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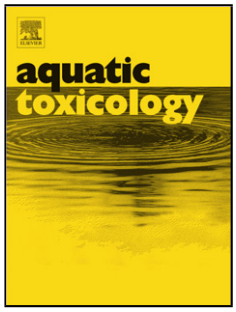

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Changes in protein expression in mussels *Mytilus galloprovincialis* dietarily exposed to PVP/PEI coated silver nanoparticles at different seasons

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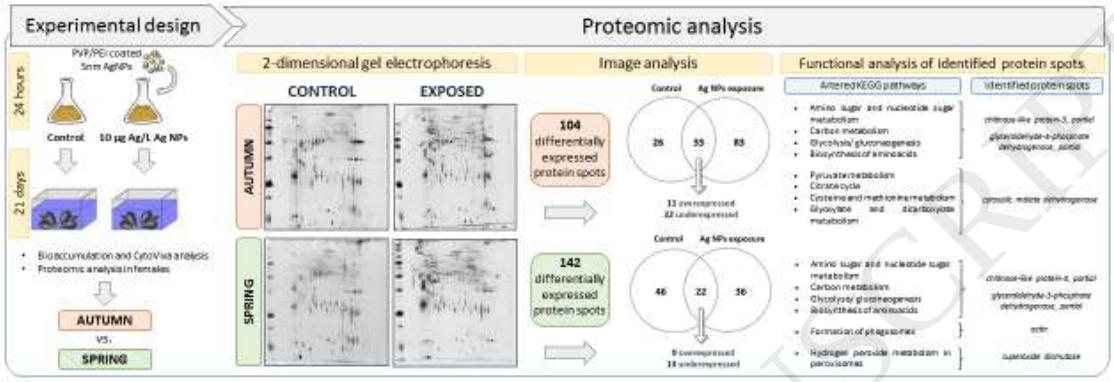

Highlights

- Protein expression profiles in mussels dietarily exposed to Ag NPs for 21 d were studied in autumn and spring.
- Mussels accumulated Ag in both seasons and Ag NPs were found within mussels digestive gland tubule cells and gills.
- Protein expression profiles and associated metabolic pathways depended on season and Ag NPs exposure.
- Chitinase like protein-3 and G3PDH were among differentially expressed spots identified in both seasons.
- Status of gamete development should be considered when assessing effects caused by NPs in mussels.

ABSTRACT

Potential toxic effects of Ag NPs ingested through the food web and depending on the season have not been addressed in marine bivalves. This work aimed to assess differences in protein expression in the digestive gland of female mussels after dietary exposure to Ag NPs in autumn and spring. Mussels were fed daily with microalgae previously exposed for 24 hours to 10 µg/L of PVP/PEI coated 5 nm Ag NPs. After 21 days, mussels significantly accumulated Ag in both seasons and Ag NPs were found within digestive gland cells and gills. Two-dimensional electrophoresis distinguished 104 differentially expressed protein spots in autumn and 142 in spring. Among them, *chitinase like protein-3, partial* and *glyceraldehyde-3-phosphate dehydrogenase*, that are involved in amino sugar and nucleotide sugar metabolism, carbon metabolism, glycolysis/gluconeogenesis and the biosynthesis of amino acids KEGG pathways, were overexpressed in autumn but underexpressed in spring. In autumn, pyruvate metabolism, citrate cycle, cysteine and methionine metabolism and glyoxylate and dicarboxylate metabolism were altered, while in spring, proteins related to the formation of phagosomes and hydrogen peroxide metabolism were differentially expressed. Overall, protein expression signatures depended on season and Ag NPs exposure, suggesting that season significantly influences responses of mussels to NP exposure.

Keywords: silver nanoparticles, dietary exposure, mussels *Mytilus galloprovincialis*, seasons, proteomic analysis.

1. Introduction

The rapid development of nanoscience and nanotechnology has led to the increasing incorporation of nanoparticles (NPs) into consumer products (Baker et al., 2014; Corsi et al., 2014; Vance et al., 2015). Due to their unique optical, catalytic and antimicrobial properties, silver NPs (Ag NPs) have gained high commercial and scientific interest (Fabrega et al., 2011; Vance et al., 2015; Zhang et al., 2016; McGuillicuddy et al., 2017). The production, use and degradation of products containing Ag NPs can provoke the potential release and input of Ag NPs into freshwater and marine environments (Baker et al., 2014; Corsi et al., 2014). Once in the environment, Ag NPs may interact with aquatic organisms and may induce toxic effects at different levels of biological organization (Rocha et al., 2015a).

Toxic effects caused by the exposure to Ag NPs through the water have been widely investigated in different freshwater organisms such as microalgae (Navarro et al., 2008; Ribeiro et al., 2014; Sendra et al., 2017a), daphnids (Ribeiro et al., 2014; Khan et al., 2015; Pakrashi et al., 2017) and zebrafish (Griffit et al., 2009; 2013; Ribeiro et al., 2014; Lacave et al., 2017; 2018; Orbea et al., 2017). In seawater, studies based on waterborne exposure to Ag NPs have mainly been focused on microalgae (Gambardella et al., 2015; Schiavo et al., 2017; Sendra et al., 2017b) and bivalves (Ringwood et al., 2010; Buffet et al., 2013; 2014; Gomes et al., 2013a; 2013b; McCarthy et al., 2013; Bebianno et al., 2015; Katsumiti et al., 2015; Jimeno-Romero et al., 2017). However, studies on the potential trophic transfer of metals, metal oxides and metal mixtures in nano-size are scarce but increasingly necessary due to their potential incidence in

human and other species health (Tangaa et al., 2016). In fact, few works have assessed the potential toxic effects of metallic NPs ingested through the food web in marine organisms (Larguinho et al., 2014; Wang et al., 2016; Manzo et al., 2017; Bhuvaneshwari et al., 2018), including Ag NPs (Buffet et al., 2013; Wang and Wang 2014; Duroudier et al., 2019).

In general, bivalve molluscs form the most studied invertebrate group since they have been identified as an important target group for NP toxicity in the marine environment (Moore et al., 2006; Canesi et al., 2012; Corsi et al., 2014; Canesi and Corsi, 2016). Most research in bivalves has been focused on understanding the effects of Ag NPs on *in vivo* waterborne exposed organisms using conventional biomarkers (Ringwood et al., 2010; Buffet et al., 2013; 2014; Gomes et al., 2013a; 2014b; McCarthy et al., 2013; Bebianno et al., 2015; Jimeno-Romero et al., 2017), but few studies based on other methods (e. g. transcriptomics, proteomics, metabolomics) have assessed the potential effects caused by Ag NPs (Rocha et al., 2015a). Proteomics-based methods, among others, have been applied to complement the information given by conventional biomarkers and to identify new protein pathways affected by the exposure to NPs (Dowling and Sheehan, 2006; Rocha et al., 2015a).

