Chemosphere 368 (2024) 143714

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Cytotoxicity assessment of HDPE microplastic on *Tetrahymena thermophila* (Protozoa, Ciliate): Assuring quality and FAIR data

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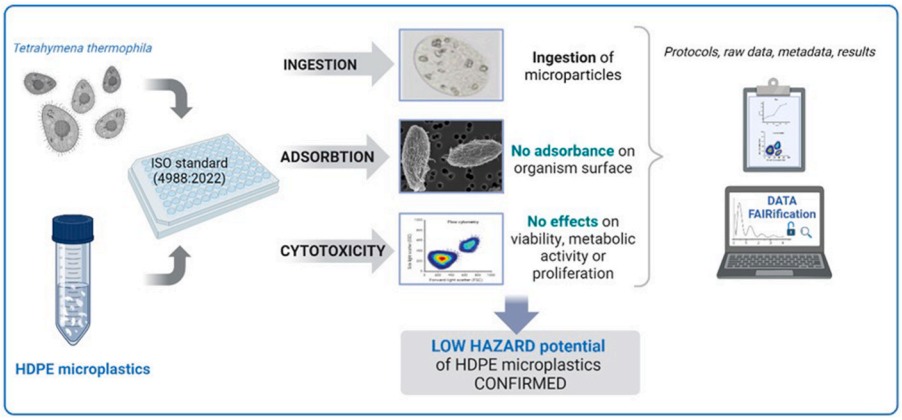
HIGHLIGHTS

- Multigeneration tests with *T. thermophila* followed ISO 4988 (2022) with additional endpoints and SEM.
- No cytotoxicity or adsorption of HDPE particles was observed up to 100 mg/L.
- HDPE particles have low hazard potential despite *T. thermophila* ingestion.
- Results are FAIR and accessible via the eNanoMapper repository.
- Detailed assay information is available in six protocols on Zenodo.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Cytotoxicity
Microplastic
HDPE
FAIR data
Tetrahymena thermophila

ABSTRACT

Microplastics is recognized as an emerging pollutant and adapting and harmonizing existing test methods is essential to advancing research. The aim of our work was to provide a case study on how to ensure quality and FAIR data in the assessment of microplastic hazards with the unicellular organism *Tetrahymena thermophila* (Protozoa, Ciliata). We selected high density polyethylene (HDPE) microplastics as a model material. In the study design we followed the quality criteria recommended for studies on particle effects, specifically emphasizing the reporting of experimental design and data. Our experimental work was based on ISO 4988 (2022) multi-generation tests with *T. thermophila* that was upgraded with additional cytotoxicity tests (protocols have been

Abbreviations: FAIR, indability, accessibility, interoperability, reusability; HDPE, high density polyethylene; ENM, engineered nanomaterials; PI, propidium iodide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; ATP, adenosine triphosphate; LDH, lactate dehydrogenase; BrdU, bromodeoxyuridine; MIMS, minimum information for publications of microplastics studies; BSA, bovine serum albumin; SOP, standard operating procedure; SEM, scanning electron microscopy; TEM, transmission electron microscopy; DLS, dynamic light scattering; MWCNT, multi-walled carbon nanotubes.

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https://doi.org/10.1016/j.chemosphere.2024.143714
Received 1 July 2024; Received in revised form 21 August 2024; Accepted 6 November 2024
Available online 15 November 2024
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made available on Zenodo). In addition, we used microscopy to inspect material-organism interaction. The results show that 24 h exposure of *T. thermophila* to HDPE microparticles did not induce changes in metabolic activity, viability, or proliferation up to exposure concentration 100 mg/L. Microscopy analyses confirmed ingestion of the test material but no adsorption of HDPE particles to the cell surfaces confirming that HDPE microplastics present a low hazard to *T. thermophila*. To maximize the impact of the generated data, we made all the produced data FAIR via the eNanoMapper repository.

1. Introduction

Plastics are an integral part of everyday life due to their low cost, versatility, durability, and strength. Global plastics production is projected to increase from 400.4 million tons in 2022 (Plastics Europe, 2023) to 1231 million tons in 2060, which will substantially worsen what is already a significant environmental burden (OECD, 2022). In the environment, larger pieces of plastic slowly break down into microplastics particles (size <5000 μm; Tavelli et al., 2022) that are already widely present in the aquatic, terrestrial, and atmospheric environment (Lamichhane et al., 2023). A key question related to micro- and nano plastic particles (MNPs) pollution is the extent to which they pose risks to the environment and human health. Despite the increasing number of studies on MNPs, research often lacks methodological harmonization and quality assurance, limiting the ability to compare studies and draw clear conclusions.

The hazard testing of MNPs is challenging due to their wide range of characteristics (e.g., size, density, shape, chemical composition, surface properties, additives, and degree of their weathering), complex transformations, and versatile interactions with biota. De Ruijter et al. (2020) reviewed the literature on the effects of organism exposure to microplastics (MPs) and identified three prevailing effect mechanisms that include: 1) inhibition of food assimilation and/or decreased nutritional value of food and physical interactions that may lead to 2) internal physical damage and 3) external physical damage. Many tests developed for engineered nanomaterials (ENMs) can be adapted for MNPs (Hüffer et al., 2017), but MNP-specific characteristics may require modifications.

Different aquatic organisms (such as fish, ciliated protozoans, crustacea, and algae) are currently used to predict the potential harmful effects of pollutants, including ENMs and MNPs, on the aquatic environment. Among these, *Tetrahymena* sp. is advantageous for aquatic toxicology studies due to its physiology and behavior, making it a valuable model for assessing toxic substances (Maurya and Pandey, 2020). *Tetrahymena* sp. tests are multigenerational growth tests, including five to six generations in 24 h, with feeding based on internalizing particulate matter up to a few microns by phagocytosis and fluid by pinocytosis (Bulannga and Schmidt, 2022; Kong et al., 2021). An ISO standard released in 2021 focuses on evaluating the toxicity and bioassimilation of nano-objects using *Tetrahymena* sp. (ISO 4988, 2022).

