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Research Paper

Adverse effects polystyrene microplastics exert on zebrafish heart – Molecular to individual level

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ABSTRACT

In the present study the effects of sublethal concentrations of polystyrene microplastics (PS-MPs) on zebrafish were evaluated at multiple levels, related to fish activity and oxidative stress, metabolic changes and contraction parameters in the heart tissue. Zebrafish were fed for 21 days food enriched with PS-MPs (particle sizes 3–12 μm) and a battery of stress indices like DNA damage, lipid peroxidation, autophagy, ubiquitin levels, caspases activation, metabolite adjustments, frequency and force of ventricular contraction were measured in fish heart, parallel to fish swimming velocity. In particular, exposure to PS-MPs caused significant decrease in heart function and swimming competence, while enhanced levels of oxidative stress indices and metabolic adjustments were observed in the heart of challenged species. Among stress indices, DNA damage was more vulnerable to the effect of PS-MPs. Our results provide evidence on the multiplicity of the PS-MPs effects on cellular function, physiology and metabolic pathways and heart rate of adult fish and subsequent effects on fish activity and fish fitness thus enlightening MPs characterization as a potent environmental pollutant.

1. Introduction

Plastics can be found even in the most remote marine and coastal habitats due to their extensive and production and usage and their slow degradation, posing potent effects on aquatic biota and causing growing environmental concerns (Jahnke et al., 2017). Microplastics (MPs), being present in oceans, sediments, rivers, and sewages at sizes ranged from 1 μm to 5 mm (Bambino and Chu, 2017; Huang et al., 2021), could operate as vectors not only of all the chemical supplements added to plastic when manufactured but also of all substances adsorbed on their surface or absorbed from the environment (Ziccardi et al., 2016), thus being a threat to aquatic organisms and human health.

Due to their small size and poor biodegradation, MPs can be ingested by organisms and accumulate for long periods of time (for review see Miloloža et al. (2021)). The degree of the response depends upon MP size, concentration tested, route of exposure, exposure time and the existence of other contaminants (Banerjee and Shelver, 2021). Generally, small particles penetrate to a greater range than larger ones (Florence et al., 1995). Field and laboratory studies reveal that MPs are ingested by crustaceans, barnacles, lugworms, mussels, amphibians (Boyero et al., 2020; Kolenda et al., 2020; Hu et al., 2016), fishes and seals and may cause damage within organisms (Boerger et al., 2010; Browne et al., 2008; Cole et al., 2013; Jantz et al., 2013; Murray and Cowie, 2011; da Costa Araújo et al., 2020). Once MPs are accumulated, they have the potential to cause a lot of adverse effects, as reduced

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Abbreviations

(AChE) Acetylcholinesterase
(acetyl-CoA) Acetyl coenzyme A
(AMR) Active metabolic rate
(ATP) Adenosine triphosphate
(ANOVA) Analysis of variance
(LC3) Autophagy Microtubule-associated protein 1 light chain 3
(BKME) Bleached kraft pulp mill effluent
(BSA) Bovine serum albumin
(CoA) Coenzyme A
(Ucrit) Critical swimming speed
(Caspases) Cysteine-dependent aspartate proteases
(DSC) Differential scanning calorimeter
(FTIR) Fourier-transform infrared spectroscopy
(EC50) Half maximal effective concentration
(MDA) Malondialdehyde
(MPs) Microplastics
(MS222) Ethyl 3-aminobenzoate methanesulfonate
(NO) Nitric oxide
(POPs) Persistent organic pollutants
(PS-MPs) Polystyrene microplastics
(PVA) Polyvinyl-alcohol
(SEM) Scanning electron microscope
(ROS) Reactive oxygen species
(RUcrit) Relative critical swimming speed
(SAM) S-adenosyl methionine
(SQSTM1/p62) Sequestosome 1
(TBARS) Thiobarbituric acid reactive substances
(TL) Total length
(TCA) Tricarboxylic acids cycle
(XRD) X-ray powder diffraction
(MTT) 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
(HEPES) 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

feeding activity, inhibited growth and development, endocrine disruption, energy disturbance, oxidative stress, immunity, neurotransmission malfunction, genotoxicity, and even mortality not only on zebrafish (*Danio rerio*) (Lu et al., 2018), but also in several aquatic and terrestrial animals. However, to our knowledge, huge gaps still remain, regarding MPs uptake and potential cause of pathological, physiological and toxicological adverse effects (for review see Huang et al. (2021) and Barría et al. (2020)).

Enyoh et al. (Enyoh et al., 2020) recently presented that the entry, migration and excretion of MPs in fish may occur via feeding, respiratory exposure and skin absorption. Hence some studies employ aquatic exposure (Enyoh et al., 2020). However, trophic transfer appears to be the predominant route of MPs uptake in aquatic environments as it has been previously demonstrated from *Poecilia reticulata* to *D. rerio* (da Costa Araújo et al., 2020), in a four-species freshwater food chain (Chae et al., 2018), in benthic and epibenthic organisms (Bour et al., 2018) and in the planktonic food web (Setälä et al., 2014). Furthermore, it is largely under investigation which factors influence MPs occurrence in biota with regards to organism habitat, trophic level as well as polymers shape and chemical composition (Bour et al., 2018) and how they affect the organisms' metabolism (Huang et al., 2021) and the interdependently associated toxicity and capacity for biomagnification (Walkinshaw et al., 2020).

Among MPs, polystyrene (PS) is extensively used in food packaging, electronics, single-used items like cutleries and bakers, several house equipment and mainly as insulating foams (Walkinshaw et al., 2020). Due to the lightness of foams and other products, it is too difficult to be recycled and thus causes detrimental effects (Chaukura et al., 2016; Schyns and Shaver, 2021) on aquatic organisms (Walkinshaw et al., 2020; Yong et al., 2020) and subsequently on human health (Barboza et al., 2018). Namely, it has been reported that PS-MPs of 44 nm size are internalized by a ATP- independent passive process in mammalian cell lines (Fiorentino et al., 2015), while in Caco-2 cells, 100 nm sized PS-MPs are transported via micropinocytosis at the apical cellular surface, following by storage before being expelled via exocytosis (Reinholz et al., 2018). Fish may also uptake very small MPs (ranging in size from <100 μm to >1000 μm) through the skin, especially when skin alterations or lesions are present (Abbasi et al., 2018). It is also reported that MPs with size of 70 nm and 50 μm can invade apart through the skin and through the muscle of the goldfish larvae (Yang et al., 2020). In case of aquatic biota, small MPs uptake could cause detrimental effects on fish species, oysters and marine copepods (Abbasi et al., 2018). Furthermore, 1 mg/L of 70 nm and 50 μm sized MPs were reported to affect heart rate, destroy intestine and nerve fibers and inhibit acetylcholinesterase (AchE) activity, altering fish growth and movement in *Carassius aurata* larvae (Setälä et al., 2014), while in tissues of adult zebrafish, 20, 200 and 2000 μg/L of MPs of 5 μm and 70 nm size induce high oxidative stress (Lu et al., 2016).

The zebrafish *D. rerio* has been widely used in ecotoxicological studies (Bambino and Chu, 2017; Dai et al., 2014; Kaloyianni et al., 2020, 2019; Bobori et al., 2020). The conservation of some physiological and developmental activities of the digestive, nervous and cardiovascular system to that of humans, as well as the low cost of housing essentials and the high fertility rate, makes *D. rerio* an ideal biological model (Howe et al., 2013; Kettleborough et al., 2013). Moreover, based on zebrafish heart responses to several pollutants (Hicken et al., 2011) and pharmaceuticals, the current species has become an essential model for biomedical, pharmacological and eco-toxicological heart studies (Heideman et al., 2005; Hodgson et al., 2018).

Given that heart morphology and function integrity could be affected by different types of pollutants (Hicken et al., 2011), the present study aimed to investigate the effects of PS-MPs (3–12 μm in size, mean diameter 8 μM) ingestion on the zebrafish heart, using a battery of stress indices and experimental procedures. Specifically, the extent of lipid peroxidation (in terms of malondialdehyde/MDA content), DNA damage, ubiquitin levels as well as apoptotic and autophagic indicators and small polar metabolites were measured in fish heart. The proper heart function was estimated, via the measurement of heart frequency and force of contraction (Chico et al., 2008; Kitambi et al., 2012; Tryfonos et al., 2009), followed by the investigation of the heart metabolic status in challenged species (Yoon et al., 2017; Zuberi et al., 2019), in order to provide useful information at the organismal level. In parallel, fish swimming performance was recorded via the sustained critical swimming speed as a suitable metric for aerobic capacity (Plaut, 2001). To our knowledge this is the first time the effects of PS-MPs on the hearts of adult zebrafish, with regards to tissue and cell function and metabolism, specific toxicological parameters and finally fish activity on the individual level are examined.

2. Material and methods

2.1. Synthesis and characterization of PS-MPs

For the preparation of microparticles, firstly a 2% w/v PS (CAS No. 9003-53-6, Batch No. 09616AC, $M_w = 230,000$, Aldrich®, USA) solution was prepared in $CHCl_3$ (Merck, Germany) at room temperature. An aqueous poly(vinyl alcohol) (PVA) (87–89%, CAS No. 9002-89-5, Lot No. MKCF7294, Mw = 13,000–23,000, Aldrich®, USA) solution was

also prepared of 1% w/v, after 1 h of magnetic stirring at 80 °C. Then, the polymer solution (5 mL of volume parts) was added with pipette Pasteur in well-separated drops into the aqueous solution (50 mL of volume intervals) and intensive mechanic stirring followed for 2 min (Ultra-Turrax®, T10 basic, IKA®, Germany) to all directions. The emulsion produced was left to be stirred magnetically for 24 h at ambient conditions to make sure that the organic solvent has been evaporated. Thus, the microparticles are dispersed into the aqueous environment and are collected by double centrifugation (Unicen 21, CE 126, Orto Alresa, Spain) at 4000 rpm for 20 min each at room temperature. The white precipitation of PS microparticles is washed and gathered with deionized H_2O and dried thereafter at 80 °C for 24 h under vacuum.

X-ray powder diffraction (XRD) patterns of PS microparticles, were recorded with a MiniFlex II XRD (Rigaku) system over the 2θ range between 5° and 45°, with steps of 0.05° and a counting time of 5 s. Thermographs of PS microparticles has been recorded by a differential scanning calorimeter (DSC), (Perkin–Elmer model Pyris Diamond). Samples of 6 ± 0.2 mg, sealed in aluminum pans, and were heated at a rate 20 °C/min up to in an inert atmosphere (N_2, 50 mL/min). Scanning electron micrographs of samples were recorded using a JEOL JSM-5600V scanning electron microscope (SEM).