Tedesco et al. (2008) first reported the use of redox proteomics in *Mytilus edulis* tissues exposed to 750 µg/L of gold-citrate-NPs (~13 nm) for 24 hours. Authors observed high carbonylation and ubiquitination of proteins in mussels gills and digestive gland, respectively (Tedesco et al., 2008). The same Au-citrate-NPs (~13 nm) as well as ~5 nm Au NPs both provoked a reduction in the amount of thiol-containing proteins in the digestive gland of *M. edulis* mussels (Tedesco et al., 2010a; 2010b), suggesting that Au

NPs caused oxidative stress. Similarly, Hu and colleagues (2014) observed a reduction in thiol groups as well as an increase in carbonylated proteins in gills of mussels *M. edulis* exposed to three different concentrations of CuO NPs for just 1 hour. Additionally, some proteins were identified as indicators of oxidation of cytoskeleton proteins and two other as specific enzymes after CuO NPs exposure (Hu et al., 2014). Short exposures (3h, 6h and 12h) to two sizes of Ag NPs (<50 and < 100 nm) also provoked protein thiol oxidation and/or protein carbonylation in mussels gills and digestive gland (Bouallegui et al., 2017). Alterations in the proteome of mussels *M. galloprovincialis* were also assessed after the exposure to CuO NPs or Ag NPs and to their ionic form (Gomes et al., 2013b; Gomes et al., 2014a). In these works, NP and ionic form-dependent protein expression signatures were reported (Gomes et al., 2013b; 2014a). In fact, *major vault protein*, *paramyosin* and *ras (partial)* were overexpressed protein spots in mussels exposed to 10 µg/L Ag NPs for 15 days (Gomes et al., 2013b) and *caspase 3/7-1*, *cathepsin L* and *Zn-finger protein* in mussels exposed to 10 µg/L CuO NPs for 15 days (Gomes et al., 2014a). Thus, such proteins were proposed as putative molecular biomarkers to assess toxicity of Ag NPs and CuO NPs, respectively.

In studies reported above, gender of mussels and developmental stage of gametes were not considered although mussels are intertidal organisms that show seasonal metabolic and enzymatic variations related both to abiotic changes in the environment, such as temperature, food availability and oxygen levels, and to biotic factors such as gender, gamete developmental stage and physiological state (Bayne and Widdows, 1978; Solé et al., 1995; Cancio et al., 1999). Some studies have identified gender-specific natural variations in gene expression patterns (Banni et al., 2011) as well

as gender-specific differences in the response to contaminants (Livingstone and Farrar, 1984; Brown et al., 2006; Riva et al., 2014; Ji et al., 2016; Banni et al., 2017) suggesting that gender of mussels should be considered in ecotoxicological studies. In fact, in many works the potential effects of different pollutants have been studied only in female mussels (Dondero et al., 2011; Negri et al., 2013; Banni et al., 2014; 2016; 2017). Thus, in the present study differences in protein expression profiles were only assessed in female organisms in order to avoid biological variability related to gender. Further, mussels sensitivity towards chemical insults may vary in some stages of their reproductive cycle such as the reproduction period (Leiniö and Lehtonen, 2005; Bocchetti and Regoli, 2006; Almeida et al., 2013). Thus, it is relevant to study season-dependent variations in mussel responses to Ag NPs as well as to other emerging pollutants.

In this context, the aim of this work was to assess differences in the expression profile of proteins in the digestive gland of female mussels fed with algae exposed to 10 µg/L Ag NPs and to compare such expression profiles at two different seasons, in autumn and spring, in which mussels were at different developmental stages. In parallel, Ag accumulation was studied in mussels soft tissues and presence of Ag NPs was assessed in tissue sections using a hyperspectral imaging system. The selected dose of Ag NPs is above levels considered environmentally relevant (Gottschalk et al., 2009; Tiede et al., 2009; Giese et al., 2018) but it has been previously used in toxicological studies of water-borne exposed bivalves (Buffet et al., 2013; 2014; Gomes et al., 2013a; 2013b; 2014b). Also, in a previous study, we reported deletereous effects of dietary exposure to 10 µg/L Ag NPs and to 1 µg/L Ag NPs, a dose close to environmentally

relevant concentrations, on spawning success of female mussels and on development of embryos descendant from exposed mussels (Duroudier et al., 2019).

2. Materials and methods

2.1. Characterization of NPs

Ag NPs coated with PVP/PEI (Poly N-vinyl-2-pirrolidone + Polyethyleneimine; 77%:23% at a concentration of 104 g/L in the final dispersion) were purchased as a stable aqueous suspension from Nanogap (O Milladoiro, Galicia, Spain). According to the manufacturers' information, PVP/PEI coated Ag NPs showed an average size of 5.08 ± 2.03 nm and a zeta potential of +18.6 ± 7.9 mV in distilled water. A transmission electron microscope (TEM) image of the same batch of NPs is provided in Orbea et al. (2017).

Particle size distribution and dissolution of PVP/PEI coated 5 nm Ag NPs in seawater (SW) were analyzed as reported in Duroudier et al. (2019). Briefly, PVP/PEI coated 5 nm Ag NPs dispersed in SW immediately reached a mean size of roughly 97 nm according to Dynamic Light Scattering using a Zetasizer Nano Z (Malvern Instruments Ltd., Worcestershire, UK). After 24 hours, particle size remained stable around 94-96 nm (Duroudier et al., 2019). Dissolution of PVP/PEI coated 5 nm Ag NPs was assessed in SW after 12, 24, 48 and 72 hours For that, 10 mL samples of the Ag NP suspensions were prepared at a concentration of 10 mg/L and filled into dialyzer tubes (Spectra/ Por® Float-A-Lyzer; MWCO 0.1-0.5 kDa). The sample-filled dialyzers were then immersed in 1 L SW and samples (3 replicates of 1 mL) were extracted at 12, 24, 48 and 72 hours from the solution and analyzed for Ag ions using Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Thermo X2 series) after 100-fold dilution using external

calibration. Differences obtained for replicate samples were consistently lower than 5 % (Relative Standard Deviation; R.S.D). Dissolution of Ag NPs in SW was detected along the 72 hours of experimentation. After 12 hours, Ag NPs released around 4% of Ag ions increasing the dissolution to around 14% at 24 hours. After 48 hours ~17% of Ag ions were released from Ag NPs and at 72 hours, dissolution increased to 20% (Duroudier et al., 2019).

2.2. Experimental design

Mussels *Mytilus galloprovincialis* 3.5-4.5 cm in shell length were collected in autumn (November 2013) and spring (March 2014) from San Felipe, Galicia (43° 27.599'N, 8° 17.904'W). Upon arrival to the laboratory, mussels were placed in an acclimation tank with natural filtered SW (temperature =15.6°C, salinity =28.7‰, conductivity =36.4 mS/cm, pH =7.7 at light regime 12 h/12 h L/D) for 10 days. Mussels were maintained 5 days without feeding and then mussels were fed with *Isochrysis galbana* microalgae (20 x 10^6 cells/ mussel-day) for other 5 days. For that, microalgae were cultured with natural filtered SW as above, additionally filtered (0.2 µm) and sterilized at 20°C under cool continuous white fluorescent light (GRO-LUX F58W) with constant aeration in reactors at a concentration of 6x10^6 cells/mL. Commercial F2 algae medium (Fritz Aquatics, USA) was supplied according to manufacturer´s instructions. Concentration of microalgae was checked every day before feeding mussels.