Since *Tetrahymena* sp. is a unicellular organism, various assays developed for non-motile *in vitro* cell cultures can be used to assess cytotoxicity with this organism. Common cytotoxicity tests include different metabolic activity assessments, cell viability and cell proliferation tests. For tracking cell viability, the propidium iodide (PI) and Hoechst differential staining assay is frequently used. PI can passively penetrate cells with a permeable cell membrane regardless of the mechanism of death and binds to DNA and RNA. PI staining is usually coupled with a universal dye, Hoechst, that can cross both damaged and intact membranes and stains the nucleic acids of all cells, allowing a total cell count to be determined (Rosenberg et al., 2019). Among the most frequently used assays to detect changes in cellular metabolic activity are resazurin, MTT, and ATP. In the fluorometric resazurin assay, resazurin, a blue, non-fluorescent, cell-permeable, soluble dye, is catalysed in viable, metabolically active cells by cellular enzymes, such as NAD(P)H-dependent reductase, to the pink, fluorescent product resorufin (Mello et al., 2020). The MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay is a commonly used colorimetric assay that also measures the metabolic activity and viability of cells. MTT determines cell viability by measuring the activity of the mitochondrial enzyme succinate dehydrogenase, which converts water-soluble tetrazolium salt into an insoluble purple formazan in metabolically active cells (Fotakis and Timbrell, 2006). The ATP (adenosine triphosphate) luciferase assay is a luminescent assay that is used to measure cellular energy production. In the ATP-luciferase assay, cells are incubated with the enzyme luciferase and the substrate luciferin. Since the luciferase reaction requires ATP, only living cells in the sample produce a bioluminescent signal. The relationship between the luminescence signal intensity and the ATP concentration in viable cells is linear (Aslantürk et al., 2018). The ISO 4988 (2022) recommends the ATP luciferase and MTT assays for cytotoxicity measurement of (nano) materials with *Tetrahymena* sp., with the LDH assay as an alternative.

Cell proliferation assays provide valuable toxicological information regarding the effects of substances on cell growth and division. Two such assays are the CyQUANT and BrdU cell proliferation assays. The CyQUANT cell proliferation assay is based on assessing the total amount of DNA in living cells. A bright green, fluorescent, cell-permeable DNA binding dye is combined with a background suppression reagent which blocks the staining of dead cells with damaged cell membranes. In this way, only viable cells are detected. This assay is commonly used to monitor cell proliferation and assess cell viability (Hanson and Hancock, 2014). In contrast to assays that measure the metabolic activity of each sample, which are dependent on the metabolic activity of individual cells as well as on the number of viable cells, the BrdU assay provides information which is limited to cell proliferation. Cells are grown in a medium containing BrdU (thymidine analog 5-bromo-2′-deoxyuridine) which is incorporated into newly synthesized DNA instead of thymidine during the S phase of the cell cycle. The incorporated BrdU can then be easily detected with labeled anti-BrdU antibodies (Bergler et al., 1993; Sivakumar et al., 2004).

As awareness of the environmental risks posed by MNPs grows, there is a concurrent rise in both funding and the volume of publications dedicated to this critical subject. Consequently, many researchers have elaborated recommendations for how to harmonize these studies. De Ruijter et al. (2020) developed 20 study quality criteria including particle characterization, experimental design, applicability in risk assessment, and ecological relevance. Cowger et al. (2020) developed documents that can be used to make studies on MPs comparable and reproducible. Dehaut et al. (2019) proposed minimum information for publications of microplastics studies (MIMS) in seafood as a starting point for the harmonization of analyses. Also, OECD guidance documents, in particular OECD 317 (2020) for nanomaterials, provide recommendations and control measurements that can be performed to better understand the assay results and avoid artefacts when testing particles.

Despite these recommendations, poorly described methods remain a challenge. Consequently, various initiatives are advocating for comprehensive protocol documentation and sharing to enhance the quality of research and minimize research inefficiency (SPIRIT, PROMaP, PRISMA) (Chan et al., 2013; Leite et al., 2023; Page et al., 2021). Besides documenting and sharing protocols, it is crucial to process and curate data in accordance with FAIR principles (findability, accessibility, interoperability, and reusability) (Dumit et al., 2023). The FAIR principles emphasize the importance of proper metadata, clear

licensing, and standards for making data findable and useable across different contexts. Reusability is facilitated by having raw and processed data in machine readable form, allowing re-use in contexts such as automated data analysis workflows (e.g., scoring, ranking, similarity). Reusability involves providing clear licensing information, provenance (who produced the data), and context for understanding how the data can be effectively reused.

In an attempt to harmonize data entry templates and address both machine readability and user friendliness, the eNanoMapper FAIR-ification workflow (Kochev et al., 2020) and Template Wizard were developed during the integration of large nanosafety data sets (Jeliazkova et al., 2024). These templates resulted from a co-creation process involving laboratory researchers and data managers and are now being reused in microplastics and advanced materials safety research.

Present study aims to ensure quality and FAIR data in assessing the cytotoxicity of high-density polyethylene microplastics (HDPE MPs) using *T. thermophila* (Protozoa, Ciliata). HDPE plastic is used in many food contact products, such as milk jugs and food storage containers and is also present in construction materials, medical devices, toys, automotive parts, and clothing. HDPE is FDA-approved, meaning it is safe for food and beverage contact (A&C Plastics, 2024). However, some recent studies report effects of HDPE on environmental organisms, in particular on their development and behaviour (Bringer et al., 2020), as well as effects of leachates from new plastic HDPE bags (Balestri et al., 2019). This reopens the discussion on the safety of plastics and underscores the necessity for reassessing existing tests. We evaluated the hazard of HDPE MPs at the organism and cell levels by conducting observational studies of material ingestion and surface adsorption and by implementing several different metabolic activity assessments, cell viability and cell proliferation tests. In addition, we made all data FAIR and all protocols open. In this way, we implemented the principles of open science in the area of MNP safety investigation.

2. Materials and methods

2.1. Tested MNPs preparation and characterisation

High-density polyethylene microparticles (HDPE MPs, 5 μm) were provided from partners in the PlasticsFatE project (BAM-Bundesanstalt für Materialforschung und-prüfung). As particulate controls, we used TiO_2 nanoparticles (NPs) purchased from a TiO_2 producer (the producer does not want to be identified) and Ag NPs purchased from Sigma Aldrich (Taufkirchen, Germany). Chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Merck (Darmstadt, Germany), or Carl Roth (Karlsruhe, Germany), unless otherwise indicated.

All particles were dispersed in water containing 0.05% (w/v) bovine serum albumin (BSA) and 25 μl/L surfactant Tween40 and sonicated in a water bath (Iskra PIO, Sonis) for 1 h. The details of this method are described in a standard operating procedure (SOP) established for different types of polymers within the InnoMat.Life project (Vogel et al., 2021).