For Fourier-transform infrared spectroscopy (FTIR) measurements the exposed zebra fish control and exposed hearts were treated in liquid form after thawing. The respective solution was mixed with 0.25 g spectroscopic grade KBr powder and was dehydrated for 48 h at room temperature in a desiccator before being pressed to a pellet form. The microspheres were mixed with KBr in 1% w for the polystyrene pellet formation. The pellets were measured by a Jasco spectrometer (Jasco FTIR-6700, Japan). Seventy-five scans were collected for each measurement in the spectral range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}, in the absorbance mode. The second derivative absorption spectrum was calculated using the Spectra Manager 2.15.12 software (Jasco Corporation, Japan) by a 17- point Savitzky-Golay algorithm according to relative processing.

2.2. Animal maintenance, exposure and sample collection

Adult *D. rerio* (ZF WT 2 F10, Wageningen Agricultural University, The Netherlands) of both sexes and similar body length and weight (33.5 ± 2.65 mm Total Length (TL), 0.25 ± 0.07 g), of 6 months old, were obtained from the Department of Biology of the University of Crete.

In the present study the following experimental approaches were followed: (i) in vivo treatment of zebrafish with several nominal concentration of PS-MPs (3–12 μm in size)/g dry food, through diet for 21 days, in order to be submitted sublethal exposure concentration, (ii) in vivo treatment of zebrafish with PS-MPs concentration according to the result of the previous exposure that is with 10 mg PS-MPs (3–12 μm in size)/g dry food, through diet for 21 days, followed by measurement of oxidative stress parameters in heart, metabolomics analysis in heart and by swimming velocity estimation, (iii) ex vivo direct exposure of isolated zebrafish heart to 26 and 260 mg/L of PS-MPs (3–12 μm in size) for measuring the frequency and force of ventricular heart contraction.

2.2.1. Fish maintenance

All zebrafish were acclimatized for 7 days in 20 L aquaria (3 individuals per liter) with dechlorinated water, under 14:10 h light – dark photoperiod, at 28 ± 0.2 °C temperature, 470–500 μS/cm conductivity, 7.5–8.2 pH and 97–99% oxygen saturation. These conditions were kept stable throughout all experiments. During the acclimatization period, fish were daily fed with commercial food (Cichlid Omni Flakes, Ocean Nutrition Europe, Essen, Belgium). Fish excrement was removed manually, every day with a net.

2.2.2. Fish treatment experiments

2.2.2.1. Investigation of the exposure concentration by EC 50 determination via MTT measurement. After the acclimatization of fish to laboratory conditions, 24 zebrafish were divided in 4 groups with 6 individuals in each group. Each group was exposed through food to nominal concentrations of 1, 25 and 50 mg PS-MPs/g of dry food respectively, for 21 days. Control fish were treated with the same food without the addition of PS-MPs. At the end of the exposure time, 20 μL of blood was collected from the tail of each fish, centrifuged at 800*g* for 10 min at room temperature and the supernatant was used for the determination of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, (MTT) levels (Nerantzaki et al., 2017). Afterwards, the mean values of MTT obtained from 6 fish, from each exposure condition, was calculated. Thereafter, EC_{50} was evaluated by IBM SPSS statistics. The EC_{50} value was estimated to be 10 mg of PS-MPs/g of dry food. The latter concentration was used for the consecutive in vivo experiments. The use of EC_{50} has been largely employed as a potent and reliable index for the estimation of the sublethal concentration of various toxicants in several studies of our laboratory (e.g. Kaloyianni et al., 2020; Bobori et al., 2020; Sidiropoulou et al., 2018; Patetsini et al., 2013). The chosen MPs concentrations was in accordance with those previously reported by relevant studies, as the study of Mazurais et al. (2015) who investigated the effect of 12 mg polyethylene MPs/g food in *Dicentrarchus labrax* larvae and that of Devriese et al. (2017), who studied dietary exposure of the Norway lobster (*Nephrops norvegicus*) to PCB-spiked PE and PS microspheres (6 μm, 500–600 μm) at 155 mg/9.64 g of gelatin cubes.

Fish food preparation followed the procedure described in Bobori et al. (2020). In particular, PS-MPs in suspension were incorporated into commercial fish food by mixing a proper amount with powder food. The mixture was well homogenized and dried in oven for about 2 h. Food for control group was prepared following the same procedure, omitting the addition of MPs. Each fish was fed once per day with food corresponding to 3% of its wet weight (Iheanacho and Odo, 2020). The exposure period of 21 days of fish treatment to MPs was chosen as a semi-long exposure period compared to other toxicity studies using either longer or shorter exposure times (Kaloyianni et al., 2020; Bobori et al., 2020; Wang et al., 2019; Rainieri et al., 2018; Kögel et al., 2020).

2.2.3. Exposure experiments

A total of 180 zebrafish of both sexes were divided into two groups, control and PS-MPs treated fish. Treated zebrafish (3 aquariums, 30 individuals each) were fed for 21 days. with food containing 10 mg PS-MPs, 3–12 μm in size/g of dry food, while control zebrafish (3 aquariums, 30 individuals each) were fed for 21 days the same amount of food lacking PS-MPs.

At the end of the exposure period, control and treated fish, were anesthetized by emerging them to cold water, transferred individually in Petri dishes and carefully dissected under stereoscope. Heart was isolated, immediately frozen in liquid nitrogen and stored at − 80 °C until further analysis. Thereafter, lipid peroxidation, protein carbonylation, DNA damage, ubiquitin levels, apoptotic and autophagic indicators, as well as metabolites were measured in zebrafish heart samples. During the experimental procedure no fish mortality was observed in both control and MPs-treated groups.

2.2.4. Zebrafish exposure and swimming performance assay

For the swimming trials, only male fish (n = 7 per condition) were selected, in order to avoid any sex-related differences (Dimitriadi et al., 2018). We chose to use only male fish, since females' swimming velocities would be masked by differences in body shape (e.g., swollen bellies) which eventually affects swimming capacity. Experimental fish were randomly assigned to 2 groups (control and exposed fish). All aquaria were connected to a common closed recirculation system (ZebTec, Standalone, 2 8.0 ± 0.2 °C, 470–500 μS/cm conductivity,

7.5–8.2 pH, 97–99%, oxygen saturation and 14/10 h light/dark (Spence et al., 2008). Then, exposed fish were fed for 21 days with MPs supplemented industrial food (Cichlid Omni Flakes, Ocean Nutrition Europe, Essen, Belgium) at a concentration of 10 mg/g while control fish were fed with the same industrial diet with no additive MPs. Water parameters were measured every day and retained stable according to species standard protocols. After 21 days of rearing at the different experimental conditions (exposed and control fish), swimming performance tests were carried out at 28.0 °C water temperature. Swimming performance was estimated by measuring the sustained critical swimming speed (Brett, 1964), using a custom designed apparatus with a swim tunnel described in Koumoundouros et al. (2009). For the swimming trials, male fish of similar size were placed in the swimming tunnel for 10 min at 2 TL s^{-1} water velocity. Then, water velocity increased every 10 min at a rate of 3 TL s^{-1}. Fatigue was determined when fish left the swimming channel, unable to react to visual or acoustic stimuli from the side or behind. Fatigued fish were anaesthetized (MS222, 100 mg/L), measured for TL, and examined for the presence of gross morphological abnormalities. Only fish with normal morphology were included in the analysis. Relative critical swimming speed (RU_{crit}) was calculated as the ratio of U_{crit} to the TL of each specimen.

2.2.5. Isolated heart ex vivo experiment

Adult zebrafish (n = 45) of both sexes and the same strain and size, as in the previous described experiment were used. Prior to the ex vivo experiments on the isolated heart, fish were fed with commercial food without PS-MPs addition (Cichlid Omni Flakes, Ocean Nutrition Europe, Essen, Belgium).

For heart excision, the procedure described in Orfanidou et al. (2013) was followed. Specifically, zebrafish were anaesthetized in cold water and the immobilized individuals were transferred to a small Petri dish half filled with Sylgard resin. Zebrafish were pinned ventral side up using fine dissecting pins. One micropin was placed by using fine forceps on the head above the gill aperture and the second one on the caudal region before the caudal fin. Then the whole fish was covered with oxygenated (100%, O_2) physiological saline (140 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4) (Tsai et al., 2011). To expose the spontaneously beating heart, a longitudinal incision was done by using fine dissecting micro scissors (FST, No. 15003-08) from the gill aperture to the pelvic fin. The whole heart was excised carefully and transferred to the recording chamber, filled also with oxygenated physiological saline. The total volume of the recording chamber was 5 mL. Fine oxygenation of the preparation in the form of small bubbles was achieved by using a NIPRO syringe needle (0.40 × 13 mm) connected to micro infusion set with flow regulator. All experiments were performed at a temperature of 22 ± 2 °C. The temperature was controlled via a circulator and chiller (VWR, International Model 1162A).

Heart beats were started to be recorded once the samples were placed within the recording chamber. Thereafter, an equilibrium period of 20–30 min was followed, during which the force and the frequency of the beats were stabilized. The end of the equilibrium period corresponded to the started time (t = 0), when measurements of the contractions were started. After the heart preparation had settled, it was treated for 2 h by directly applying on the heart PS-MPs at the concentrations of 26 and 260 mg/L (diluted in physiological saline within the 5 mL recording chamber). Fresh stocks of the MPs examined were prepared daily in physiological saline. To our knowledge, relevant ex vivo studies investigating direct effects of MPs on isolated heart preparation are lacking. Hence, MPs concentrations were selected that were similar to those used in other in vivo studies on fish organisms. Sheepshead minnow (*Cyprinodon variegates)* was exposed for 96 h to 50–250 mg/L, of MPs suspended in the water tank (Choi et al., 2018). In other in vivo studies, the effects of lower concentrations, 0.002–10 mg/L of MPs (tank water concentration) were assessed on the heart rate of *Danio rerio* embryos and *Oryzias melastigma* offsprings for longer period of times 48 h post exposure – 132 h post hatching and after 1440 h of parental exposure respectively (Barría et al., 2020; Zhang et al., 2020). Thus, the relatively higher concentrations in relation to the shorter time (24–720 times) of exposure of the conditions applied in the present study in comparison to the latter studies, favors the faster response of the excised heart, since our study measures the spontaneous activity of atrial pacemaker (frequency) and subsequent contractility of working ventricular cells (force), after MPs treatment. Furthermore, the possible implication of MPs to heart function in vivo after their entrance to the fish organism is not taken into account by the present ex vivo bio-assay. In addition any interactions of MPs with other systems of the fish which could potentially affect the response of the heart by MPs, are excluded.