After the acclimation period, mussels were distributed in two high density polypropylene containers (250 L) containing 240 mussels per tank. Mussels in the control tank were fed for 21 days with the microalgae *Isochrysis galbana* (20 x 10^6 cells/

mussel-day) and mussels in the treatment tank were fed for 21 days with the same ration of microalgae *I. galbana* (20 x 10^6 cells/ mussel-day) previously exposed for 24 hours to 10 µg Ag /L Ag NPs. Microalgae exposure was performed in constantly aerated two beakers (control and exposed) that contained the same volume of 6x10^6 cell/mL of microalgae. A stock suspension of PVP/PEI coated Ag NPs was daily diluted in MilliQ water, vortexed and spiked to microalgae at the selected nominal exposure concentration. This concentration was lower than Ag NP concentrations causing growth inhibition (0.1 mg Ag/L Ag NPs) in 72 hours bioassays (Schiavo et al., 2017). According to Duroudier et al. (2019), Ag was significantly accumulated in microalgae exposed to 10 µg Ag/L Ag NPs for 24 hours, reaching a mean value of 21.3±2.1 µg Ag/g d.w. After 24 hours, the beaker content with non-exposed or exposed microalgae was transferred to the corresponding mussel tank. Water in the mussel tanks was renewed every day before animal feeding. No mussel mortality was recorded along the experimentation time.

After 21 days of exposure, whole soft tissues of 20 mussels per experimental group were collected for chemical analysis. For Ag NPs localization, whole soft tissues of 3 mussels were fixed in 10% neutral buffered formalin. A piece of gonad of 20 mussels per experimental group were dissected out and fixed in 10% neutral buffered formalin in order to select only female mussels for the proteomic study. Digestive glands of the same 20 mussels per group were stored at -80°C until processing. Then, digestive glands of 5 female mussels per experimental group were used for the proteomic analysis.

2.3. Accumulation of Ag in mussel soft tissues

Soft tissues of 20 mussels per experimental group were pooled in 4 groups (4 pools of 5 individuals each) and then lyophilized by a freeze-dryer (Telstar Cryodos) for 5 days. Afterwards, samples (20–150 mg) were mineralized using 1.4 ml of HNO_3 (14 M, PlasmaPur) and 2 ml of HCl (12 M, PlasmaPur), according to Daskalakis (1996). Briefly, closed tubes were digested for 3 hours on a hot plate at 90°C (DigiPREP MS; SCP SCIENCE). After cooling, digestates were diluted and analyzed for Ag concentrations by ICP-MS (Thermo, X Series II) using external calibration (made of commercially available standard solutions PLASMACAL, SCP Science). Accuracy (>90%) and precision (<5%, Relative Standard Deviation) were controlled during each analytical session by parallel analysis of international referenced certified materials (TORT 2, IAEA 407). Results are expressed as µg Ag/g d.w. Significant differences ($p<0.05$) with respect to controls as well as among seasons were set based on the 2-way ANOVA followed by the Tukey multiple comparison test using the statistical package SPSS v.22 (SPSS Inc., IBM Company, Chicago, USA).

2.4. Histology of samples

After 24 hours of fixation in 10% neutral buffered formalin, whole mussels and gonad samples were routinely processed for paraffin embedding using a Leica Tissue processor ASP 3000 (Leica Instruments, Wetzlar, Germany). Histological sections (5 µm in thickness) were cut in a Leica RM2255 microtome (Leica Instruments) and placed on microscope glass slides.

2.4.1. Localization of Ag NPs

Dewaxing of mussel samples was performed using either xylene or Roti-histol. After 3 washes in xylene or Roti-histol, samples were hydrated with decreasing concentrations of ethanol (100%, 95% and 70%) followed by a final wash in deionized water before being dehydrated with increasing concentrations of ethanol (70%, 95% and 100%). Then, slides were washed in xylene or Roti-histol and mounted on cover slips. Samples were let to dry for 48 hours at room temperature before CytoViva® analyses.

Slides were visualized using CytoViva® hyperspectral imaging system (CytoViva Inc., Auburn, Alabama, USA) mounted on an Olympus BX-43 optical microscope as described in Mehennaoui et al. (2018). Briefly, images of the digestive gland and gills of non-exposed and exposed mussels were captured at 60x oil immersion magnification using hyperspectral camera controlled by environment for visualization ENVI software (version 4.8 from Harris Corporation, Melbourne, FL, USA and modified by CytoViva, Inc.). Spectral libraries of exposed mussels were generated manually with the acquisition of about 200 spectra per individual mussel. Acquired libraries were filtered against non-exposed samples to filter out all spectra non-related to Ag NPs using a spectral angle mapper (SAM) algorithm with a 0.05 radians tolerance. Filtered libraries were mapped onto images of exposed samples using SAM with 0.1 radian tolerance which allows highlighting similarities between the spectra in the image and in the spectral library (Mehennaoui et al., 2018).

2.4.2. Gonad histology

Gonad sections from the same individuals used for proteomics were stained with hematoxylin-eosin (Gamble and Wilson, 2002) and examined under the light microscope (Nikon Eclipse Ni; Nikon Instruments, Tokyo, Japan) in order to identify and select female mussels. A gonad index (GI) value was assigned to each selected female gonad as described by Kim et al. (2006). A mean GI was then calculated for controls and for mussels dietarily exposed to Ag NPs.

2.5. *Proteomic analysis*

2.5.1. Cell-free extract preparation and protein assay

A pool of five female digestive glands per experimental group was weighed, suspended in 20% (w/v) HEPES-saccharose buffer (10 mM HEPES and 250 mM saccharose) containing 1 mM DTT, 1 mM EDTA, 1 mM PMSF and 10% protease inhibitor mixture and homogenized at 4°C. Then, homogenates were centrifuged at 15000 *g* for 2 hours and cell free extracts of crude cytosolic fractions were collected. Protein content was determined following the Bradford method (Bradford, 1976) with bovine serum albumin (BSA) as standard. 100 µg of protein content of each sample was suspended in 9 volumes of a precipitation solution (20 mM DTT, 10% trichloroacetic in cold acetone) for 2 hours at -20°C, centrifuged at 10000 g for 30 minutes (4°C) and washed with cold acetone. The residual acetone was removed by air drying before rehydrating the samples for the separation of proteins by two-dimensional gel electrophoresis.

2.5.2. Two-dimensional gel electrophoresis (2-DE)

Proteins were first separated by isoelectric focusing (IEF) followed by SDS-PAGE. Each sample (containing 100 µg of protein) was incubated for 30 minutes in 300 µL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.8% pharmalyte, 65 mM DTT and bromophenol blue traces), centrifuged at 14000 *g* for 10 minutes (4°C) and loaded on 6 different Immobiline®DryStrip (pH 3-10; 18 cm). After 6 hours of passive and 6 hours of active (50 V) rehydration, IEF was carried out (20°C, 50 µA/strip) in a Ettan IPGphor II (GE Healthcare) using a four step program: 1000 V for 1 hour; 4000 V for 1 hour; 8000 V for 1 hour and 8000 V until 50000 V/h were reach. Before the second dimension protein separation, 6 strips per sample were equilibrated in SDS equilibration buffer (6 M urea, 75 mM Tris-HCl, 4% SDS, 29.3% glycerol, 0.1 mM EDTA and 0.25% bromophenol blue) first with 2% DTT and second with 2.5% iodoacetamide. After equilibration, SDS-PAGE was performed in 10% polyacrylamide gels using a Ettan Daltsix Electrophoresis Unit (GE Healthcare). The separation in 6 gels was run in two steps; first, at 120 V for 30 minutes and then at 500 V for 5 hours until separation was finished. Gels were silver stained using a protocol compatible with MS analysis (Blum et al., 1987). Four gel replicates out of 6 gels for each pool were analyzed to ensure the reproducibility of 2D electrophoresis.