After dispersion, the particles (HDPE MPs, TiO_2 NPs, and Ag NPs) were diluted in a semi-defined proteose-peptone based, nutrient poor medium (PM), as described by Schultz (1997), to obtain 100 mg/L of particle suspension for characterization. The hydrodynamic diameter of the particles in suspension was measured using dynamic light scattering (DLS; Litesizer 500, Anton Paar, Graz, Austria) and the zeta potential was measured with a ZetaPALS zeta potential analyser (Brookhaven Instruments Corporation, Holtsville, NY). The form of the NPs (TiO_2 and Ag) was observed with transmission electron microscopy (TEM) JEOL 2100 (Tokyo, Japan), while the morphology was determined using field emission scanning electron microscopy (FE-SEM JEOL JSM-6500F). For SEM, the particles were fixed on an aluminium holder using adhesive carbon tape and sputtered with a 10 nm layer of platinum-palladium.

2.2. T. thermophila growth and exposure conditions

A culture of *T. thermophila* from PROTOXKIT F (MicroBioTests) was grown for 3 days in dark conditions at 32 °C in a nutrient rich medium (RM) containing D-glucose, yeast, Trisma-base, chlorides, and sulphates, according to Schultz (1997). The *T. thermophila* were then centrifuged at 60 rcf for 3 min, washed, and resuspended in PM and seeded in flat-bottomed 96-well plates. To each well, 100 μL of the cell suspension (15 x 10^3 TTH/well) and 50 μL of the test material were applied and incubated for 24 h. HDPE-MP, Ag NP, and TiO_2 NP suspensions were prepared in PM for final concentrations of 1, 10, 25, 50, 75, and 100 mg/L for each type of particle. As a positive control for cytotoxicity, 2% Triton X-100 was used. Wells containing only the treatment with particles and an assay reagent (without *T. thermophila*) were used as a background control. After incubating the plates for 24 h at 32. °C, cytotoxicity was determined using the resazurin, ATP, MTT, CyQUANT proliferation, BrdU proliferation, and PI and Hoechst differential staining assays.

2.3. Imaging of MNPs uptake and adsorbtion by T. thermophila

Particle uptake into the food vacuoles of *T. thermophila* was investigated after exposure to 100 mg/L each of HDPE MPs, TiO_2 NPs, and Ag NPs. The food vacuoles formed by phagocytosis were observed after 4 h of exposure using a light microscope (LM; Axio Imager Z1, Zeiss). For this purpose, the *T. thermophila* was fixed with Karnovsky fixative (2.5% glutaraldehyde and 0.4% paraformaldehyde in Na-phosphate buffer) and 10 μL of the sample was transferred from the well to a glass slide. Two replicates were performed for each exposure concentration.

Adsorption of HDPE MPs, Ag NPs, and TiO_2 NPs on the surfaces of *T. thermophila* was observed with SEM. After 24 h of exposure, 50 μL of suspension containing *T. thermophila* from the 50 and 100 mg/L exposure groups were washed with PBS and chemically fixed in modified Karnovski fixative (2.5% glutaraldehyde and 0.4% paraformaldehyde in phosphate buffer) at 4 °C for 24 h. The cells were then dehydrated in a series of alcohols (30%, 50%, 70%, 80%, 90%, and absolute ethanol) and chemically dried with hexamethyldisilazane according to Hočevar et al. (2020). Between each step, the samples containing *T. thermophila* were centrifuged at 800 rcf for 3 min. The organisms were then transferred to aluminium holders on a carbon tape disc, sputtered with 7 nm gold-palladium (Sputter coater SCD 050, BAL-TEC), and investigated with SEM (Jeol JSM-6500F).

2.4. Cell viability assays

The assays were performed according to ISO 4988 (2022), with four additional viability tests (resazurin, CyQUANT direct cell proliferation, BrdU cell proliferation, and PI and Hoechst differential staining). Instead of the positive controls suggested in the standard, 2% Triton X-100 (final concentration 0.1%) was used as a positive control.

2.4.1. Resazurin assay

After a 24 h treatment, the resazurin assay was performed as described in Kononenko and Drobne (2019) with slight modifications. In brief, 50 μL of resazurin (0.15 mg/mL) was added to each well and incubated at 32 °C for 4h as described in the relevant protocol Perc et al.

(2024a). The fluorescence intensity of the formed resorufin was measured (560/590 nm ex/em) using a spectrofluorimeter (BioTek, Cytation 3). For each treatment condition, three independent experimental repetitions were performed, for a total of six replicates.

2.4.2. ATP assay

ATP was quantified using the ATP Cell Viability Luciferase Assay (Millipore), a highly sensitive firefly luciferase cell-based assay used to measure cell viability in cell cultures.

T. thermophila was treated as described in Chapter 2.2, with the exception that the positive control for cytotoxicity (2% Triton X-100) was added after a 24 h treatment incubation and before the measurement. The ATP Detection Cocktail was prepared as described in the relevant protocol Perc et al. (2024). A microplate reader was set up with a delay time of 10 s and an integration time of 10 s. Subsequently, 100 µL of the ATP Detection Cocktail was added to each well by automated injection. Luciferase activity measurements started after a 1 s delay and an integration time of 10 s. The resulting luminescence was measured using a Spectrofluorimeter (Biotek, Cytation 3) microplate reader. For each treatment condition, three independent experimental repetitions were performed, each with three replicates.

2.4.3. MTT

After a 24 h treatment, 15 µL MTT (5 mg/mL) was added to each well and incubated for 4 h at 32 °C, as described in the relevant protocol Perc et al. (2024d). After 4 h, a solution for solubilizing the formed formazan (10% SDS and 0.01 M HCl) was added according to Jo et al. (2015). The metabolic activity of the exposed *T. thermophila* was determined using absorbance measurements at 570 nm using a spectrophotometer. For each treatment condition, three independent experimental repetitions, each with six replicates, were performed.

2.4.4. CyQUANT™ cell proliferation assay

The CyQUANT™ Direct Cell Proliferation Assay kit (Invitrogen, Carlsbad, CA, USA) was used to determine cell growth and viability in accordance with the manufacturer's instructions, which are described in detail in the relevant protocol Perc et al., (2024e). After addition of the detection reagent (a combination of a nucleic acid stain and background suppressor), the *T. thermophila* were incubated for 60 min and fluorescence was measured at 480/535 nm using a plate reading spectrofluorimeter. For each treatment condition, three independent experimental repetitions were performed, each with three replicates.