Furthermore, in order to investigate the possible implication of MPs with molecules which modulate heart activity, the response of the heart to 10 μM adrenaline (Orfanidou et al., 2013; Joyce et al., 2019) (DEMO S.A, Pharmaceutical Company) and to 0.1 μM ouabain (according to Xie et al. (2008)) was initially examined. Then, heart preparations were preincubated for 2 h with PS-MPs (26 or 260 mg/L), followed by addition of either adrenaline (10 μM) or ouabain (0.1 μM) for 30 min and then washed out using physiological saline (Joyce et al., 2019).

2.3. Molecular and biochemical analyses

2.3.1. Estimation of lipid peroxidation

Lipid peroxidation in fish heart was determined by malondialdehyde (MDA) measurement according to Niehaus and Samuelsson (1968). In brief, malondialdehyde (MDA) which is formed by polyunsaturated fatty acids' decomposition mediated by free radicals, was quantified as thiobarbituric acid reactive substances (TBARS). Three batches of 10 zebrafish hearts from each, control and PS-MPs treated, group were homogenized in phosphate buffer (50 mmol L^{-1}, pH 7.4) and centrifuged (2000*g*, 4 °C, 15 min). In 250 μL of supernatant, 250 μL TCA (20%) and 500 μL thiobarbituric acid (0.67%) were added. Thereafter, the mixture was boiled (60 min), cooled (room temperature), 2 mL of butanol added and subsequently centrifuged (3000*g*, 15 min). TBARS, were spectrophotometrically (535 nm) measured ($\varepsilon = 1.5 \times 10^5$ L mol^{-1} cm^{-1}). The results are expressed as nmol MDA/mg protein.

2.3.2. Alkaline single-cell gel electrophoresis (comet assay)

The DNA comet assay was employed as modified by Dailianis et al. (2005). In brief, hearts of 30 individuals of control and 30 individuals of treated fish were dissected and divided into 3 batches per group. Each batch was processed with dissociating solution (0.01% collagenase type CLS IV, 175 U/mg) in calcium-magnesium -free saline in order for heart cells to be isolated. The resulting pool from each group, was processed for electrophoresis according to the procedure described by Dailianis et al. (2005). An inverted fluorescent microscope (Zeiss Axovert) and an epifluorescence microscope (WANG BioMedical, The Netherlands) were employed to examine the presence of comets. In order to exclude abnormal comets from counting, comet scoring was conducted according to established criteria (Ritter and Knebel, 2009). Four slides per pool were measured, in order to represent technical replicates. Randomly selected 100 cells were scored from each slide (TritekCometscoreTM 1.5, TriTek Corporation, USA). The results are expressed as % DNA in tail (percentage of DNA in comet tail). The comet assay method electrophoresis conditions were validated by cells treatment for 1 h with 1 μM of H_2O_2 (positive control) as previously mentioned (Binelli et al., 2009; Chatziargyriou and Dailianis, 2010). % DNA in tail and Olive moment in positive control data (1 μM H_2O_2) were 28.3 ± 5.2 and 40 ± 6.3, respectively.

2.3.3. Autophagy and apoptosis

Quantification of autophagic and apoptotic indicators were assessed with SDS/PAGE and immunoblot analysis. The preparation of tissue samples for SDS-PAGE and the immunoblot analysis are based on well-

established protocols. Specifically, in the present study, hearts of 30 individuals of control and 30 individuals of treated fish were dissected and divided into 3 parts for each group. Each part of dissected hearts from 10 zebrafish from each, control and PS-MPs treated, group were lysed, and thereafter equivalent amounts of proteins (50 μg) were separated either on 10% and 0.275% or 15% and 0.33% (w/v) acrylamide and bisacrylamide respectively (depending on the molecular weight of the detected proteins) slab gels and electrophoretically transferred onto nitrocellulose membranes (0.45 μm, Schleicher & Schuell, Keene N. H. 03431, USA). All nitrocellulose membranes were dyed with Ponceau stain in order to assure a good transfer quality and equal protein loading. Subsequently, the membranes were incubated overnight with the appropriate primary antibodies. Antibodies used were as follows: monoclonal rabbit anti-LC3B (3868, Cell Signaling), polyclonal rabbit anti-p62/SQSTM1 (5114, Cell Signaling), anti-Bcl2 (7973, Abcam), anti-Bax (B-9) (2772, Cell Signaling). Antibodies were diluted (1/1000) according to the manufacturer guidelines in TBST [20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20] containing 5% (v/v) bovine serum albumin (BSA). Membranes were then washed with TBST (3 × 5 min) and thereafter incubated for 1 h with a horseradish peroxidase linked secondary antibody (7074, 7076, Cell Signaling). Secondary antibodies were diluted (1/3000) according to the manufacturer guidelines in TBST containing 1% (v/v) BSA. Membranes were again washed with TBST (3 × 5 min). The blots were detected using enhanced chemiluminescence (Chemicon) on Fuji Medical X-ray film and quantified by densitometry scanning laser (GelPro Analyzer Software, GraphPad).

2.3.4. Ubiquitin and cleaved caspases conjugates

Quantification of caspases and ubiquitinated proteins was assessed in a solid- phase immunochemical assay as described by Kaloyianni et al. (2019). In short, hearts of 30 individuals of control and 30 individuals of treated fish were dissected and divided into 3 parts for each group. Each part was homogenized, lysed and immersed in a nitrocellulose membrane (0.45 mm), set in a dot blot (BioRad) vacuum apparatus. Finally, the antibodies used were a polyclonal anti-ubiquitin rabbit antibody (Cat. No. 3936, Cell Signaling, Beverly, MA, USA) and anti-cleaved caspase antibody (Cat. No.8698 Cell Signaling, Beverly, MA, USA). Antibodies were diluted (1/1000) according to same procedure described above in 2.3.3 section. The dots were detected using enhanced chemiluminescence (Chemicon) on Fuji Medical X-ray film and quantified by densitometry scanning laser (GelPro Analyzer Software, GraphPad).

2.4. Metabolomics

2.4.1. Sample preparation

Polar metabolite extraction was performed by adding 1.7 mg of heart tissue (corresponding to a pool of hearts of 10 individuals) to 50 μL of an ice cold methanol:water (1:1) mixture. The tissue was then grounded using a chilled mortar and pestle. The ground extract was transferred quantitatively into an eppendorf tube and further subjected to ultrasonic treatment in a sonication bath for a total of 6 times, with 30 s intervals and a 2 min pause between intervals (total ultrasonic exposure of 3 min). Following sonication, the extract was centrifuged at 5000 rpm for 10 min at room temperature and was then stored in − 20 °C until analysis. Before analysis, each extract was allowed a brief thaw time and was then filtered throw a 20 μm syringe filter. L-Alanine-3,3,3-d_3 was added as an injection standard to each sample to a concentration of 10 ppm before analysis.

2.4.2. LC-MS/MS analysis

Each sample preparation was analyzed on a Thermo Scientific™ TSQ Quantum™ Access MAX Triple Quadrupole Mass Spectrometer employing a Waters™ ACQUITY UPLC BEH Amide Column (1.7 μm, 2.1 mm × 150 mm). The applied analysis method was based on previously developed methods (Virgiliou et al., 2015; Zisi et al., 2017). Briefly, the flow rate was set to 300 μL/min. Solvent A was 95:5% Acetonitrile:H_2O, 10 mM CH_3COONH_4 and solvent B was 30:70% Acetonitrile:H_2O, 10 mM CH_3COONH_4. A gradient elution program was applied as follows: 100% A (hold for 4 min), then to 60:40% A:B (over 21 min), then to 15:85% A:B (over 4 min and then hold for 3 min) then to 100% A (hold for 15 min). The injection volume was 5 μL.

Sample data was analyzed using Thermo Scientific™Qual Browser, Thermo Xcalibur version 3.063. Sample comparison was performed using response ratios of the analyte peak area to the area of the injection standard.

2.5. Recording and analyzing the electromechanical activity of the heart

To record the tension generated by the spontaneously contracting ventricle, an isometric force-displacement transducer (FDT) (FT-03C, Grass Instrument Company, USA) was used. Then, the proximal part of the ventricle was attached to FDT probe while the distal ventricle's end was fixed on the substrate covered the bottom of the recording chamber. Thereafter, the ventricle was stretched after employing a 0.9 mN force, using a micromanipulator, thus ensuring that approximately the same force, as that produced during contraction due to various manipulations, is monitored. The analog signal (voltage) from the FDT was digitized, stored and analyzed, using standard software (Labview 5.1, National Instruments, USA) and hardware (Keithley KPCI 3102, Keithley Instruments, Cleveland, OH, USA). To estimate the spontaneous contractions, the strength of the ventricle (from the baseline to the peak of the intensity) and the frequency of the ventricle contractions (as assessed between two consecutive contractions), were measured. Force contraction (μN) and frequency (Hz) measurements of 5–10 s duration were recorded every 2 or 5.0 min. Results are presented as mean ± standard error, after standardization at time = 0, corresponding to 100%.

2.6. Statistical analyses

The results derived from molecular and biological analyses are presented in the form of mean ± standard error of the mean (SEM). Since several of the parameters examined were expressed in arbitrary units, all data were converted in ratios (e.g. measured value divided by the respective control value), quantifying thus the response of heart tissue in relation to the biomarkers examined. Significant differences ($p < 0.05$) of the effects of PS-MPs on control and exposed zebrafish heart samples as well as on fish RU_{crit} were tested by the non-parametric Mann-Whitney U test ($p < 0.05$). All analyses were considered significant at p-level < 0.05 and were performed using the SPSS software (ver. 25, Inc. Chicago, USA).

3. Results and discussion

Plastic release in the environment has already become a significant universal concern, due to their slow degradability rate, their ingestion by aquatic organisms and to the fact that they serve as transporters of synthetic organic molecules from the environment to aquatic organisms (Jiang, 2018). The effect of plastic pollution on aquatic organisms has been increasingly examined recently (Lu et al., 2018).