2.5.3. Image acquisition and analysis

The four gels per experimental group were scanned using a GS-800 densitometer (BIORAD, Hercules, CA) and analyzed using the PDQuest Advanced 8.0 software (BIORAD, Hercules, CA). All the 2-DE maps were performed with identical background subtraction after spot detection. The normalized volume of each spot was used for

quantitative analyses by dividing its volume by the total volume of detected spots on the image in order to reduce experimental errors related to protein loading and staining. The normalized volumes from the different spots obtained from exposed samples were matched against the corresponding spots from control gels. The protein intensity of each spot was normalized to the total intensity of each gel image. Significant differences in regulated protein spots between control and mussels dietarily exposed to Ag NPs were set based on the Mann–Whitney U-rank test. Only spots regulated at least in 3 of the 4 gels for each group were included in the statistical analysis. Significance level was set at 5% ($p<0.05$). Principal Component Analysis (PCA) was conducted using the PRIMER-E v6 software after the normalization method described by Apraiz et al. (2009). Briefly, volume data (vol. %) was normalized according to the following equation:

$$NVol\ \% = \ln (vol.\ \% + 1)$$

where NVol.% is the normalized vol.% obtained for each spot. After normalization of volume data and to reduce the inter-replicate variability, only protein spots with a coefficient of variation smaller than 40% were considered for the PCA.

2.5.4. Digestion of proteins and protein identification

Protein spots from control and exposed mussels including spots with changes in protein expression above or below 4-fold were manually excised from silver stained gels. Digestion and analysis through mass spectrometry were performed in the Bioscope Bio-Analytics and Proteomics Lab from the New University of Lisbon. Briefly, picked spots were digested with trypsin, as described in Shevchenko et al. (2007). Proteins were

subjected to peptidemass fingerprint (PMF) and mass spectra were acquired using an Ultraflex II MALDI–TOF (Bruker Daltonics) operating with positive polarity in reflectron mode. Spectra were acquired in the range of m/z 900–3500. A total of 3000 spectra were acquired at each spot position at a laser frequency of 50 Hz. Data acquisition and processing was performed with Flex Analysis software 3.0 (Bruker Daltonics) with the SNAP peak detection algorithm. The obtained peptide mass list was sent to the MASCOT search engine using the NCBI Database. Searches were performed using the following parameters: taxonomy: *M. galloprovincialis* or Bivalvia, proteolytic enzyme: trypsin, peptide tolerance: up to 100 ppm, fixed modifications: carbamidomethyl, variable modification: oxidation, peptide charge state: +1, missed cleavages allowed: up to 2. The significance threshold was set to a minimum of 95%.

Biological processes of proteins were identified according to UniProt database (UniProt Consortium, 2017). Then, the KEGG: Kyoto Encyclopedia of Genes and Genomes database (Kanehisa et al., 2017) was used to decipher the altered metabolic pathways. Briefly, identified sequences were annotated against the Ostreidae family using the BlastKOALA tool in order to get the KEGG identifiers. Then, assigned KEGG identifiers were introduced in the KEGG pathways mapping tool to identify the different metabolic pathways in which the identified protein spots were involved.

3. Results

3.1. Accumulation of Ag in mussels soft tissues

Ag was significantly accumulated in mussel soft tissues after 21 days of dietary exposure to 10 µg /L Ag NPs both in autumn and in spring (Table 1). Although microalgae

and mussel exposure conditions were identical in both seasons, higher levels of Ag were accumulated in autumn (0.73 µg Ag/g d.w.) compared to spring (0.35 µg Ag/g d.w.) (Table 1).

3.2. Localization of Ag NPs

CytoVIVA® analyses confirmed the presence of Ag NPs in the sections of the digestive gland of mussels dietarily exposed to 10 µg /L Ag NPs both in autumn and in spring (Figure 1A, B). Ag NPs were mainly located inside the digestive tubule cells and in the lumen in both seasons (Figure 1A, B). Ag NPs were also found in the gills of mussels dietarily exposed both in autumn and in spring (Figure 1C, D).

3.3. Gonad histology

All mussels selected for proteomic analysis were confirmed to be female. In autumn, female mussels showed gonads in early gametogenic stage (Figures 2A, B). GI mean value was 2.2±1.20 for non-exposed females and 1.9±0.89 for dietarily exposed mussels. In spring, female gonads were in an advanced gametogenic stage or in a mature stage (Figures 2C, D). Non-exposed females GI was 4.75±0.67 and exposed females GI was 4.1±0.82.

3.4. Proteomic analysis

3.4.1. 2-DE image analysis

After 2-DE, different protein expression profiles were obtained in the digestive gland of unexposed mussels and in mussels dietarily exposed to 10 µg /L Ag NPs for 21 days in two different seasons (Figure 3). Image analysis of the gels revealed 104 differentially expressed protein spots in autumn (Figure 4A) and 142 differentially expressed protein spots in spring (Figure 4B).

In autumn, among the 104 protein spots differentially expressed, 46 were present only in control mussels and 36 protein spots were present only in exposed ones (Figure 4A). 22 protein spots differentially expressed were common for both groups and showed at least a 2-fold or a higher fold change in comparison to controls. 9 of them were overexpressed and 13 were underexpressed (Figure 4A).

Of the 142 protein spots differentially expressed in spring, 26 protein spots appeared significantly expressed only in non-exposed mussels, while 83 protein spots were significantly expressed only in exposed digestive glands (Figure 4B). 33 protein spots showing a fold change higher than 2 were expressed in both treatments. Among them, 11 protein spots were overexpressed and 22 protein spots were underexpressed (Figure 4B). Overall, more protein spots were differentially expressed in spring than in autumn (Figures 4A,B).

Comparing control samples among seasons, 121 protein spots were differentially expressed (Figure 4C). 62 protein spots were only expressed in autumn while 22 protein spots were only expressed in spring (Figure 4C). 37 protein spots differentially expressed were common to both seasons being 19 of them overexpressed and 18 underexpressed (Figure 4C).

Finally, 156 protein spots were differentially expressed between seasons in the digestive gland of mussels dietarily exposed to 10 µg/L Ag NPs (Figure 4D). Among them, 50 protein spots were only found in autumn and 63 protein spots were only found in spring (Figure 4D). 43 protein spots differentially expressed were common to both seasons; 9 of them were overexpressed and 34 were underexpressed (Figure 4D).

Therefore, the PCA showed differences in protein expression profiles based on the dietary exposure to Ag NPs and the season. PC1, which explained 36,8% of variation, clearly separated mussels exposed to 10 µg /L Ag NPs in autumn from the rest of mussels (Figure 5). PC2 (34,6% of variation) discriminated the two seasons and PC3 (28,4% of variation) grouped non-exposed mussels belonging to both seasons together. The analysis revealed that the protein expression profiles were affected by the exposure treatment and that mussels showed differences in the response depending on the season (Figure 5).