2.4.5. BrdU cell proliferation assay

For the evaluation of *T. thermophila* proliferation, the BrdU Cell Proliferation Assay from Sigma Aldrich was used. A BrdU label was added to the *T. thermophila* medium together with the treatments. After a 24 h incubation period, the assay was performed according to the manufacturer's instructions. In brief, the samples were centrifuged, fixed, and denatured, after which the anti-BrdU antibody was added. The samples were then washed and perixidase-goat anti-mouse IgG-HRP was added, as detailed in the relevant protocol Perc et al., (2024c). After 30 min of incubation, a stop solution was added and absorbance was measured at dual wavelengths of 450–540 nm (or 450–595) using a plate reading spectrophotometer. For each treatment condition, three independent experimental repetitions were performed, each with three replicates.

2.4.6. Propidium iodide (PI) and Hoechst - differential staining cell viability assay

After 24 h of exposure, the *T. thermophila* were stained with 5 µg/mL propidium iodide and 5 µg/mL Hoechst 33342 for 20 min. The amounts of dead and viable *T. thermophila* were determined with a flow cytometer (FACSMelody™, BD Bioscences) using a propidium iodide (excited at 488 or 561 nm, with a 610/10 bandpass filter) and Hoechst stain (excited with a UV laser at 350 nm, measured with a 450/20 BP and 675 EFLP optical filter), as described in the relevant protocol Perc et al., (2024b). For each treatment condition, two independent series of experiments with two replicates each were performed.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.03 software. The results from all cytotoxicity assays (resazurin, ATP, CyQUANT, MTT, BrdU, and PI and Hoechst differential staining) were expressed as the arithmetic mean ± standard deviation (SD) and were statistically analyzed using a one-way ANOVA with Dunnett's multiple comparison test. A p value lower than 0.05 was considered statistically significant. Flow cytometry results were analyzed with FlowJo_V10 software and the statistics were performed with GraphPad Prism 5.03.

3. Results

3.1. MNPs characteristics

SEM observation revealed that the HDPE particles have a non-uniform shape and range in size from app. 2–5 µm (Fig. 1a). Both NPs, TiO2 NPs and Ag NPs are round shaped and in nanosizes (Fig. 1b and c). The hydrodynamic diameter was 360 nm for the TiO_2 NPs, 62 nm for the Ag NPs, and 434 nm for the HDPE MPs. The ζ-potential was −21,0 mV for the TiO_2 NPs, −21,1 mV for the Ag NPs, and −25,5 mV for the HDPE MPs. TEM showed a crystalline form of the TiO_2 NPs with sizes ~150 nm (See Supplementary material, Fig. S1). The Ag NPs were found to possess a crystalline surface layer and were present as large aggregates of few hundred nm (See Supplementary material, Fig. S2).

3.2. MNPs uptake and adsorbtion by T. thermophila

Using light microscopy, we showed that micro-sized HDPE particles, as well as nano-sized TiO_2 and Ag, were ingested by *T. thermophila.* As

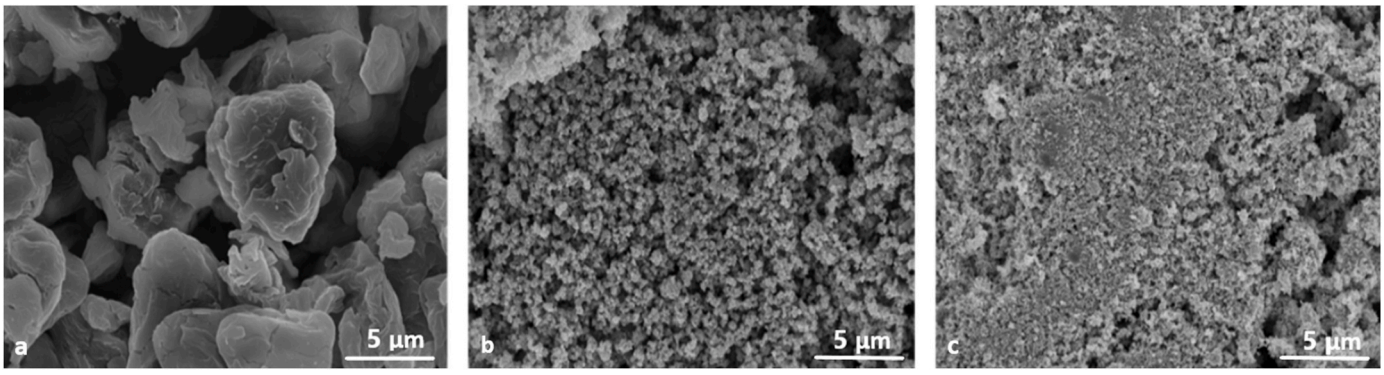

Fig. 1. Scanning electron micrographs of (a) HDPE MPs, (b) TiO_2 NPs, and (c) Ag NPs at same magnification.