3.1. PS-MPs characterization

In the present study, the effects of PS-MPs were evaluated over a variety of stress indices, metabolic profile, cardiac function and fish aerobic capacity. Specifically, on the first set of experiments several ecotoxicological biomarkers were evaluated in the heart of zebrafish fed with 10 mg of PS-MPs per gram of dry food for 21 days. PS-MPs with sizes ranged from 3 to 12 μm (average diameter about 8 μm, Fig. 1a) and completely amorphous, as was found by XRD and DSC studies (Fig. 1b and c – with a glass transition value at 102 °C). In addition, apoptotic,

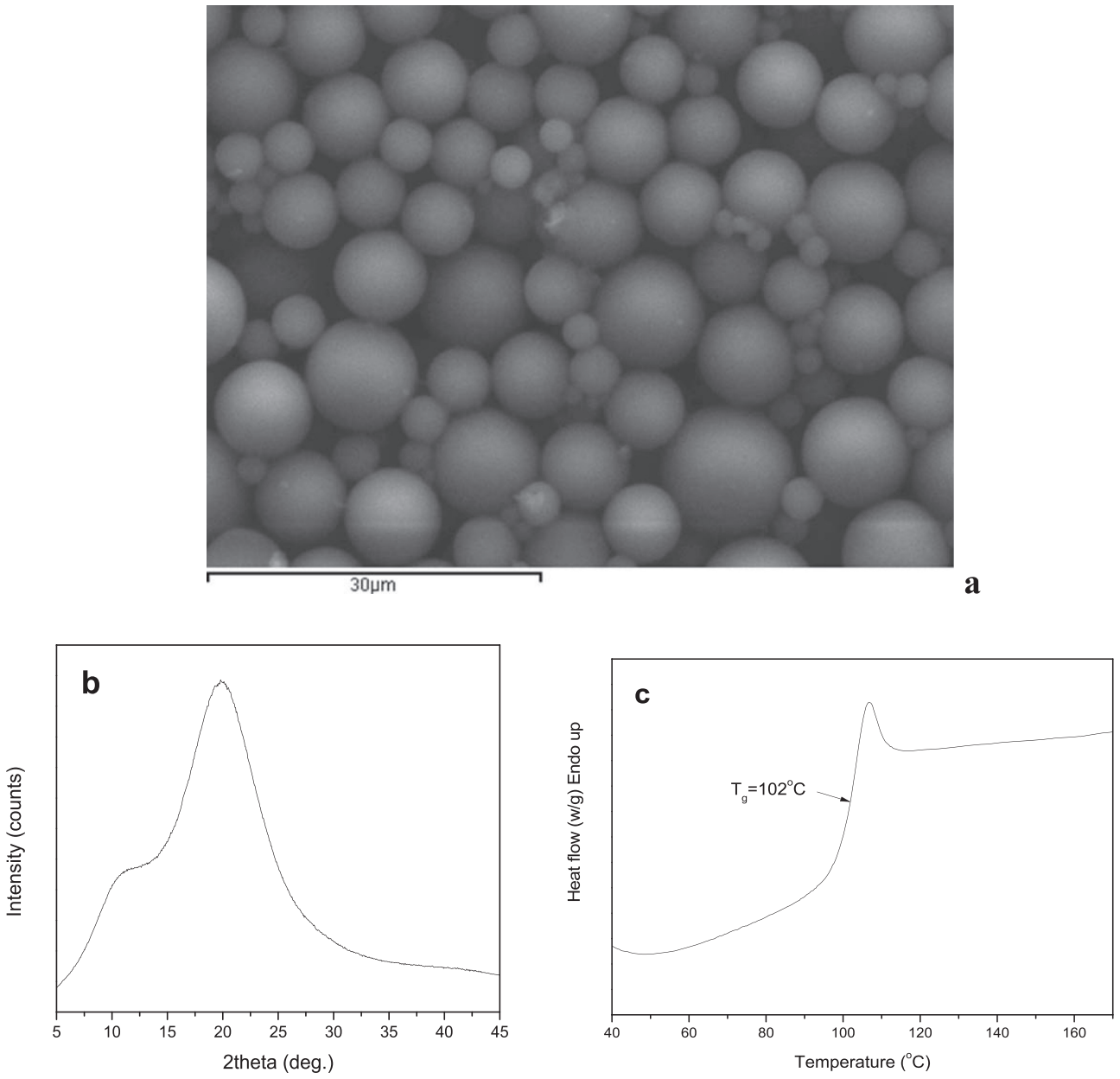

Fig. 1. (a) SEM micrographs, (b) XRD patterns and (c) DSC thermographs of prepared PS-MPs.

autophagic and metabolomics parameters as well as swimming velocity measurements were conducted. On the second set of experiments, the effect of ex vivo together with other effectors was studied on the force and frequency of the isolated zebrafish heart.

The FTIR spectrum of the heart exposed samples (n = 5) and PS microspheres samples (n = 5) are exhibited in Fig. 2a. The PS peaks mainly at 542, 698 and 759 cm^{-1} (out-of-plane bending vibration of C-H), at 1454, 1493, 1645 cm^{-1} (correspond to aromatic C=C stretching vibration absorption), at 2849 and 2922 cm^{-1} assigned to CH_2 groups and at 3028 and 3059 cm^{-1} due to aromatic C-H stretching vibration of phenyl rings, while the heart exposed spectrum corresponds to the heart tissue of the challenged zebrafish exposed for 21 days to 10 mg/g PS-MPs. The heart control spectrum is similar to the exposed spectrum, exhibiting peaks corresponding to proteins; between 900 and 1300 cm^{-1} are phosphates mainly associated with RNA and DNA related nucleic acids, in the 1300 and 1800 cm^{-1} region are protein (Amide I, II) bonds while in the 2700–3900 wavenumbers are peaks related to N-H stretching vibration of proteins (Parker, 1971).

Presence of PS in the exposed heart sample could be identified with the exhibition of PS characteristic peaks e.g. 1454, 1493 cm^{-1}, as it has been reported in other FTIR studies of particles in biological media (Kastrinaki et al., 2015). In the current heart exposed spectrum though such peaks are not exhibited. This can be related either to absence of the PS material in the exposed heart, or possibly due to the low exposure concentration and very limited presence in the heart spectrum. The second derivate of the control and exposed heart spectra, as shown in Fig. 2b, can determine minor changes in the spectra peaks; the exposed heart spectrum (orange line) exhibits higher second derivative peaks which can be attributed to enhancement of the spectrum in absorbance values due to PS presence, which also exhibit peaks at 400–1900 and at 2790–3800 cm^{-1} regions. Thus, the concentration of PS in the exposed heart may not be that high in order to exhibit the PS characteristic peaks but a further analysis of the exposed sample and comparison with the control heart spectrum can show an increase of the second derivative peaks which can be related to PS presence.

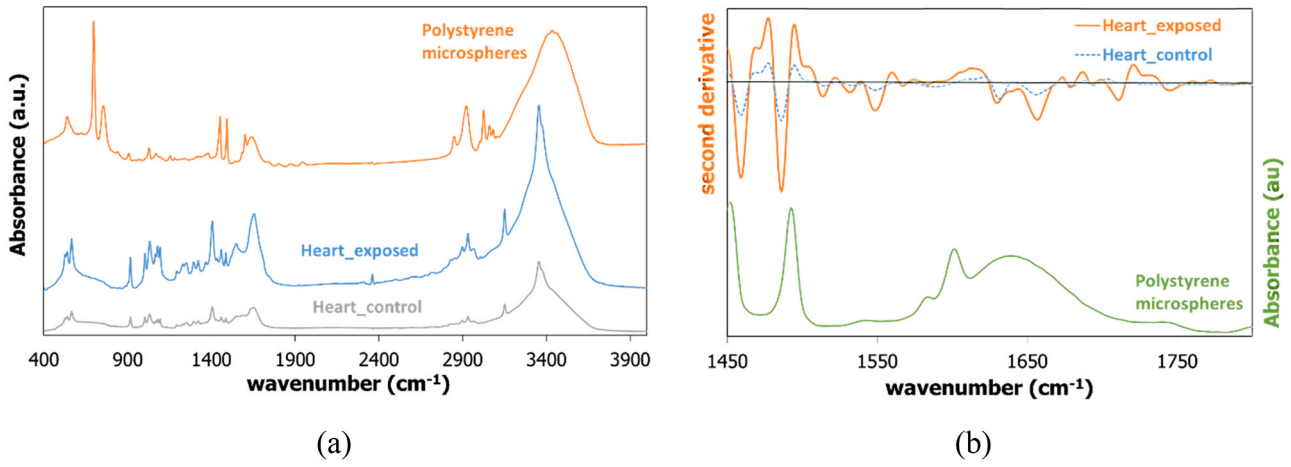

Fig. 2. (a) FTIR spectrum of the control and exposed heart sample and the PS microspheres, (b) second derivative analysis of the exposed and control heart samples and the absorbance spectrum of the PS microspheres.

3.2. Molecular and biochemical indicator assays

3.2.1. Lipid peroxidation

MDA is the main biomarker of lipid peroxidation and is widely used as an oxidative stress biomarker in many ecotoxicological studies on aquatic animals (Kaloyianni et al., 2020; Bobori et al., 2020). Our results revealed that treatment with PS-MPs resulted to a significant increase of heart lipid peroxidation, as measured by MDA production compared to untreated control animals (Fig. 3). In specific, a significant augmentation in MDA levels was recorded in challenged species, approaching the percentage of 528.5% compared to the control ones. The latter increase was attributed to reactive oxygen species (ROS) increase after PS-MPs exposure. According to Lu et al. (2016) PS-MPs caused both significantly increased activities of superoxide dismutase and catalase and lipid accumulation, indicating the production of oxidative stress in zebrafish liver. In line with our results, several studies on the effect of MPs on different fish species and different fish tissues have been published. Specifically, MPs internalization resulting to lipid peroxidation has been depicted in the liver and the gills of *Dicentrarchus labrax* juveniles (Barboza et al., 2018), in catfish juveniles (Ferreira et al., 2016), in cichlid juveniles (Wen et al., 2018), as well as for other aquatic species (Ribeiro et al., 2017; Guilhermino et al., 2018; Oliveira et al., 2018; Yu et al., 2018).

3.2.2. DNA damage

DNA damage is an important biomarker in ecotoxicological studies in the terrestrial and aquatic environment (Kaloyianni et al., 2020; Bobori et al., 2020; Sidiropoulou et al., 2018; Feidantsis et al., 2020). In Fig. 4 is presented the percentage of DNA found in comets' tails in treated zebrafish hearts with the examined PS-MPs. The percentage measured on 21st day of fish exposed to PS-MPs, was 100 times higher compared to control. To our support, exposure of medaka embryos and larvae to DMSO-extract of MPs induced increased DNA breaks (Pannetier et al., 2019). In another study, exposure of mussels' hemocytes to MPs resulted in an increase of DNA strand breaks (Gómez-Mendikute et al., 2002). Moreover, apart from MPs, exposure of marine and terrestrial organisms to several pollutants like NPs also provoked DNA damage (Kaloyianni et al., 2020; Sidiropoulou et al., 2018; Feidantsis et al., 2020).

