemented with 25 mM sodium bicarbonate, 22 mM lactate, 1 mM pyruvate, 6 mg mL-1 fatty acid–free BSA, 100 U mL-1 penicillin and 100 μg mL-1 streptomycin (FERT) as overlaying medium. After 1 h, the supernatant was recovered, and sperm concentration was calculated using a Neubauer chamber. A total of 5x106 sperms mL-1 was used for incubation. For the incubation with microplastic, five groups were made: 1) a control group of FERT media without beads; 2) FERT media containing 0.047 μm polystyrene beads; 3) FERT media containing 0.100 μm polystyrene beads; 4) FERT media containing 0.304 μm polystyrene beads; and 5) FERT media containing 1.112 μm polystyrene beads. The concentration of beads in all groups was 1.2 million beads mL-1. Sperm were incubated at 38.5ºC in a humidified atmosphere of 5% CO2 and 95% O2 for 2 h, with samples being collected for motility, acrosome integrity, and bead attachment analysis at 0, 1, 1.5 and 2 h.

**Sperm motility, bead attachment, and acrosome integrity assessment**

At each of the time points mentioned above, a 5 μL aliquot of sperm was either placed in a pre-warmed slide, covered with a coverslip and a video of at least 5 distinct areas for motility analysis was recorded, using an EVOS M7000 microscope; or added to a SuperFrost™ slide (Expredia), fixed with an equal volume of paraformaldehyde 4% and smeared across the slide. Those slides were left to dry overnight before subsequent analysis of bead attachment and acrosome integrity. The sperm motility (N = 4 bulls) was calculated by analyzing each video and counting total sperm, number of sperm moving, and number of sperm not moving. For the bead attachment, the confocal Raman microscope (WITec, Germany) was used, the slides were imaged using a ×100 DIC NA 0.9 objective, and random areas selected and analyzed for the presence of sperm with microplastic attached to its surface. Each slide had, at least, 100 sperm cells counted (N = 4 bulls). Due to difficulties in visualizing the smallest 0.05 and 0.1 μm polystyrene beads, bead attachment was only quantified for the 0.3 and 1.1 μm beads.

For the acrosome integrity analysis (N=3 bulls), fixed slides were washed 3x with PBS, and stained using a Hoechst 33342 (5 μg mL-1, Thermo Fisher Scientific, USA) and FITC-PNA Alexa 493 (5 μg mL-1, Thermo Fisher Scientific, USA) solution for 15 min. Slides were then washed 3x with PBS and mounted using an anti-fade solution (ProLong™ Gold Antifade Mountant, Thermo Fisher Scientific, USA) and a cover slip. The samples were imaged using a Leica Thunder DMi8 Microscope. The images were captured and processed using the Thunder software. The stained sperm cells were visualized with a ×20 NA 1.25 objective. At least 100 sperm cells/group were counted to determine percentage of cells with intact acrosome.

**Sperm oxidative stress analysis**

We were also interested in assessing the extent to which bovine spermatozoa experienced oxidative stress when exposed to MPs. To this end, for all of the 5 treatment groups described above, 2 μL mL-1 of CellRox™ Green Reagent (Thermo Fisher Scientific, USA) was added (N = 4 bulls). A positive control group was also created by adding 50 μM of H2O2 to stimulate sperm oxidative stress (*53*) Sperm were incubated at 38.5ºC in a humidified atmosphere of 5% CO2 and 95% O2 for 3 h. Every 30 min, a 5 μL aliquot was taken and mounted in a RT slide and cover slip. Slides were let to cool down for 1 min (to stop sperm movement) and images were taken using an EVOS M7000 fluorescence microscope using ×20 NA 0.45. At least 100 sperm cells were counted, and midpiece CellRox™ stained sperm were considered positive.