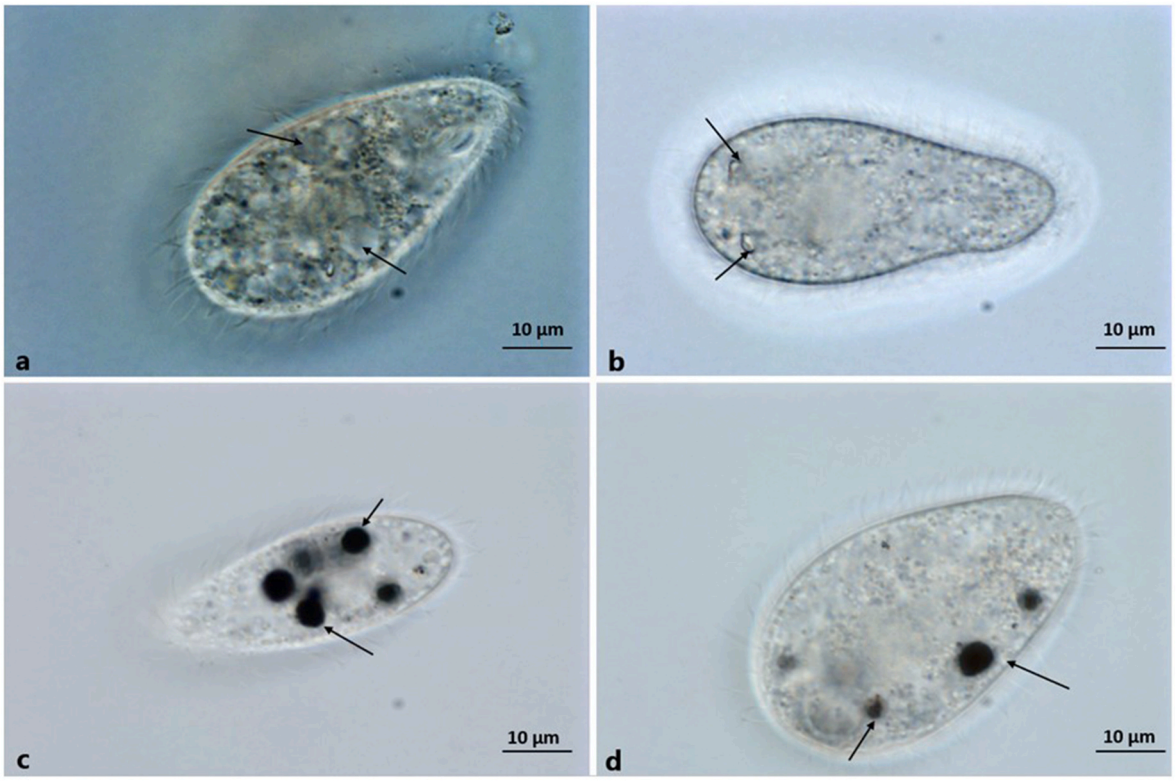

Fig. 2. Micrograph of *T. thermophila*; Bright field LM after 4 h of exposure. The arrows indicate empty vacuoles in (a) untreated *T. thermophila,* (b) ingested HDPE MPs, (c) TiO_2 NPs, and (d) Ag NPs.

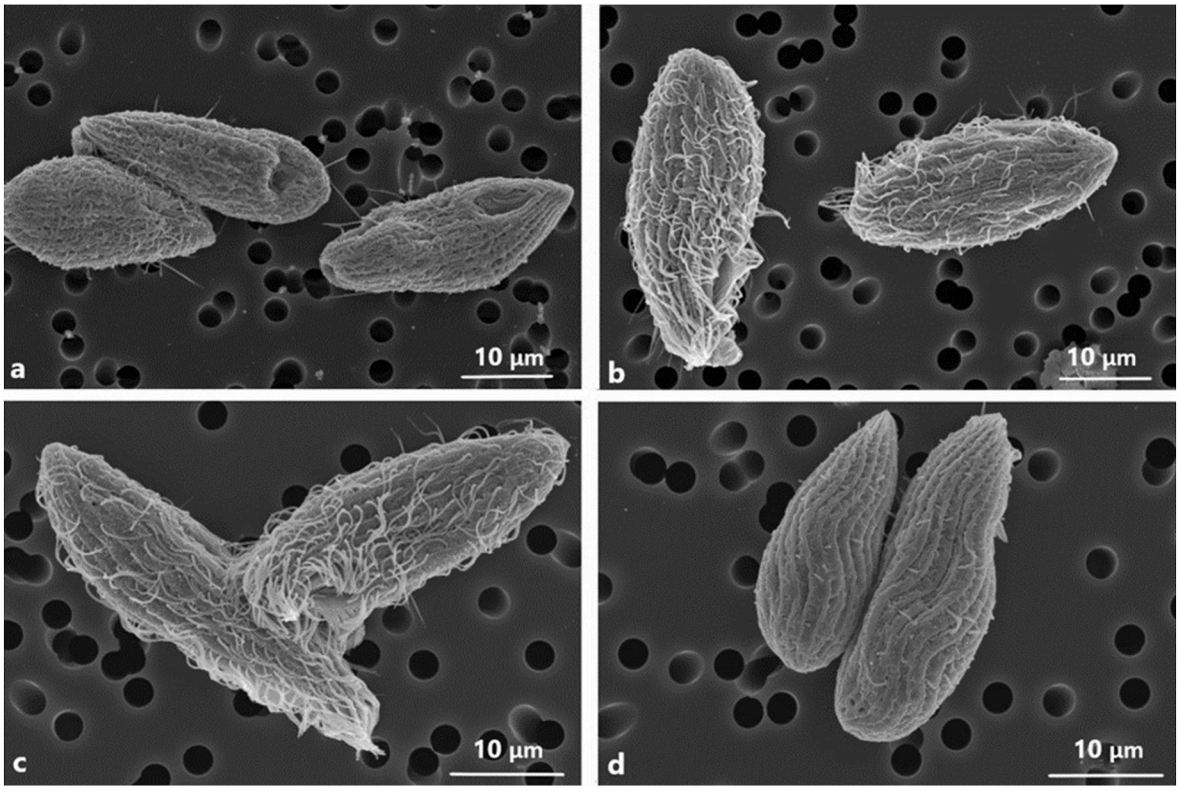

Fig. 3. Scanning electron micrograph of (a) control *T. thermophila* and after exposure to (b) HDPE MPs with concentration 100 mg/L, (c) TiO_2 NPs with concentration 100 mg/L, and (d) Ag NPs with concentration 50 mg/L.

expected, after a 4 h exposure period, the particles were present in food vacuoles (Fig. 2b–d).

After a 24 h exposure to HDPE MPs and TiO_2 NPs, no material was adsorbed onto the body surfaces of the *T. thermophila* when exposed to concentrations up to 100 mg/L. As exposure to the highest concentration of Ag NPs (100 mg/L) was already very cytotoxic, here animals exposed to 50 mg Ag NPs/L were inspected with SEM, and although no material was observed on the surfaces in this sample either, the microvilli were clearly damaged and/or absent, which is consistent with the results of cell viability testing showing toxicity at this concentration (Fig. 3).

3.3. Cytotoxicity

Cytotoxicity was assessed using assays that measured changes in cell viability, metabolic activity, and proliferation. In addition to HDPE MPs, the cytotoxicity of two particulate controls, TiO_2 Ag NPs, was also evaluated.

With none of the assays statistically significant effects of HDPE MPs on *T. thermophila* (Fig. 4a–d, g, j, m, p) were showed. The lowest standard deviations were measured in the case of the resazurin and PI and Hoechst differential staining assays (Fig. 4a–p), and in the measurement of cytotoxicity with the BrdU assay, the standard deviations were the highest when concentrations greater than 25 mg/L were tested (Fig. 4m).