3.2.3. Apoptosis, autophagy and ubiquitination

Both apoptosis and autophagy account for self-destructive processes by which excess damaged or aged cells and organelles are abolished. The choice which response is going to be followed depends on the stimulus potency (Cooper, 2018). However, the successful corporation between apoptosis and autophagy is complex. Specifically, while under undefined conditions, autophagy prevents cell death and therefore eliminates apoptosis, while, in other cellular schemes, it stands as another cell

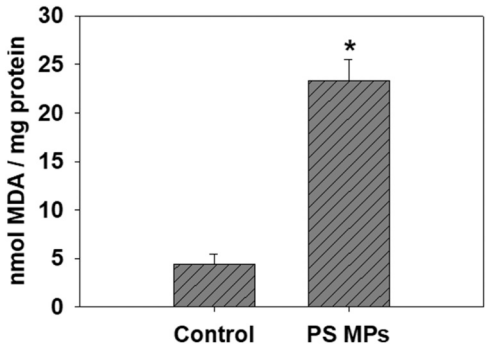

Fig. 3. MDA concentrations (mean ± SEM nmol/mg protein) in the heart of zebrafish (n = 3 pools of 10 individuals per pool) after exposure to PS-MPs. * $p < 0.05$ compared with control group (n = 3 pools of 10 individuals per pool).

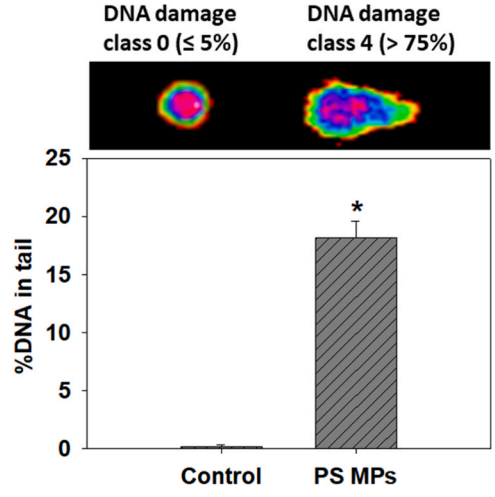

Fig. 4. Percentage (%) of DNA damage in tail (mean ± SEM) in the heart of zebrafish (n = 3 pools of 10 individuals per pool) after exposure to PS-MPs. * $p < 0.05$ compared with control group (n = 3 pools of 10 individuals per pool). % DNA in tail and Olive moment in positive control data (1 μM H_2O_2) were 28.3 ± 5.2 and 40 ± 6.3, respectively.

death possibility.

A number of pollutants as heavy metals and NPs have been shown to induce apoptotic as well as autophagic processes in fish (Gao et al., 2014). LC3 is an Autophagy Microtubule-associated protein 1 light chain 3 and is an indicator of autophagosome formation and therefore is regarded as a marker that is upregulated in the process of autophagy in diseased cells (Dhingra et al., 2018). On the other hand, Sequestosome 1 (SQSTM1/p62) is a multifunctional protein associated with signal transduction and has been also widely used as indicator of autophagy (Kabeya et al., 2004).

Ubiquitin on the other hand, is a ubiquitously expressed 76-amino-acid protein that can be covalently connected to selected proteins, and tags proteins for degradation by 26S proteasome or by lysosomes (Varum et al., 2007).

Our results showed that exposure to PS-MPs significantly increased both ubiquitination as well as the autophagic indicator levels in zebrafish heart. Specifically, when compared to the control animals, PS-MPs treatment resulted to a significant, 2.45-fold increase in the ubiquitin conjugates levels (Fig. 5) in zebrafish hearts. LC3 II/I ratio (Fig. 6a) and SQSTM1/p62 (Fig. 6b) were both affected by the presence of PS-MPs compared to control. Specifically, LC3 II/I ratio exhibited a significant increase of 2.2-fold in zebrafish hearts exposed to PS-MPs, while levels of SQSTM1/p62 were significantly decreased. Specifically, this decrease was 2.8 times lower compared to the control hearts. In agreement with our results, exposure of aquatic organisms to several pollutants have exhibited initiation of autophagy, as was reported for zebrafish embryos (Cheng et al., 2009; He et al., 2014) and for common carp *Cyprinus carpio* (Gao et al., 2014).

Caspases (a cascade of cysteine-dependent aspartate proteases) dissect many essential cell substrates and are considered the main component of the apoptotic machinery (Chandra et al., 2000; Kratz et al., 2006; Pyati et al., 2007). Concerning the apoptotic markers, our results showed that both Bax/Bcl-2 ratio and caspases levels were increased in exposed to PS-MPs zebrafish compared to control fish, indicating apoptotic phenomena. Specifically, Bax/Bcl-2 ratio exhibited a 5.1-fold increase (Fig. 7a), while caspases levels exhibited a 2.5-fold increase (Fig. 7b) in the PS-MPs treated animals compared to the control. In agreement with our results, exposure of several marine organisms to MPs leads to increased apoptotic levels (Koehler et al., 2008; von Moos et al., 2012). In addition, pollutants other than microplastics, as metals (aluminum, arsenic, cadmium, chromium, cobalt, zinc, copper, mercury and silver) as well as other chemicals including bleached kraft pulp mill effluent (BKME), persistent organic pollutants (POPs), and pesticides (organo-phosphated, organo-chlorinated, carbamates, pyrethroids and biopesticides) have been also shown to induce the apoptotic pathways in several fish species (AnvariFar et al., 2018).

Autophagy and apoptosis can be simultaneously take place, due to the fact that both processes can be triggered by similar stressors activating the upstream signaling pathway (Mariño et al., 2014). The present results revealed that PS-MPs ingestion and their transfer through the circulation can activate stress responses via signaling pathways that lead to stimulation of both autophagy and apoptosis processes in the zebrafish heart. In agreement with the latter, other stressors such as heat stress can lead to induction of both autophagy and apoptosis in several organs, such as the kidney, duodenum and abomasum in calves (Zhai et al., 2015). Moreover, both processes, autophagy and apoptosis are conserved and coexist in the ovaries of the tropical fish species *Astyanax altiparanae,* which is probably attributed to ameliorate the ovarian remodeling potency (Cassel et al., 2017).

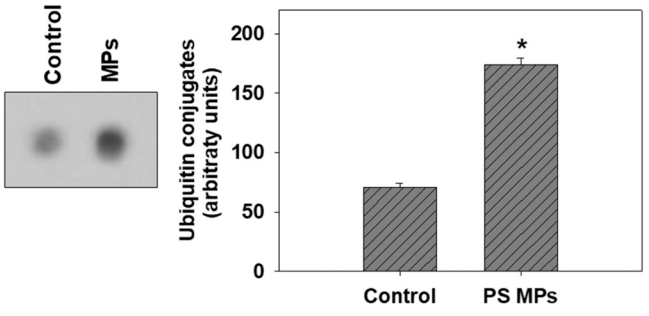

Fig. 5. Ubiquitin conjugates (mean ± SEM) in the heart of zebrafish (n = 3 pools of 10 individuals per pool) after exposure to PS-MPs. * $p < 0.05$ compared with control group (n = 3 pools of 10 individuals per pool).

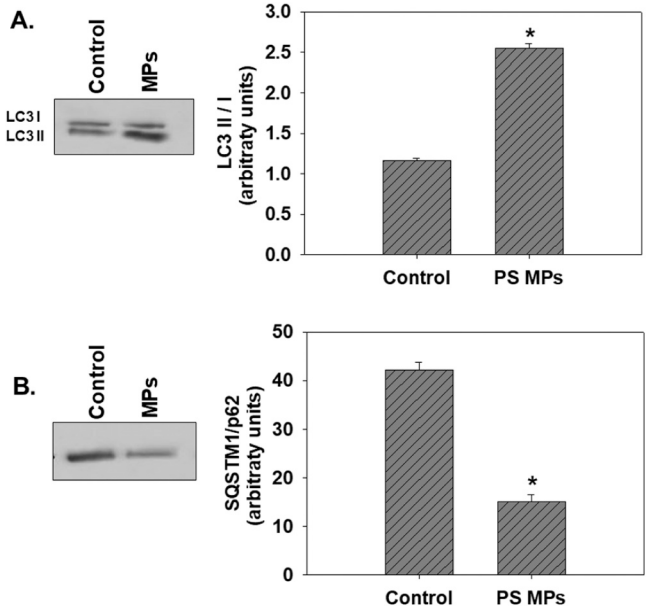

Fig. 6. (a) Autophagic indicators LC3 II/I ratio and (b) SQSTM1/p62 (mean ± SEM) in the heart of zebrafish (n = 3 pools of 10 individuals per pool) after exposure to PS-MPs. * $p < 0.05$ compared with control group (n = 3 pools of 10 individuals per pool).

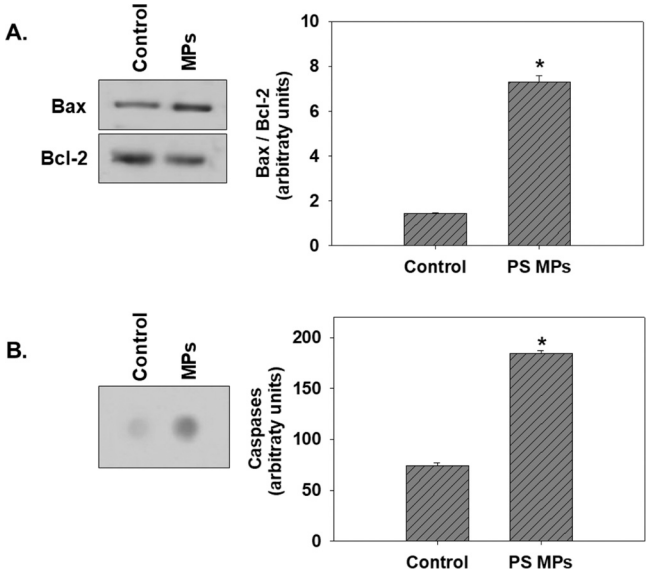

Fig. 7. Bax/Bcl-2 ratio (a) and caspases levels (b) (mean ± SEM) in the heart of zebrafish (n = 3 pools of 10 individuals per pool) after exposure to PS-MPs. * $p < 0.05$ compared with control group (n = 3 pools of 10 individuals per pool).