3.4.2. Protein identification by mass spectrometry

Based on the *Mytilus galloprovincialis* database (2666 sequences), 16 out of the 60 picked and differentially expressed protein spots were identified by MALDI-TOF. In autumn, 10 differentially expressed protein spots could be identified (Table 2). Among them, 4 different overexpressed protein spots (spots 7524, 8555, 8556, 8558) were identified as *chitinase like protein-3, partial* and 2 different overexpressed protein spots (spots 6325, 7304) as *glyceraldehyde-3-phosphate dehydrogenase, partial* (Table 2). The rest of protein spots were identified as *LKD-rich protein-1*, *nuclear receptor subfamily 1 DEF, partial*, *paramyosin* and *cytosolic malate dehydrogenase* (Table 2). In spring, 6

protein spots were identified based on the *M. galloprovincialis* database (Table 3). *Chitinase like protein-3, partial, superoxide dismutase* and *glyceraldehyde-3-phosphate dehydrogenase, partial* were significantly underexpressed, while *actin* (spots 3420, 2419) and *putative C1q domain containing protein MgC1q52* were significantly regulated only in dietarily exposed mussels (Table 3).

The identification search was extended to the Bivalvia database (120604 sequences) allowing the classification of 6 new protein spots in autumn and other 9 protein spots in spring (Tables 2, 3). In autumn, *matrix metalloproteinase-19*, two *hypothetical proteins* (CGI_10016468 and *CGI_10001770,* respectively) and *PREDICTED: inorganic pyrophosphatase-like isoform X2* were significantly underexpressed while *putative transcription factor PML* and *PREDICTED: vinculin-like isoform X7* were only significantly expressed in non-exposed and exposed mussels, respectively (Table 2). Among overexpressed protein spots in spring *putative transcription factor PML* (spots 8206, 5201) and c*onnector enhancer of kinase suppressor of ras 2* were identified (Table 3). *PREDICTED: GRIP and coiled-coil domain-containing protein 2-like* and *3-hydroxybutyryl-CoA dehydrogenase* were identified among underexpressed protein spots in spring (Table 3). *PREDICTED: lipoxygenase homology domain-containing protein 1-like isoform X3* appeared expressed only in control mussels and *selenoprotein H* only in dietarily exposed ones. *PREDICTED: centrosomal protein of 152 kDa-like* (spots 8602, 1506) was identified separately in control mussels as well as in dietarily exposed ones (Table 3).

The number and sequences of the found peptides and the overall sequence coverage have been provided for each identified protein as Supplementary material.

KEGG pathway analysis successfully related some of the identified protein spots to different metabolic pathways in which they are involved (Tables 2, 3). Similar metabolic pathways such as *amino sugar and nucleotide sugar metabolism*, *carbon metabolism*, *glycolysis/gluconeogenesis* and the *biosynthesis of amino acids* in which *chitinase like protein-3, partial* and *glyceraldehyde-3-phosphate dehydrogenase* protein spots are involved in were altered in both seasons (Tables 2, 3). In addition, other specific pathways were altered in each season. In autumn, the *pyruvate metabolism*, *citrate cycle*, *cysteine and methionine metabolism* and *glyoxylate and dicarboxylate metabolism* were altered due to the under expression of the *cytosolic malate dehydrogenase* protein spot (Table 2). In spring, the overexpression of *actin* (2 protein spots) was associated to alterations in the *formation of phagosomes* and the under expression of *superoxide dismutase* to alterations in the *hydrogen peroxide metabolism of the antioxidant system of peroxisomes* (Table 3).

4. Discussion

Proteomic analysis has been already applied in mussels after the waterborne exposure to different metallic NPs (Tedesco et al., 2008; 2010a; 2010b; Gomes et al., 2014a; Hu et al., 2014), including Ag NPs (Gomes et al., 2013b; Bouallegui et al., 2018) in order to identify significant alterations in protein expression patterns. In the present study, differentially expressed protein signatures were investigated in the digestive gland of female mussels exposed to Ag NPs through the diet at two different seasons, autumn and spring.

In both seasons, mussels soft tissues significantly accumulated Ag after 21 days of dietary exposure to 10 µg /L Ag NPs, but higher levels of Ag were accumulated in autumn than in spring. Differences in metal concentration in mussels can result from changes in the physiology of animals related to the season, rather than from changes in metal exposure conditions (Mubiana et al., 2005). For instance, gonadal growth decreased concentrations of Cu and Zn in oysters *Crassotrea iridiscensis* (Paéz-Osuna et al., 1995) and modified Ag and Cd concentrations in wild oysters of the Gironde estuary due to the weight losses and gains during the reproduction cycle (Lanceleur et al., 2011). In mussels collected from a metal polluted area during one year, the decrease in metal concentrations in the digestive gland was linked to the penetration of gonadic tissues into the digestive gland during gametogenesis, which biologically diluted metal concentrations (Regoli and Orlando, 1994). Thus, the lower concentration of Ag measured in mussel soft tissues in spring could be related to the increase of body weight caused by the development of gametes observed in spring. For future studies, the use of the metal/shell-weight index could be useful as it avoids variability in metal accumulation due to variations in soft-body weight related to the season (Fischer, 1983; Soto et al., 1995).

Silver has an unusually wide range of bioaccumulation as reviewed by Luoma and Rainbow (2005), but marine bivalves exposed to different types of Ag NPs usually accumulate low concentrations of Ag in their tissues, as observed in the present study (0.73 and 0.35 µg Ag/g d.w. in autumn and spring, respectively). Bivalves *Scrobicularia plana* waterborne exposed to 10 µg Ag/L of lactate-stabilized Ag NPs for 14 days accumulated around 0.753 µg/g w.w. of Ag in their tissues. When clams were exposed to the same dose of Ag NPs through the diet the concentration of accumulated Ag in

their tissues was lower (~0.4 µg/g w.w.) (Buffet et al., 2013). The same authors observed even a lower accumulation of Ag (~0.25 µg/g w.w.) in clam tissues after waterborne exposure to 10 µg/L of maltose-Ag NPs for 21 days in comparison to their previous work (Buffet et al., 2014). Jimeno-Romero and colleagues (2017) also reported low values of Ag accumulated in *M. galloprovincialis* mussels soft tissues after their exposure to 0.75 µg/L of different sized maltose-stabilized Ag NPs. After 1 day of exposure to 20 nm and 100 nm Ag NPs, around 0.101 ± 0.049 µg/g d.w. and 0.172 ± 0.112 µg/g d.w. of Ag were accumulated in mussels soft tissues, respectively, while accumulation of Ag significantly decreased at day 21 in both cases (Jimeno-Romero et al., 2017).

According to Ag NP dissolution results, most Ag (around 86%) remained in nanoparticulate form during the 24 hours exposure period (Duroudier et al., 2019). Thus, the main part of the Ag measured was in NP form and the presence of Ag NPs was confirmed in the digestive gland of mussels in both seasons, indicating a successful transfer of Ag NPs from microalgae to mussels. Ag NPs were mainly located inside the digestive tubules and in their lumen in both seasons, as previously observed by light and electron microscopy in mussels exposed to Ag NPs via water (Jimeno-Romero et al., 2017). In fact, the digestive gland of bivalves is considered the main organ for accumulation of NPs (Canesi and Corsi, 2016). However, Ag NPs were also localized in mussel gills, which are considered the first barrier to surrounding water and a vulnerable tissue to NP interactions (Corsi et al., 2014; Rocha et al., 2015a).