Cytotoxicity of TiO_2 NPs was not detected by the resazurin, ATP, CyQUANT, BrdU, and PI and Hoechst assays (Fig. 4b, h, k, n,q). With the CyQUANT assay, an increase in cell proliferation was observed at an exposure concentration of 50 mg/L. However, this increase was not statistically significant at any of the three highest tested concentrations (Fig. 4k). In the case of TiO_2 NPs due to interferences of the MTT assay with particles, negative values were measured at concentrations $\geq$50 mg/L, which were statistically significantly different compared to the untreated control (Fig. 4e).

The cytotoxic effect of Ag NPs on *T. thermophila* was confirmed with all the selected assays (Fig. 4c,f, i, l, o, r). However, statistically significant effects occurred at different exposure concentrations, depending on the test used. In the resazurin assay, statistical differences occurred at concentrations $\geq$25 mg/L (Fig. 4c). A dose-dependent decrease in the metabolic activity of the exposed cells was measured, with the highest doses showing the greatest decreases compared with the control. With the MTT and CyQUANT Proliferation assays, statistically significant effects were found already at 10 mg/L (Fig. 4f,l), whereas with the ATP and BrdU assays, they occurred at 50 mg/L (Fig. 4i,o). With the ATP assay, high standard deviations were observed at concentrations of 10 and 25 mg/L (Fig. 4i). Also, the PI and Hoechst differential staining assay showed a statistically significant difference in the number of dead cells compared with the control at concentrations of 50 mg/mL and above (Fig. 4r).

3.4. Data sharing

Six detailed protocols for the cytotoxicity assays used in this study were produced and uploaded on the Zenodo repository (resazurin (Perc et al., 2024a); ATP (Perc et al., 2024); MTT (Perc et al., 2024d); CyQUANT cell proliferation assay (Perc et al., 2024e); BrdU Cell Proliferation assay (Perc et al., 2024c); Propidium iodide (PI) and Hoechst - Differential staining cell viability assay (Perc et al., 2024b).The metadata and results were uploaded to the eNanoMapper database (Enanomapper, 2024).

4. Discussion

Here, we have provided a case study on how to ensure quality and FAIR data on microplastics hazards. We tested effect of HDPE microplastics according to modified ISO 4988 (2022) multigeneration tests with *T. thermophila* (Protozoa, Ciliate). We have successfully adopted different *in vitro* assays to provide information on cell metabolic activity, viability and proliferation upon exposure to microplastics to allow comprehensive assessment of hazard. Our results show that exposure of *T. thermophila* to HDPE MPs, did not provoke any adverse effects up to exposure concentration of 100 mg/L. In addition, microscopy analyses confirmed ingestion of the tested material, but no adsorption of HDPE MPs was found on the surfaces.

Based on these evidences we conclude that tested HDPE particles have a low potential for hazard, which aligns with the FDA-approved utilization of HDPE (SyBridge Technologies, 2021) and many *in vitro* cytotoxicity studies (Choi et al., 2021; Dusza et al., 2022; Zimmermann et al., 2019). However, some other studies show effects of HDPE MPs on environmentally relevant organisms, such as zebrafish embryos, seaworms, sea urchin, oysters, different bivalves, and various plant species (Aishwarya et al., 2024; Bringer et al., 2020; Green et al., 2019; Kim et al., 2020; Martínez Gomez et al., 2017; Missawi et al., 2021; Niemcharoen et al., 2022; Von Moos et al., 2012). These effects could be explained by inhibition of food assimilation and/or decreased nutritional value of food and internal and external physical damage (Bringer et al., 2020; De Ruijter et al., 2020) as well as indirect effects, such as adsorption to body surfaces.

In our study, none of these modes of interaction lead to adverse effects. Despite ingestion of HDPE, it did not cause adverse events. Our results show that HDPE MPs (5 μm) were present in the food vacuoles, which could make these particles bioavailable for organisms at higher trophic levels. In case of *T. thermophila*, ingested food is defecated and any incidental material does not accumulate inside the cells with time. Nilsson (1977) reported that the digestive cycle lasts roughly 2 h and that egestion of undigestible material is a random process. Mortimer et al. (2016) provide evidence that the mass of MWCNTs retained in protozoa correlates positively with cell number. This means that material does not accumulate inside individual cells. However, the bigger the population, the more material is concentrated in vacuoles and has the potential to be transferred along the food chain. Dong et al. (2018) studied the bioaccumulation of 14C-labeled graphene in an aquatic food chain through direct uptake and trophic transfer. They reported high food chain transfer from *E. coli* to *T. thermophila*, however, food chain transfer from *T. thermophila* to *D. magna* and from *D. magna* to *D. rerio* was much lower, indicating that biomagnification is unlikely to occur in these food chains. This suggests that the presence of material in the vacuoles of *T. thermophila* is confirmation that the material is bioavailable, but has a low potential for significant food web transfer (Mondellini et al., 2024).

Also, we did not observe any surface adsorption of HDPE MPs by SEM. This may be due to the fact that *T. thermophila* are covered by cilia that may prevent adherence of particles to their surfaces. In addition, SEM investigation of *T. thermophila* can identify deciliation, i.e., cilia shedding that is a typical response of ciliates to stress, which can occur even if material is not ingested (Gogendeau et al., 2020). We have confirmed that effect when the particulate control with known toxic effect, Ag NPs was tested. As part of validating our experimental setup, we also tested the adsorption of spherically shaped PS microparticles on *Tetrahymena*. We confirmed that there was no adsorption in this case as well (Results available in Supplementary Information, Fig. S4).