3.3. Sequence of events

All the parameters tested, after fish treatment with PS-MPs showed statistically significant differences from their respective control group. According to Mattsson et al. (2017), MPs can penetrate the blood-to-brain barrier in fish and cause behavioral disorders. It is likely PS-MPs to be taken up and induce toxicity by the following sequence of events: after MPs ingestion they are transferred through circulation to the heart tissue where loss in plasma/endo-lysosomal/nuclear membrane integrity occurs (increase lipid peroxidation of heart), generation of reactive oxygen species (ROS), causing proteolysis, elevated DNA damage, triggering release of pro-apoptotic factors from the mitochondria and finally activation of cell death pathways leading to apoptosis and/or autophagy. All these processes are in a complex manner inter-connected. This is also supported by the highly significant intercorrelations found between Bax/Bcl-2 and SQSTM1/p62 ($r_s = -0.999$, $p < 0.001$) and caspases and ubiquitin ($r_s = -0.986$, $p < 0.001$) levels. From all the parameters examined DNA damage seems to be more vulnerable biomarker to PS-MPs treatment. Our data suggests that these substances exert high toxicity on animal cells, which implies the vulnerability of fish physiology to PS-MPs. These responses probably exert detrimental effects on fish health and fitness and hence to human health.

3.4. Metabolomics analysis

The metabolic profiles of the heart tissues of zebrafish exposed to PS-MPs were altered in relation to control, as it is shown in Table 1. The relative concentrations of most metabolites measured were decreased whereas only pyruvic acid and acetylcarnitine exhibited a relative concentration higher than 10% in exposed zebrafish heart. The metabolites that exhibited the greatest reduction were ornithine, salicylic acid, succinic acid, citrulline and deoxyadenosine.

Table 1
Chromatography retention times (t_R), response ratio of measured metabolites over the injection standard and respective change values (%) of the PS-MPs exposed sample in comparison to the control. The change values provide an indication of the increase or decrease of the levels of each metabolite in the exposed heart tissue sample.

	Metabolite	t_R	Response ratio		Change %
			Control	Exposed	
TCA cycle	α-Ketoglutaric acid	21.12	0.295	0.130	-56
	Succinic acid	18.00	0.605	0.152	-75
Glycolysis	Acetylcarnitine	17.56	52.5	59.9	14
	Carnitine	19.65	120	65	-46
	Creatine	19.78	167	156	-7
	Pyruvic acid	10.35	0.016	0.022	38
Nucleic acid metabolism	Adenine	5.62	3.92	4.05	3
	Adenosine	6.47	354	318	-10
	Deoxyadenosine	4.54	0.740	0.285	-61
	Guanine	14.32	0.238	0.110	-54
	Hypoxanthine	7.51	1.727	1.178	-32
	Uridine	7.37	5.37	2.55	-53
Arginine metabolism	Citrulline	22.55	1.153	0.366	-68
	Ornithine	29.21	5.24	0.75	-86
Amino acids	Arginine	28.62	27.4	12.8	-53
	Alanine	19.92	0.935	0.549	-41
	Asparagine	22.59	0.356	0.305	-14
	Glutamic acid	23.72	9.99	7.83	-22
	Lysine	21.87	73.5	44.4	-40
	Phenylalanine	16.47	5.72	2.99	-48
	Proline	17.79	7.93	3.90	-51
	Tyrosine	18.52	1.120	0.999	-11
	Valine	17.93	1.684	1.060	-37
Sugars	Raffinose	24.22	0.253	0.100	-60
Others	Betaine	15.97	3.82	1.56	-59
	Butyric acid	11.11	0.096	0.053	-45
	Choline	10.53	98.5	46.2	-53
	Nicotinamide	1.83	12.7	13.3	5
	Salicylic acid	2.50	8.77	2.13	-76

It is observed that pyruvic acid and acetylcarnitine were increased by 38% and 14%, respectively, while carnitine exhibited a reduction of 46%. In mitochondria the acetyl group of acetyl coenzyme A (acetyl-CoA) is reversibly transferred to carnitine forming acetylcarnitine and releasing CoA, thus acetylcarnitine is regarded as an indicator of mitochondrial metabolism of acetyl-CoA (Rosca et al., 2009). Although Acetyl-CoA is difficult to be directly determined due to its instability, its indirect estimation is thus achievable. Acetyl-CoA is a key metabolite, end product of the catabolic pathways of carbohydrates, fatty acids, most amino acids and ketone bodies, but also the initiating metabolite of tricarboxylic acids cycle (TCA), which combined with the respiratory chain delivers in aerobic cells most of the cell's energy, and other anabolic processes (Fig. 8).

According to the presented data the combined higher concentration of acetylcarnitine and lower concentration of free carnitine indicate that the acetyl-CoA concentration in the heart cells of exposed zebrafish was considerably higher. This is also in accordance with the observed higher pyruvic acid levels, the carbohydrates metabolic precursor of acetyl-CoA. However, TCA cycle metabolites, succinic acid and α-ketoglutaric, were reduced by 75% and 56% of their respective control values, indicating that the TCA cycle is impeded, causing inadequate ATP production (Fig. 8). Since TCA cycle rate is reduced, the major routes of metabolism following acetyl-CoA accumulation are fatty acid biosynthesis and ketone bodies formation, however these pathways are not predominant in the heart, even though ketone bodies contribute significantly in ATP formation in the heart in most animals (Karwi et al., 2020). The fate of accumulated acetyl-CoA remains to be investigated. Moreover, the accumulation of pyruvic acid could indicate increased glycolysis rate.

All amino acids measured were found in smaller relative concentrations in the exposed than in the control pooled sample, an observation that could be linked to reduced protein metabolism. Ornithine and citrulline are greatly reduced in exposed heart cells to 14% and 32% respectively of the relative concentration in the control sample. Although both are well known urea cycle metabolites their function may not attributed to amino acid deamination in the urea cycle since this pathway operates mostly in the liver. Ornithine and citrulline may be related to arginine metabolism, which are also related to glutathione metabolism and the activity of nitric oxide synthases which lead to the production of nitric oxide (NO), an important cellular signaling molecule. All these metabolites were reduced in the exposed fish sample indicating a rate reduction of the pathways involved. Moreover, ornithine and arginine in humans are believed to attenuate fatigue (Wan et al., 2017). Hence, their great reduction could also be related to the reduced aerobic capacity of zebrafish, as our results indicated (described below).

Most relative concentrations of purine and pyrimidine nucleobases metabolites were reduced in exposed cells indicating the probable reduction of nucleic acid metabolism.

Finally, the considerable reduction of salicylic acid by 76% may be indicative of inflammation. Moreover, decreased levels of choline by 53% may be related to muscle damage, stunting and neural tube defects and that of betaine by 57% to reduced S-adenosyl methionine levels a potent signaling molecule (Wortmann and Mayr, 2019).

The effects of MPs on the metabolome of zebrafish have also been studied recently (Wan et al., 2017; Zhao et al., 2021, 2020; Wan et al., 2019; Qiao et al., 2019). However, to our knowledge this is the first metabolomics study of the effect of MPs on zebrafish heart.

The results obtained highlight the potential posed by the metabolomics approach to elucidate cellular mechanisms, providing further adjustments are made in order to specifically determine key metabolites and potential biomarkers.

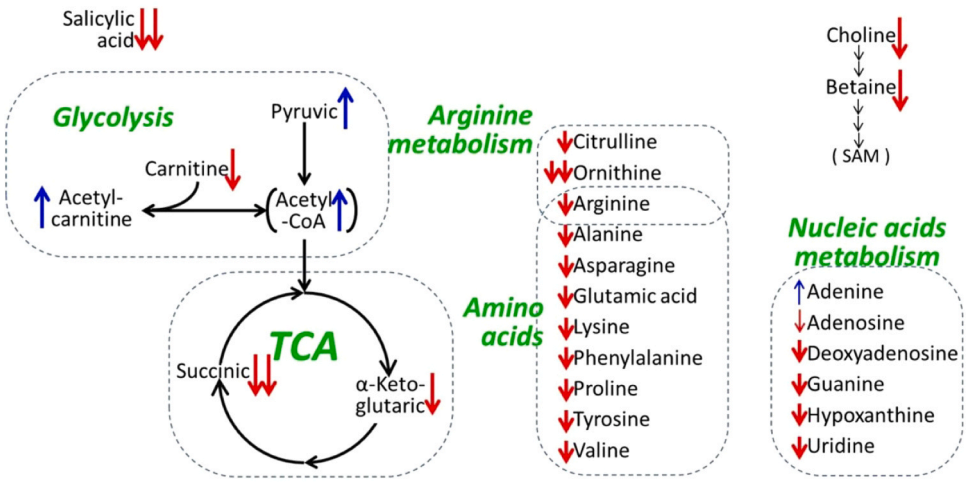

Fig. 8. Pathway analysis of the metabolome of heart tissues. TCA; S-adenosyl methionine (SAM); coenzyme A (CoA). Metabolites in brackets were not chromatographically determined.

3.5. Isolated ventricular heart contraction

Subsequently, we studied heart frequency and force of contraction, that are strong indicators of the vitality and function of zebrafish heart, in order to give more information of the cardiotoxic effects of the investigated materials.

3.5.1. Effects of microplastics on the frequency and force of ventricular contractions of isolated zebrafish heart

3.5.1.1. The response of the isolated heart to physiological saline. The cardiotoxic effects of various xenobiotics and pollutants on the isolated heart of fish have been previously assessed by measuring the force and frequency of spontaneous heart contractions (Tryfonos et al., 2009; Orfanidou et al., 2013). In the present study, following the equilibration of the preparation, continuous recordings of the ventricular contractions driven by the pacemaker of the heart located in the auricle at successive time intervals of 2.5 min were taken. Representative recordings are given in Fig. 9.

Then the % time-response curve for the frequency of ventricular contractions was produced (Fig. 10a, curve a). The % value of frequency remained constant for over 2 h, without significant ($p > 0.05$) changes. This is an indication of the proper functioning of the pacemaker cells generating the electrical impulses for the stimulation and subsequent contraction of ventricular cells. The % time-response of the force of ventricular contractions in shown in Fig. 10b (curve a). In this case there was a gradual, minor decrease to 80% of its initial value, within 100 min after incubation in physiological saline.

3.5.1.2. Effects of microplastics on the frequency and force of ventricular contractions. The effects of 26 mg/L PS-MPs on the frequency of ventricular contractions are shown in Fig. 10A, curve B. There was a $36 \pm 7\%$ gradual decrease ($n = 5$, $p < 0.05$) on the frequency, which was maintained until the end of the experiments. The effects of PS-MPs on the frequency were dose-dependent, since a 10 times higher concentration of 260 mg/L PS-MPs induced a $52 \pm 8\%$ ($n = 5$, $p < 0.05$) decrease (Fig. 10A, curve c), which was irreversible during application of PS-MPs.