Even if accumulation of Ag was higher in autumn than in spring, the proteome level response was more marked in spring than in autumn. Season and exposure dependent alterations in the protein expression profile of mussels dietarily exposed to

Ag NPs were shown by the PCA plots. These findings could be related to the sensitivity shown by mussels in some stages of their reproductive cycle such as the reproduction period (Leiniö and Lehtonen., 2005; Almeida et al., 2013) together with the effect of the treatment since other confounding factors such as gender and size of mussels can be discarded.

Based on the *M. galloprovincialis* and Bivalvia databases, out of 60 picked protein spots, 31 protein spots coding for 22 different proteins were identified. Peptide identification using MS spectra can be challenging and limiting in non-model organisms such as mussels, whose genomes are not yet fully sequenced or available to the public (Gomes et al., 2017). The identified sequences were set to KEGG pathway analysis in order to understand which metabolic pathways were affected after the dietary exposure of mussels to Ag NPs. C*hitinase like protein 3, partial* and *glyceraldehyde-3-phosphate dehydrogenase, partial* were involved in the same KEGG pathways that included the *amino sugar and nucleotide sugar metabolism*, *carbon metabolism*, *glycolysis/gluconeogenesis* and the *biosynthesis of amino acids* in both seasons. In mussels, chitinase enzymes are released by the crystalline style during digestion to break down glycoside bonds in chitin present in the exoskeleton of certain zooplankton species and in the cell wall of some microalgae (Birkbeck and McHenery, 1984). Chitinases seem also to have immune functions (Gooday, 1999) and a role in the formation of the shell of molluscs (Weiss and Schönitzer, 2006). Alterations of the chitin metabolism in *Mytilus* spp. have been previously observed under hypoxic condition (Woo et al., 2011) and after the exposure to different contaminants such as metals, polycyclic aromatic hydrocarbons and fluorinated substances, among others (Díaz de Cerio et al., 2013; Dondero et al., 2006; 2010; 2011; Maria et al., 2013; Negri et al., 2013). On the other

hand, Banni and colleagues (2011) proposed that alterations in chitinase-related activities should be considered as a typical response in bivalve molluscs during periods of intense feeding activity as consequence of higher food metabolism. In the present study, mussels were fed daily with microalgae during the experimental period, but *chitinase like protein 3, partial* was differentially expressed in exposed organisms suggesting that the exposure of mussels to Ag NPs through the diet could interfere in the metabolism of food.

Glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) is a glycolytic enzyme involved in glucose degradation and energy yield (Romero-Ruiz et al., 2006). Two protein spots (6325, 7304) of *glyceraldehyde-3-phosphate dehydrogenase, partial* were overexpressed in autumn while the same protein was underexpressed in spring (spot 7202) after the dietary exposure to Ag NPs. In *S. plana* clams inhabiting sites with high metal content, *G3PDH* was significantly more expressed and authors linked such overexpression with a heavy oxidative response (Romero-Ruiz et al., 2006). However, inactivation of *G3PDH* allows glucose metabolism to change temporally to the pentose phosphate pathway, enabling the cell to generate the antioxidant cofactor NADPH (Bernard et al., 2011). Underexpression of *G3PDH* was observed in mussels digestive gland after the exposure to diclofenac (Schmidt et al., 2014; Jaafar et al., 2015) and gemfibrozil (Schmidt et al., 2014), as a response to oxidative stress.

Oxidative stress has been described as one of the major modes of action of engineered metallic NPs, including Ag NPS, in bivalve tissues (reviewed in Baker et al., 2014; Rocha et al., 2015a; Canesi and Corsi, 2016). The burden of reactive oxygen species (ROS) production is largely counteracted by an antioxidant defense system that

among others, includes enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase (Finkel and Holbrook, 2000). Several studies have reported an increased activity of SOD in the digestive gland of mussels exposed to CuO NPs for 15 or 21 days (Gomes et al., 2012; Ruiz et al., 2015), to CdTe quantum dots for 14 days (Rocha et al., 2015b) or to Ag NPs for 15 days (Gomes et al., 2014b) as a response to ROS production. An increase in SOD activity was also described in *S. plana* clams exposed to Ag NPs or ionic Ag for 14 days through the diet (Buffet et al., 2013). In this work, the metabolism of the antioxidant system of peroxisomes was affected in spring due to the under expression of the *superoxide dismutase* protein spot after the exposure of mussels to Ag NPs through the diet. Oxidative stress caused by the inhibition of enzymes such as SOD, catalase and peroxidases, after the exposure to Ag has been linked to the interaction of ionic Ag with the thiol groups found in such enzymes (Lapresta-Fernández et al., 2012). Thus, the under expression of *superoxide dismutase* suggests a situation of oxidative stress in mussels exposed in spring.

Oxidative stress has been observed to induce cytoskeleton disorganization in mussels hemocytes (Gómez-Mendikute et al., 2002; Gómez-Mendikute and Cajaraville, 2003) and clams (Rodríguez-Ortega et al., 2003) and this effect could be linked to increasing tubulin and actin levels (Clarkson et al., 2002). The integrity of actin cytoskeleton of mussel hemocytes can be disrupted by ionic Ag, Ag NPs and bulk Ag (Katsumiti et al., 2015) as well as by Cd and Cu (Gómez-Mendikute and Cajaraville, 2003). At proteome level, actin isoforms were under expressed in gills and digestive gland of mussels exposed to CuO NPs (Gomes et al., 2014a) or after the exposure to ionic Ag (Gomes et al., 2013b). Hu and colleagues (2014) also observed expression changes in four cytoskeletal components, including actin, after the exposure of mussels to CuO NPs.

In this study, two protein spots identified as *actin* were overexpressed only in the digestive gland of mussels exposed to Ag NPs through the diet in spring, which was associated to alterations in phagosomes according to KEGG pathway analysis. Additionally, another cytoskeletal protein not classified by KEGG analysis but significantly under expressed in autumn was *paramyosin*. Previous studies have reported an induction of *paramyosin* after Ag NPs exposure in mussel gills (Gomes et al., 2013b) as well as an under expression of the same protein in mussels digestive gland after the exposure to CuO NPs (Gomes et al., 2014a). The disturbance of structural proteins of cytoskeleton such as *actin* and *paramyosin*, could be mediated by ROS formation (Gomes et al., 2013b; 2014a; Hu et al., 2014).

Further, the pyruvate metabolism, citrate cycle, cysteine and methionine metabolism, glyoxylate and dicarboxylate metabolism were altered in autumn associated to the under expression of the *cytosolic malate dehydrogenase* protein spot after the dietary exposure to Ag NPs. C*ytosolic malate dehydrogenase* is an enzyme that plays an important role in energy metabolism including the malate–aspartate (or NADH) shuttle, the acetate shuttle in lipogenesis and gluconeogenesis (Dahlhoff and Somero, 1993; Fields et al., 2006). Alteration in the expression of this protein has been already reported in juvenile mussels as well as in adult mussels *M. galloprovincialis* after the exposure to arsenate for 48 hours (Yu et al., 2016) and under acute heat stress (Tomanek and Zuzow, 2010), respectively, which was associated with the disturbance of energy metabolism in both cases. Thus, the exposure of mussels to Ag NPs through the diet seem to alter the energy metabolism in mussels digestive gland in autumn.