**Confocal Raman spectroscopy of isolated microparticles and spiked polystyrene beads**

A confocal Raman (alpha300 R, WITec, Germany) with a spectrometer (UHTS 300, WITec, Germany), and a 532 nm laser was used for the characterization of the microplastics. The microscope was operated by using the Control Five software, and the acquired data was processed by the Project Five software for compensation of background noise or cosmic radiation signal. Initially, the microscope was focused on the samples by using a ×50 NA 0.75 Zeiss objective, and the whole area of the membrane was imaged using the ‘Area Stitching’ option and the Z-stack auto-focus. Then, the Particle Scout software was used to identify all the particles present in the imaged membrane. The Raman spectra of each identified particle was then measured with a ×100 DIC NA 0.9 Zeiss objective, using an autofocus function, with a laser power of 10 mV, an accumulation of 4 and integration time of 0.45 s. The presence of microplastics was confirmed by matching the spectra found using the True Match- Integrated Raman Spectra Database Management software, with the S.T. Japan database (S.T. Japan Europe GmbH, Germany). Only matches with a value higher than 75% were considered for further analysis. Poly(tetrafluoroethylene-co-perfluoro-(alkyl vinyl ether)) – PTFE – and Perfluoroalkoxy alkane – PFA – particles were excluded from the analysis, since they are components of the pore membrane used for imaging.

All identified particles were classified as: 1) non-plastic related particle; 2) plastic polymer (PP); 3) plasticizer (PZ); 4) pigment (PG); 5) coating, solvent or filler (CSF); and 6) unknown. Data S1 shows a list of all identified particles and their classification. Particle number was converted to weight by using the formula:

where Pw is particle weight, *ρ* is the particle density and *v* is the particle volume. See Table S1 for data on the density of the particles analyzed here.

To determine the PS nature of B0.3 and B1.1 attached to sperm cells, the Raman spectra of identified PS beads was measured with a ×100 DIC NA 0.9 Zeiss objective, with a laser power of 5 mV, an accumulation of 10 and integration time of 0.5 s. The PS nature of the beads was confirmed by matching the spectra found using the True Match- Integrated Raman Spectra Database Management software, with the S.T. Japan database.

**Statistical analysis**

For data analysis and visualization, R 4.1.2 was used. Data was analyzed using a general linear model in which bull and replicate were considered as random effects. The significance was checked using a Tukey HSD. Differences were considered significant when p < 0.05. All the statistical analysis and the R code used for statistic are described in the supplementary material (GITHUB – MIKE).

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**Figures and Tables**

**Chart, box and whisker chart

Description automatically generated**

**Fig. 2.** **Polystyrene MP effects on sperm function *in vitro*.** (**A**) Percentage of sperm cells with PS beads attached to its surface over time with picture examples of B0.3 and B1.1 attached sperm. Time did not affect attachment, and PS beads were not internalized by the spermatozoa. B0.3 attached more to the sperm surface than B1.1 **(**p = 0.003). (**B**) Boxplot depicting no effect of PS MPs of different sizes (0.05, 0.1, 0.3 and 1.1 μm) on sperm motility in comparison to the control. (**C**) PS MPs significantly reduced acrosome integrity in all groups, independently of the size of the bead (p<0.001). (**D**) Increased ROS production was observed in all MPs groups when compared to the negative control, with B0.05 and B0.1 reaching levels similar to the positive control.

**~~Table 1.~~****~~Short title of the first table.~~** ~~Start table legends with a title (short description of the table). Format tables using the Word Table commands and structures. Do not use spaces, tabs or hard returns to create tables. Every vertical column should have a heading, followed by a unit of measure (if any) in parentheses. Units should not change within a column. Centered headings of the body of the table can be used to break the entries into groups. Footnotes should contain information relevant to specific cells of the table; use the following symbols in order, as needed:\*, †, ‡, §, ||, ¶, #, \*\*, ††, etc. (Don’t use footnotes in column heads; include any such details in sentence form after the table itself.)~~

**Supplementary Materials**

Data S1. Classification of all particles detected in follicular fluid and water samples.

Data S2. Summary of Raman spectroscopy data for all samples.

Table S1. Density values of identified MPs.