The % time response curves of the force of the ventricular contractions show that there was no significant ($p > 0.05$) effect of PS-MPs on the force, for both concentrations, 26 and 260 mg/L, examined (Fig. 10B, curves b and c correspondingly). In conclusion, our results indicated that PS-MPs at the concentrations tested caused only a significant chronotropic decrease, inhibiting heart rate by 0.3–0.5 times

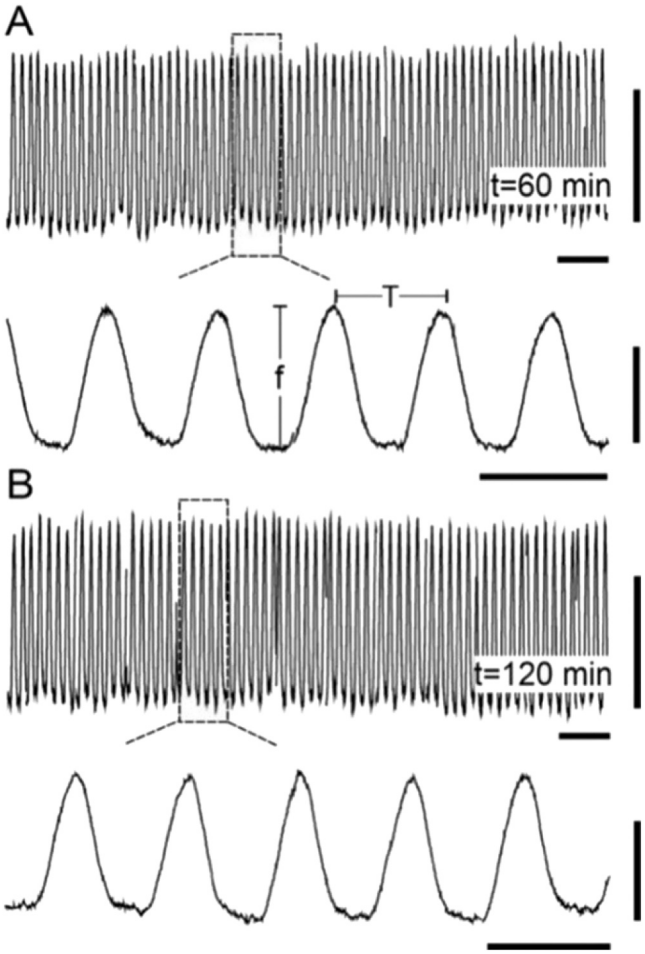

Fig. 9. (A, B) Recordings of heart contractions at constantly oxygenated physiological saline. In lower traces, parts of upper recordings were expanded in time scale (dotted box). Horizontal scale bars in upper and lower trace 5 and 1 s correspondingly, vertical 1 mN. (f) indicates force of contraction and (T) indicates the period of successive contractions used for measurements of frequency.

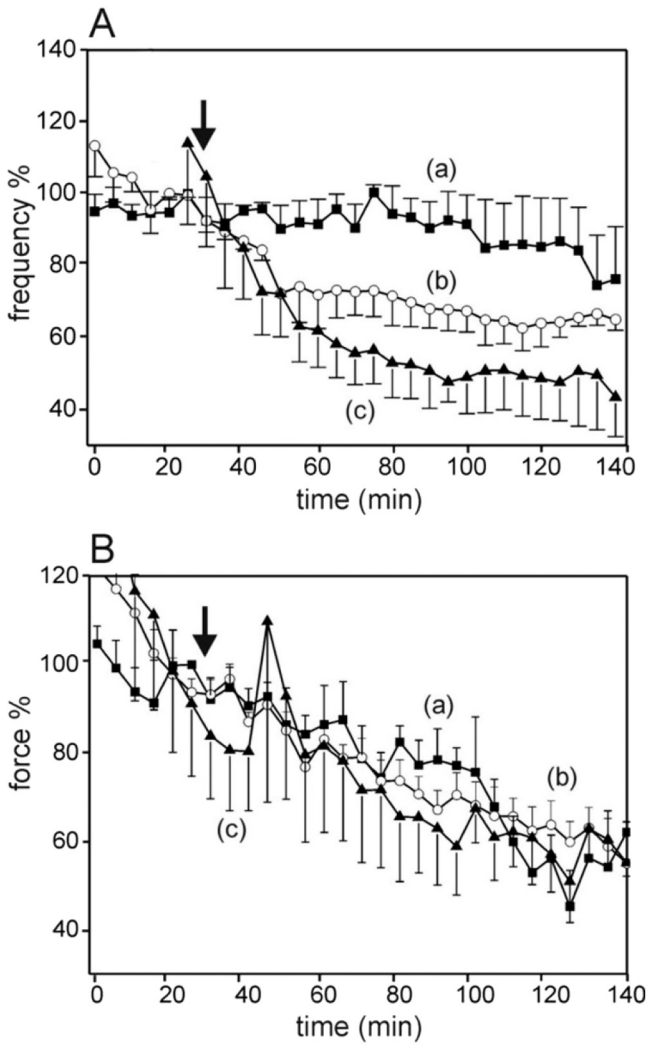

Fig. 10. (A) Time course of the mean value of the frequency of the spontaneous ventricular contractions during incubation of the preparation with physiological saline only (curve a) and after application of 26 (b) and 260 mg/L PS-MPs (c). Each point in the curves is the mean from six experiments. Vertical bar represents SEM. Arrow indicates application of PS-MPs (B) Same as in (A) but for the force of ventricular contractions.

compared to control.

The above only chronotropic decrease (Fig. 10A, curves b and c) suggests that atrial pacemaker cells which control the heart rate, are affected by 26 and 260 mg/L PS-MPs, while the ventricular cardiomyocytes which mainly contribute to the generation of the contractile force are not affected by the same concentrations of PS-MPs (Fig. 10B, curves b and c). The chronotropic decrease and the lack of inotropic decrease implies also that the propagation of the electrical depolarizing stimulus derived from sinus venosus pacemaker cells (Sedmera et al., 2003) to the ventricle of the heart is not affected by the presence of PS-MPs. In accordance with the above, the lack of a significant inotropic decrease in the ventricle excludes a possible deactivation (not stimulation) of ventricular cardiomyocytes. Similar chronotropic decreases were recently detected in the heart rate of zebrafish embryos and marine medaka (*Oryzias melastigma*) embryos, following long term in vivo exposure to concentrations of PS-MPs ranging between 0.02 and 0.2 mg/L (Zhang et al., 2020).

3.5.1.3. Effects of microplastics on the adrenaline induced excitation of heart contractility. In order to further study the effect of PS-MPs on fish cardiac tissue, our results were compared with those of the effect of

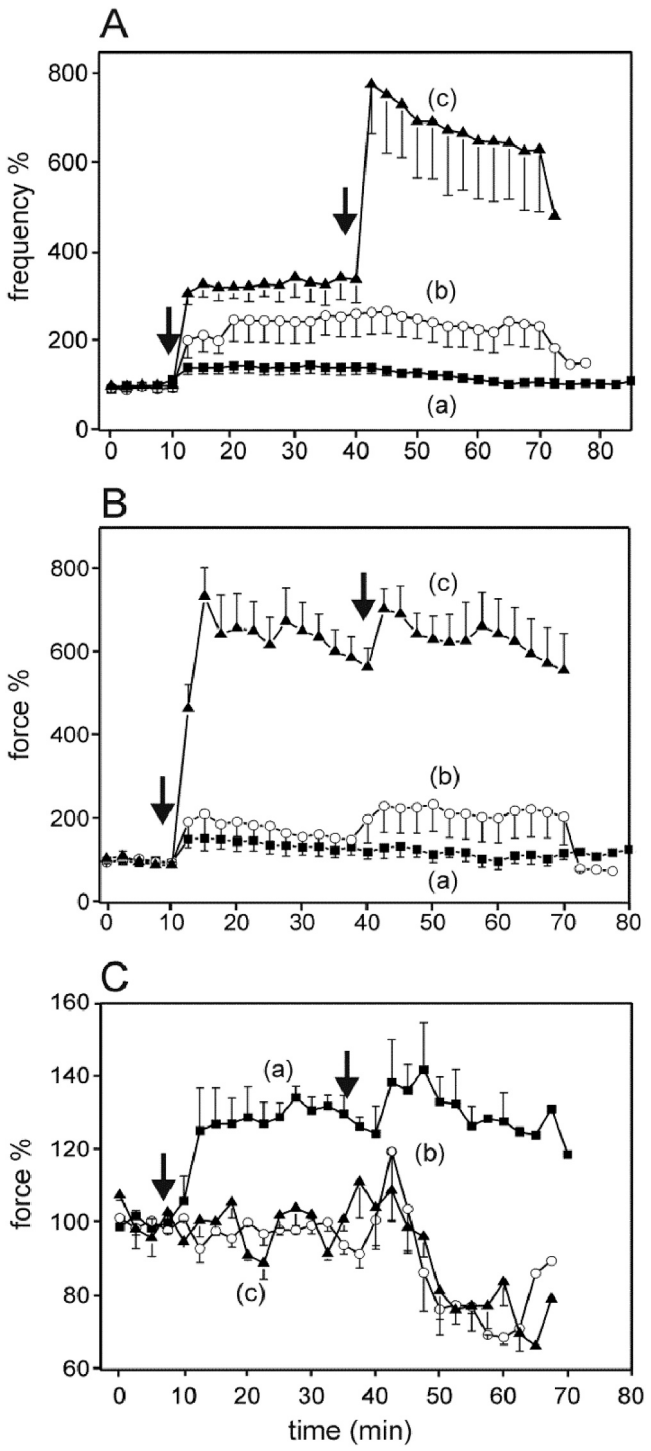

Fig. 11. (A) Time course of the mean value of the frequency of the spontaneous ventricular contractions after application of 10 μM adrenaline alone (curve a) and after application of 26 (b) and 260 mg/L PS-MPs (c) at the presence of adrenaline. Each point in the curves is the mean from six experiments. Vertical bar represents SEM. First and second arrow indicates application of either adrenaline or adrenaline and PS-MPs and second arrow wash out with physiological saline. (B) Same as in (A) but for the force of ventricular contractions. (C) As in (B), but the heart was exposed to 0.1 μM ouabain and after application of 26 (b) and 260 mg/L PS-MPs (c) in the presence of ouabain. Each point in the curves is the mean from four experiments. First and second arrow indicates application of either ouabain or PS-MPs and second arrow wash out with physiological saline.

positive inotropic agents such as adrenaline as well as of ouabain on zebrafish heart (Fig. 11A).

In spite of the fact that PS-MPs induced a reduction in frequency of heart contraction, the presence of 26 mg/L PS-MPs together with 10 μM of adrenaline induced a significant 160 ± 38% (n = 6, $p < 0.05$) increase in the frequency of ventricular contractions (Fig. 11A, curve b), which was maintained during the 30-min incubation of the preparation. Following wash out with physiological saline (Fig. 11A, second arrow in curve b) the positive chronotropic action of adrenaline at the presence of PS-MPs was persistent for at least 30 min.