Moreover, the *nuclear receptor subfamily 1DEF* and *putative C1q domain containing protein MgC1q52* were also significantly altered after the dietary exposure of mussels to Ag NPs, although no KEGG pathway was associated in the analysis. The *nuclear receptor subfamily 1DEF, NR1DEF* was specifically expressed in exposed mussels in autumn. Gomes and co-authors (2013b) reported the overexpression of another nuclear receptor, the *nuclear receptor subfamily 1G,* in mussel gills after the exposure both to Ag NPs and to Ag^{+}, suggesting that both Ag forms are able to interfere with the regulation of gene transcription.

In spring, the *putative C1q domain containing protein MgC1q52* was only expressed in exposed mussels, suggesting that the immune response of mussels was affected by the dietary exposure to Ag NPs. The large family of C1q domain containing proteins participates in several metabolic processes, such as tissue homeostasis, protein activation, immune response, apoptosis, phagocytosis, cell adhesion and cell growth modulation (Gestal et al., 2010; Gerdol et al., 2011; Gerdol and Venier, 2015). Under expression of one spot identified as *C1q domain protein* after the exposure to CuO NPs and Cu^{2+}, as well as the overexpression of the *C1q domain containing protein 60* after the exposure to Ag^{+} were reported in mussel gills (Gomes et al., 2013b; Gomes et al., 2014a). In both cases, authors suggested that the immune capacity of exposed mussels was disrupted since cell-mediated immunity represents a significant target for NPs in bivalve molluscs (Canesi et al., 2012; Katsumiti et al., 2014; 2015; 2018).

5. Conclusions

Dietary exposure to PVP/PEI coated 5 nm Ag NPs significantly altered the proteome of digestive gland in mussels both in autumn and in spring. Treatment and season dependent protein signatures were found. Dietary exposure to Ag NPs altered common metabolic pathways such as amino sugar and nucleotide sugar metabolism, carbon metabolism, glycolysis/gluconeogenesis and the biosynthesis of amino acids in both seasons associated to the differential expression of *chitinase like protein-3, partial* and *glyceraldehyde-3-phosphate dehydrogenase.* The specific protein expression profile observed for each season showed that the dietary exposure to Ag NPs in autumn altered proteins related to the pyruvate metabolism, citrate cycle, cysteine and methionine metabolism and glyoxylate and dicarboxylate metabolism, while in spring proteins involved in the formation of phagosomes and hydrogen peroxide metabolism of peroxisomes were differentially expressed. Other affected processes were the transcriptional regulation (nuc*lear receptor subfamily 1DEF*) in autumn, the immune response (*putative C1q domain containing protein MgC1q52*) in spring and the organization of the cytoskeleton (*actin* and *paramyosin*) in both seasons. For future studies, season and related gamete developmental stage are factors that should be considered for assessing the potential effects caused by engineered NPs in marine bivalves since different protein expression profiles were found for autumn and spring after the dietary exposure to Ag NPs.

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References

Almeida C., Pereira C.G., Gomes T., Cardoso C., Bebianno M.J., Cravo A. 2013. Genotoxicity in two bivalve species from a coastal lagoon in the south of Portugal. Marine Environmental Research 89: 29-38.

Apraiz I., Cajaraville M.P., Cristóbal S. 2009. Peroxisomal proteomics: Biomonitoring in mussels after the Prestige´s oil spill. Marine Pollution Bulletin 58: 1815-1826.

Baker T.J., Tyler C.R., Galloway T.S. 2014. Impacts of metal and metal oxide nanoparticles on marine organisms. Environmental Pollution 186: 257-271.

Banni M., Negri A., Mignone F., Bousetta H., Viarengo A. and Dondero F. 2011. Gene expression rythms in the mussel *Mytilus galloprovincialis* (Lam.) across an annual cycle. PLoS ONE 6 (5): e18904. doi:10.1371/journal.pone.0018904.

Banni M., Attig H., Sforzini S., Oliveri C., Mignone F., Boussetta H., Viarengo A. 2014. Transcriptomic responses to heat stress and nickel in the mussel *Mytilus galloprovincialis.* Aquatic Toxicology 148: 104-112.

Banni M., Sforzini S., Balbi T., Corsi I., Viarengo A., Canesi L. 2016. Combined effects of n-TiO_2 and 2,3,7,8-TCDD in *Mytilus galloprovincialis* digestive gland: A transcriptomic and immunohistochemical study. Environmental Research145: 135-144.

Banni M., Sforzini S., Arlt V.M., Barranguer A., Dallas L.J., Oliveri C., Aminot Y., Pacchioni B., Millino C., Lanfranchi G., Readman J.W., Moore M.N., Viarengo A., Jha A.N. 2017. Assessing the impact of benzo[*a*]pyrene on marine musels: application of a novel targeted low density microarray complementing classical biomarker responses. PLoS ONE 12 (6): e0178460.

Bayne B.L., Widdows J. 1978. The physiological ecology of two populations of *Mytilus edulis* L. Oecologia 37: 137-162.

Bebianno M.J., Gonzalez-Rey M., Gomes T., Mattos J.J., Flores-Nunes F., Bainy A.C.D. 2015. Is gene transcription in mussel gills altered after exposure to Ag nanoparticles? Environmental Science and Pollution Research 22: 17425-17433.

Bernard K.E., Parkes T.L., Merritt T.J.S. 2011. A Model of Oxidative Stress Management: Moderation of Carbohydrate Metabolizing Enzymes in SOD1-Null *Drosophila melanogaster*. PloS ONE 6 (9): e24518.

Bhuvaneshwari M., Thiagarajan V., Nemade P., Chandrasekaran N., Mukherjee A. 2018. Toxicity and trophic transfer of P25 TiO_2 NPs from *Dunaliella salina* to *Artemia salina*: Effect of dietary and waterborne exposure. Environmental Research 160: 39-46.

Birkbeck T.H., McHenery J.G. 1984. Chitinases in the mussel (*Mytilus edulis*, L). Comparative Biochemistry and Physiology, Part B 77: 861–865.

Blum H., Biere H., Gross H.J. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8: 93–99.

Bocchetti R., Regoli F. 2006. Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic Sea. Chemosphere 65: 913-921.

Bouallegui Y., Ben Younes R., Oueslati R., Sheehan D. 2018. Role of endocytotic uptake routes in impacting the ROS-related toxicity of silver nanoparticles to *Mytilus galloprovincialis*: A redox proteomic investigation. Aquatic Toxicology 200: 21-27.

Bradford M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry 72: 248-254.

Brown M., Davies I.M., Moffat C.F., Craft M.A. 2006. Application of SSH and macroarray to investigate altered gene expression in *Mytilus edulis* in response to exposure to benzo[*a*]pyrene. Marine Environmental Research 62: S128-S135.

Buffet P.E., Pan J.F., Poirier L., Amiard- Triquet C., Amiard J.C., Gaudin P., Risso. De Faverney C., Guibbolini M., Gilliland D., Valsami- Jones E. and Mouneyrac C. 2013. Biochemical and behavioural responses of the endobenthic bivalve *Scrobicularia plana* to silver nanoparticles in seawater and microalgal food. Ecotoxicology and Environmental Safety 89: 117-124.