Studies on the effects of MNPs on *Tetrahymena* sp. are scarce. To date, no study has investigated the effect of PE-MNPs on the viability of ciliates, although some authors have reported effects of PS-MNPs. While the primary aim of our study was to propose a strategy for testing MNPs on *Tetrahymena* sp., we also validated the results obtained with HDPE-MNPs using PS-MNPs. We concluded that there was no effect on the viability of *T. thermophila* up to the highest tested concentration. Wu et al. (2021) studied the effects of PS nanoplastics on *T. thermophila* and found that particles induced the release of Ca into the cytosol, leading to growth inhibition due to increased mitochondrial permeability and ROS generation. The ingestion of PS microparticles by *Tetrahymena* sp. was confirmed by Bulanga and Schmidt (2022), however they did not

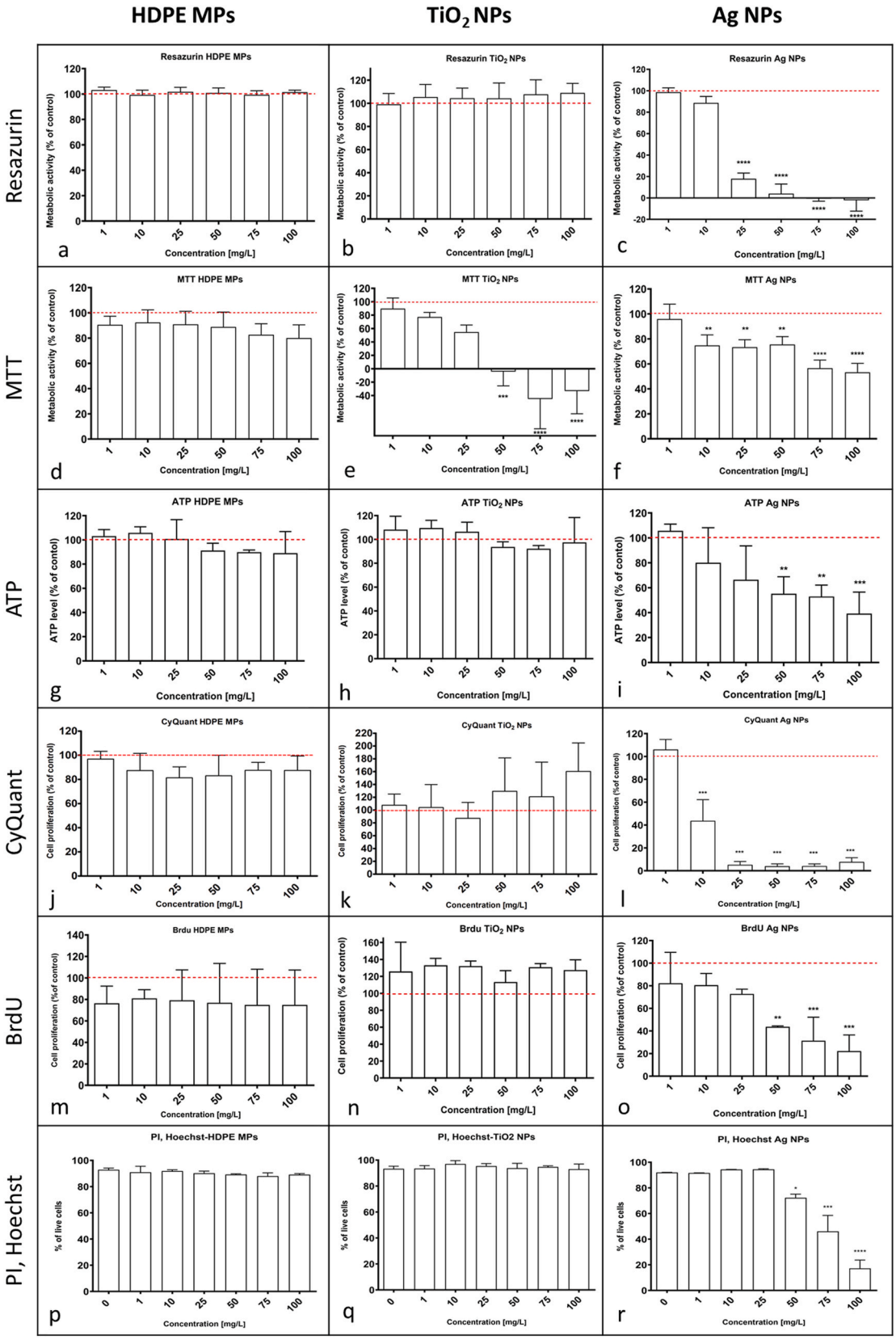

Fig. 4. Cytotoxicity after 24h exposure to HDPE MPs (a, d, g, j, m, p), TiO_2 NPs (b, e, h, k, n, q), and Ag NPs (c, f, i, l, o, r). Cell viability assay (PI and Hoechst differential staining, p-r), cell proliferation assays (CyQUANT and BrdU assays, j-o), and metabolic activity assays (resazurin, MTT and ATP, a-i) were performed with *T. thermophila.* Data are presented as average percentages of untreated controls (+SD) except for PI, Hoechst (p, q, r). An asterisk represents a significant difference with respect to the untreated control cells (dashed line; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; one-way ANOVA with Dunnett's post-test).

investigate the effects of the ingested material on the organisms. Mondellini et al. (2024) demonstrated uptake and trophic transfer of PS microplastics, emphasizing the need to study the effects of MNPs on unicellular eukaryotes when assessing the hazard of MP contamination in aquatic environments. The rate and uptake of PS MPs have also been studied in other ciliated protozoa (Fyda and Budziak, 2023; Nugroho and Fyda, 2020; Zhang et al., 2021). In a recent study by Budziak and Fyda (2024), they examined the population growth rate of selected ciliate species (*Blepharisma japonicum*, *Euplotes* sp., and *Spirostomum teres*) and confirmed that MPs have variable, species-specific effects. They also found that the ingestion of MPs depends on MP concentration and size. Additionally, Zhang et al. (2021) confirmed that PS MPs significantly decreased the biomass of the marine protozoa *Uronema marinum*.

Various cytotoxicity assays are employed to test effects of different substances on the motile cell cultures depending on the objectives of a particular study and the feasibility of specific methodologies within a research group (Maurya and Pandey, 2020). ISO 4988 (2022) proposes that the MTT or LDH assay, ATP assay, cell viability assay with trypan blue staining, and population growth impairment test be used to assess particle toxicity. In the study presented here, we modified and expanded the list of tests that can be used to evaluate various outcomes of MNPs exposure. We used the MTT, resazurin, and ATP assays for evaluating metabolic activity. We further selected the BrdU and CyQUANT assays to provide us with more precise information on cell proliferation than the ones proposed in the standard, i.e., population growth impairment tests based on measuring the optical density of *T. thermophila*. Also, limitation of metabolic assays is that cellular metabolic activity varies greatly throughout the lifecycle of cells (Quent et al., 2010). To assess cell viability, we used differential staining with PI and Hoechst instead of the less sensitive trypan blue staining. Additionally, as direct visual cell counting using haemocytometer is very time consuming and subject to operator errors, we have measured the viable cells with more accurate and reproducible method, flow cytometry.