The presence of 10-times higher concentration of PS-MPs together with 10 μM of adrenaline, induced a further significant 220 ± 35% (n = 4, $p < 0.05$) increase in the frequency of ventricular contractions (Fig. 11A, curve c), which was also maintained during the exposure period. Interestingly the positive chronotropic action of adrenaline was further augmented, when the preparation was washed out with physiological saline (Fig. 11, second arrow in curve c).

Similar results to those observed in frequency were obtained on the force of ventricular contractions (Fig. 11B). It is known that after adrenaline effect, influx of Ca^{2+} in cardiac myocytes is increased and as a result the force of heart contraction is also amplified, a phenomenon that is characterized as positive inotropic effect. Positive inotropism and chronotropism through a β-adrenoceptor mediated mechanism was found also at the isolated hearts of other fish species (Sandblom et al., 2010). The augmentation of adrenaline effects at the presence of PS-MPs suggests a possible perturbation of the signaling of zebrafish ventricular cardiac cell surface β-adrenergic receptors. This is reinforced by the fact that the positive inotropic and chronotropic effects of adrenaline in the presence of microplastics are irreversible, indicating a longer lasting intracellular effect during the wash out (removal) of PS-MPs. Perturbation of cell surface receptors was also proposed as a plausible mechanism for PS-MPs induced mammalian toxicity (Yong et al., 2020).

3.5.2. *Effects of microplastics on the ouabain-induced positive inotropic action*

The response of the zebrafish heart preparation was examined after ouabain treatment, since the latter cardiac glycoside is a well-known positive inotropic agent in mammalian heart (Bai et al., 2013). 0.1 μM ouabain induced a 36 ± 6% (n = 4, $p < 0.05$) maximum increase in the force of ventricular contraction, which was maintained during the 30-min incubation of the preparation in the presence of ouabain (Fig. 11C, curve a). When the preparation was washed out with physiological saline, the positive chronotropic effect of ouabain was not reversed (Fig. 11c, second arrow in curve a). The presence of either 26 mg/L PS-MPs (Fig. 11C, curve b) or 260 mg/L PS-MPs (Fig. 11C, curve c) abolished the above positive effect of ouabain. When the preparation was washed out with physiological saline, there was a further significant decrease of 20–30% on the force (Fig. 11C, second arrow in curves b and c). It is known that treatment with ouabain, an increase of intracellular Ca^{2+} occurs, due to K^+/Na^+ ATPase inhibition (Shu et al., 2003) and as a result increase of heart force is observed. This is the most probable mechanism for ouabain enhanced cardiac contractility in zebrafish, suggested also elsewhere (Nesher et al., 2010; Buzaglo et al., 2018). Our results showed that PS-MPs abolished the positive inotropic effect of ouabain, indicating that PS-MPs probably bind to the enzyme K^+/Na^+ ATPase and prevent ouabain from binding to it; as a result, changes in ionic gradients of zebrafish ventricular cells takes place that inhibit ouabain function.

3.6. *Swimming performance*

Our results showed that zebrafish fed on PS-MPs supplemented diet for 21 days exhibited a significant decrease ($p < 0.05$) in swimming velocity in relation to the control fish (26% decrease in RU_{crit}) (Fig. 12). Swimming performance has been a reliable indicator for assessing environmental impacts on fish fitness due to its high ecological

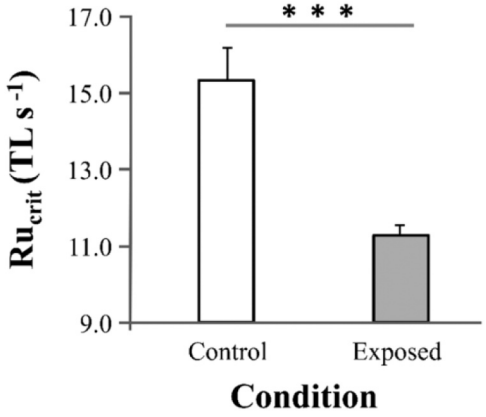

Fig. 12. Effect of PS-MPs on the aerobic swimming performance of male zebrafish. Mean RU_{crit} was measured in males of control (n = 7) and exposed (n = 7) fish. Asterisks (***) indicate significant statistical differences between the two groups ($p < 0.001$, Mann-Whitney *U* test). Error bars equal to 1 SEM.

importance in natural populations (Plaut, 2001; Reidy et al., 2000). In this framework, swimming performance tests have been used to study the effects of several contaminants, such as metals (Atchison et al., 1987; Puga et al., 2016; Vieira et al., 2009), MPs (Chen et al., 2017; Tosetto et al., 2017), PAHs (Lucas et al., 2016), NPs (Kaloyianni et al., 2020; Bobori et al., 2020; Chen et al., 2014; Truong et al., 2012), pesticides among others and represent a good indicator to measure the toxic effects of environmental contaminants (Tierney, 2011).

In agreement with our results, exposure of zebrafish to PS microspheres MPs has been found to decrease swimming competence of fish larvae (Qiang and Cheng, 2019). In goldfish, PS nMPs had a stronger impact on the larvae swimming speed than mMPs at the same concentration, with slower swimming speed of the larvae following high dose MPs exposure (Yang et al., 2020). To our knowledge, this study is the first to investigate the effects caused by exposure to sublethal doses of PS-MPs ingestion on zebrafish aerobic swimming capacity.

Our results reinforce the emerging rationale of the alteration of aerobic swimming capacity in response to pollutants and specifically to MPs exposure through digestion. Swimming performance reduction could be attributed to ROS overproduction that induces muscle damage and influence the metabolism (Close et al., 2005). Our metabolomic results are consistent with the latter. In specific, the reduced protein metabolism, the reduction in the rate of the pathway of arginine metabolism as well as the reduced TCA cycle rate that denotes inadequate ATP production, may be related to the reduced fish aerobic capacity that designates the inter-relation of the measured responses at multiple levels, against MPs treatment. Secondly, since ROS generation is mainly governed by mitochondria (Zorov et al., 2006), the damaged mitochondria might cause a decrease in energy supply which could affect zebrafish RU_{crit}. The damage of membrane lipids as a result of increased ROS production, through the observed elevation of lipid peroxidation, is possibly the cause of fish reduced velocities. Moreover, swimming performance reduction might be due to acetylcholinesterase (AChE) inhibition which is implicated in various behavioral alterations like deficient movement and swimming (Choi et al., 2018; Pan et al., 2017). Finally, the observed reduction in the swimming velocities could also be attributed to energy imbalance resulting from the inflammatory response of MPs entrance in the organism (Rist and Hartmann, 2018; Wright et al., 2013a, 2013b).

Furthermore, reduction in swimming capacity may be related with reduced frequency of cardiac ventricular contraction of as seen above. To our support, previous studies have already correlated maximum cardiac performance with active metabolic rate (AMR) and critical swimming speed (U_{crit}) (Claireaux et al., 2005). In zebrafish, fish that grew up at elevated temperatures were correlated with reduced

swimming velocities and rounder hearts in adulthood (Dimitriadi et al., 2018). In response to pollutants, sublethal exposure to crude oil during the embryonic stage was correlated with reduced swimming capacity and altered heart shapes in zebrafish adults (Hicken et al., 2011). Our results reinforce the already established link between heart form and function and exposure to pollutants. The evaluation of swimming capacity could also be suggested together with the oxidative stress parameters to be used as a package of biomarkers in biomonitoring studies against PS-MPs.

4. Conclusions

Adverse effects of PS-MPs on zebrafish were profound in the macroscopic, cellular and metabolic level. Decrease in heart rate, increase in oxidative stress through the induction of related parameters leading to autophagy and to apoptosis, metabolic adjustments in the heart and fish activity reduction, after treatment of zebrafish to sublethal concentrations of PS-MPs was observed. DNA damage seems to be the most vulnerable parameter to PS-MPs effect from the rest oxidative stress parameters examined. Metabolomics analysis of tissue extracts using LC/MS providing evidence that aerobic capacity is reduced as a result of hampered ATP production due to a slower TCA cycle rate, and also for the presence of inflammation, muscle damage and reduced nucleic acids and protein metabolism probably hindering muscle regeneration. The results obtained also highlight the potential posed by metabolomics to elucidate cellular mechanisms, providing further adjustments are made to specifically determine key metabolites and potential biomarkers. The decrease of swimming velocity also affects fish survival and well-being. The decrease in swimming velocity could be related with the observed fall in frequency of ventricular heart contraction after PS-MPs treatment. The decrease in the frequency compared to lack of inotropic effects, indicates a higher sensitivity of atrial pacemaker cells, to PS-MPs, compared to the ventricular cardio myocytes. These materials possibly interact with cardiac cell surface β-adrenergic receptors and cause a perturbation of the ventricular signaling. In addition the elimination of ouabain positive inotropic effect at the presence of PS-MPs suggests an effect on K^+/Na^+ ATPase. Furthermore, the elevated membrane lipid peroxidation, denotes heart membrane damage that occurs after PS-MPs exposure, a fact that could also be linked to decreased aerobic capacity as observed by decreased swimming velocity. All of these parameters are interrelated in a way, since all the systems constitute part on the organism and all functions are interconnected. The current findings provide a holistic approach in identifying the biological effects of small sized PS-MPs in fish species, like the zebrafish, thus aiming to promote field studies, regarding the environmental impact of PS-MPs on fish population dynamics.

Ethics statement

All the experimental procedures involving handling and exposure of fish were performed in accordance with Greek (PD 56/2013) and EU (Directive 63/2010) legislation for animal experimentation and welfare. All protocols were approved by the Animal Care Committee of the Biology Department of the University of Crete (Permit Number: 285586 (2020).

CRediT authorship contribution statement

Anastasia Dimitriadi: Investigation, Writing - review & editing, Visualization. **Chrisovalantis Papaefthimiou:** Investigation, Supervision, Writing. **Eleni Ginizegini:** Investigation, Methodology. **Ioannis Sampsonidis:** Investigation, Writing. **Stavros Kalogiannis:** Conceptualization, Writing - review & editing. **Konstantinos Feidantsis:** Investigation, Writing. **Dimitra Bobori:** Conceptualization, Writing - review & editing. **Georgia Kastrinaki:** Investigation, Writing. **George Koumoundouros:** Resources, Validation. **Dimitra A. Lambropoulou:** Conceptualization, Validation, Formal analysis. **George Z. Kyzas:** Writing - review & editing, Methodology, Validation. **Dimitrios N. Bikiaris:** Formal analysis, Writing - review & editing, Supervision.

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.

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