We have concluded that all used assays are suitable for evaluating the cytotoxicity of MPs to a motile culture of *T. thermophila* during 24 h multigeneration tests. However, performing an excessive number of tests with similar methodologies that measure similar outcomes can be both time- and resource-consuming and may not yield significant benefits. Therefore, we advocate for a thoughtful selection of tests when assessing the hazards associated with MNPs to *T. thermophila*. We propose the use of differential staining with PI and Hoechst as an initial screening method for assessing the adverse effects of MNPs on *T. thermophila*. This method is straightforward and can be performed using either fluorescence microscopy or flow cytometry. In the case of a positive result (statistically significant effect of tested material on the viability) at environmentally relevant concentrations, we can conclude that the tested material at the given concentration is hazardous and could be classified as a material of concern. In the case of negative result, we recommend further testing with at least one of the tests for assessing metabolic activity (Resazurin, MTT, or ATP assay) and at least one test for measuring cell proliferation (CyQUANT or BrdU assay). If the results of these cytotoxicity tests are positive at environmentally relevant concentrations, the material should be classified as potentially hazardous. If the results of these cytotoxicity tests are negative, the material could be classified as a material of low hazard, i.e., low concern. To validate our findings, we have also tested PS microplastics (of the same size as the HDPE tested) and conducted all three proposed assays to compare the effects of different types and shapes of microplastics (PS and HDPE). PS microplastics did not cause any cytotoxicity (Characterization and results available in Supplementary Information, Figs. S3, S4, S5).

We have also selected two particles with known modes of action as particulate controls. Both controls served as reference points, ensuring that the test was working correctly and allowing for comparison of the response of the test system to the known effects of a particulate substance. As expected, TiO_2 NPs did not cause cytotoxicity, while Ag NPs reduced viability in a dose dependant manner. In the case of TiO_2 NPs and the MTT assay, we detected interferences with the assay. This has already been shown in the work of Kroll et al. (2012), Guadagnini et al. (2015) and Holder et al. (2012). Consequently, when TiO_2 NPs are used as a particulate control, the MTT assay should be replaced with another cytotoxicity test.

In the work presented here, we adhered to the quality criteria recommended for studies on particle effects, specifically emphasizing the reporting of experimental design and data, and also comply with Open Science principles. We provide an example on how to make data FAIR and protocols opened. Extensive information on the experimental setup and assays can be found in six protocols that are available online (See Results 3.4). The protocols are linked to 18 sets of FAIR data (Enanomapper, 2024) that make the data from this study findable and reusable. In addition to making our protocols openly accessible, we have also ensured that all toxicity data regarding microplastics adheres to the FAIR (Findable, Accessible, Interoperable, and Reusable) principles. By making data into a machine-readable form, we facilitate its reusability. Both open protocols and FAIR data are essential aspects of study quality and open science principles, fostering an open and collaborative scientific environment.

At present, data sharing differs across scientific communities, and while required by some funders and publishers, it is still perceived to be an added workload and outside of routine procedures. Open-science communities are starting to play a role in building a research culture where sharing data and laboratory materials is normal and expected behaviour (Pascu and Burgelman, 2022). Although many researchers wholly embrace open-access publishing, the open sharing of protocols and data is also very slow to be adopted due to a lack of know-how and incentives (NASA, 2023; Gentemann, 2023; Mons et al., 2020). The website *protocols.io* has sought to address some of these shortcomings by providing an option for users to vouch for the reliability of different protocols (Tay, 2024). The multitude of data formats and reporting standards and their transience also discourages researchers from data sharing. The data reported in this paper have been entered into templates available through the eNanoMapper PlasticsFatE Template Wizard and imported into the PlasticsFatE database using the FAIR-ification workflow.

Our study was also an attempt to inspire more scientists to adopt open science principles with the objective of ensuring that scientific research is readily accessible to everyone, thereby benefiting both scientists and society at large.

5. Conclusions

We demonstrate the applicability of ISO 4988 (2022) using *Tetrahymena* sp. for MPs hazard assessment, exemplified with HDPE. *Tetrahymena* sp. combines characteristics of both whole organisms and single eukaryotic cells, making it a suitable model for rapid multigeneration ecotoxicity tests (Gerhardt et al., 2010). This multilevel testing approach helps establish causal links between biochemical responses and ecological effects (Nederstigt, 2022). We showed that various cytotoxicity assays are appropriate for assessing the effects of MPs on the motile cell culture *T. thermophila*; however, potential interferences from particulate matter should be considered in each study. Even if no cytotoxicity is detected, indirect effects such as adsorption to body surfaces must be monitored. A final confirmation of material hazard should be done by assessing particle adsorption on other environmentally relevant organism surfaces as adhesion of materials on surface of ciliates is not likely to occur, even with nanomaterials. To our knowledge, no prior research has investigated the effects of PE particles on ciliates. Additionally, our study is the first to assess a range of commonly used cytotoxicity assays on motile cell cultures. The protocols used in this study are openly available, and all generated data are curated following FAIR principles, maximizing the impact of our findings and promoting

data reuse. Reusing shared data facilitates the development and validation of new algorithms – e.g., creating predictive models and novel and more efficient data analyses. With that we have demonstrated how to put an open science stance into practice to enhance the transparency, accessibility, reproducibility, and rigor of scientific research (Center for open science, 2023).

Funding information

This research was funded by the Slovenian Research and Innovation Agency J1-50014, by a Young Researcher grant from the Slovenian Research and Innovation Agency to Valentina Perc, by the EU Horizon project (g. a. 965367) and by Infrastructural Centre for Microscopy of Biological samples (I0-0022-0481-08) at Biotechnical faculty, UL.

CRediT authorship contribution statement

Valentina Perc: Writing – original draft, Methodology, Investigation, Formal analysis. **Veno Kononenko:** Writing – review & editing, Validation, Methodology. **Nina Jeliazkova:** Data curation. **Matej Hočevar:** Methodology. **Slavko Kralj:** Methodology, Data curation. **Darko Makovec:** Methodology, Data curation. **Maja Caf:** Methodology, Data curation. **Damjana Drobne:** Writing – review & editing, Conceptualization. **Sara Novak:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143714.

Data availability

All the produced data are FAIR via the eNanoMapper repository

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