Buffet P.E., Zalouk-Vergnoux A., Châtel A., Berthet B., Métais I., Perrein-Ettajani H., Poirier L., Luna-Acosta A., Thomas-Guyon H., Risso-de Faverney C., Guibbolini M., Gilliland D., Valsami-Jones E., Mouneyrac C. 2014. A marine mesocosm study on the environmental fate of silver nanoparticles and toxicity effects on two endobenthic species: The ragworm *Hediste diversicolor* and the bivalve mollusc *Scrobicularia plana*. Science of the Total Environment 470-471: 1151-1159.

Cancio I., Ibabe A., Cajaraville M.P. 1999. Seasonal variation of peroxisomal enzyme activities and peroxisomal structure in mussels *Mytilus galloprovincialis* and its relationship with the lipid content. Comparative Biochemistry and Physiology, Part C 123: 135-144.

Canesi L., Ciacci C., Fabbri R., Marcomini A., Pojana G. and Gallo G. 2012. Bivalve molluscs as a unique target group for nanoparticle toxicity. Marine Environmental Research 76: 16-21.

Canesi L. and Corsi I. 2016. Effects of nanomaterials on marine invertebrates. Science of the Total Environment 565: 933-940.

Clarkson M.R., Murphy M., Gupta S., Lambe T., Mackenzie H. S., Godson C., Martin F., Brady H.R. 2002. High glucose-altered gene expression in mesangial cells. Actin regulatory protein gene expression is triggered by oxidative stress and cytoskeletal disassembly. The Journal of Biological Chemistry 277: 9707-9712.

Corsi I., Cherr G.N., Lenihan H.S., Labille J., Hassellov M., Canesi L., Dondero F., Frenzilli G., Hristozov D., Puntes V., Della Torre C., Pinsino A., Libralato G., Marcomini A., Sabbioni E., Matranga V. 2014. Common Strategies and Technologies for the Ecosafety

Assessment and Design of Nanomaterials Entering the Marine Environment. ACS Nano 8: 9694-9709.

Dahlhoff E., Somero G.N. 1993. Kinetic and structural adaptations of cytoplasmic malate dehydrogenases of eastern Pacific abalone (genus *Haliotis*) from different thermal habitats: Biochemical correlates of biogeographical patterning. The Journal of Experimental Biology 185: 137-150.

Daskalakis K.D. 1996. Variability of metal concentrations in oyster tissue and implications to biomonitoring. Marine Pollution Bulletin 32: 794-801.

Díaz de Cerio O., Hands E., Humble J., Cajaraville M.P., Craft J.A., Cancio I. 2013. Construction and characterization of a forward subtracted library of blue mussels *Mytilus edulis* for the identification of gene transcription signatures and biomarkers of styrene exposure. Marine Pollution Bulletin 71: 230-239.

Dondero F., Piacentini L., Marsano F., Rebelo M., Vergani L., Venier P., Viarengo A. 2006. Gene transcription profiling in pollutant exposed mussels (*Mytilus* spp.) using a new low-density oligonucleotide microarray. Gene 376: 24-36.

Dondero F., Negri A., Boatti L., Marsano F., Mignone F., Viarengo A. 2010. Transcriptomic and proteomic effects of a neonicotinoid insecticide mixture in the marine mussel (*Mytilus galloprovincialis*, Lam.). Science of the Total Environment 408: 3775-3786.

Dondero F., Banni M., Negri A., Boatti L., Dagnino A., Viarengo A. 2011. Interactions of a pesticide/heavy metal mixture in marine bivalves: a transcriptomic assessment. BMC Genomics 2011; 12: 195-212.

Dowling V.A., Sheehan D. 2006. Proteomics as a route to identification of toxicity targets in environmental toxicology. Proteomics 6: 5597-5604.

Duroudier N., Katsumiti A., Mikolaczyk M., Schäfer J., Bilbao E., Cajaraville M.P. 2019. Dietary exposure of mussels to PVP/PEI coated Ag nanoparticles causes Ag accumulation in adults and abnormal embryo development in their offspring. Science of the Total Environment 655: 48-60.

Fabrega J., Luoma S.N., Tyler C.R., Galloway T.S., Lead J.R. 2011. Silver nanoparticles: Behaviour and effects in the aquatic environment. Environment International 37: 517-531.

Fields P.A., Rudomin E.L., Somero G.N. 2006. Temperature sensitivities of cytosolic malate dehydrogenases from native and invasive species of marine mussels (genus *Mytilus*): sequence–function linkages and correlations with biogeographic distribution. The Journal of Experimental Biology 209: 656-667.

Finkel T., Holbrook N.J. 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408: 239-247.

Fischer H. 1983. Shell weight as an independent variable in relation to cadmium content of molluscs. Marine Ecology Progress Series 12: 59-75.

Gambardella C., Costa E., Piazza V., Fabbrocini A., Magi E., Faimali M., Garaventa F. 2015. Effect of silver nanoparticles on marine organisms belonging to different trophic levels. Marine Environmental Research 111: 41-49.

Gamble M. and Wilson I. 2002. The hematoxylins and eosin. In: Brancfort J.D., Gamble M. (Eds.), Theory and Practice of Histological Techniques. Churchill Livingstone- Elsevier Science Ltd., London, UK. pp. 125.

Gerdol M., Manfrin C., De Moro G., Figueras A., Novoa B., Venier P., Pallavicini A. 2011. The C1q domain containing proteins of the Mediterranean mussel *Mytilus galloprovincialis*: A widespread and diverse family of immune-related molecules. Developmental & Comparative Immunology 35: 635-643.

Gerdol M., Venier P. 2015. An updated molecular basis for mussel immunity. Fish and Shellfish Immunology 46: 17-38.

Gestal C., Pallavicini A., Venier P., Novoa B., Figueras A. 2010. MgC1q, a novel C1q-domain-containing protein involved in the immune response of *Mytilus galloprovincialis*. Developmental & Comparative Immunology 34: 926–934.

Giese B., Klaessig F., Park B., Kaegi R., Steinfeldt M., Wigger H., von Gleich A., Gottschalk F. 2018. Risks, release and concentrations of engineered nanomaterial in the environment. Scientific Reports 8: doi: 10.1038/s41598-018-19275-4.

Gomes T., Pereira C.G., Cardoso C., Pinheiro J.P., Ibon Cancio I., Bebianno M.J. 2012. Accumulation and toxicity of copper oxide nanoparticles in the digestive gland of *Mytilus galloprovincialis.* Aquatic Toxicology 118-119: 72-79.

Gomes T., Araújo O., Pereira R., Almeida A.C., Cravo A., Bebianno M.J. 2013a. Genotoxicity of copper oxide and silver nanoparticles in the mussel *Mytilus galloprovincialis*. Marine Environmental Research 84: 51-59.

Gomes T., Pereira C.G., Cardoso C., Bebianno M.J. 2013b. Differential protein expression in mussels *Mytilus galloprovincialis* exposed to nano and ionic Ag. Aquatic Toxicology 136- 137: 79-90.

Gomes T., Chora S., Pereira C.G., Cardoso C., Bebianno M.J. 2014a. Proteomic response of mussels *Mytilus galloprovincialis* exposed to CuO NPs and Cu^{2+}: An exploratory biomarker discovery. Aquatic Toxicology 155: 327-336.

Gomes T., Pereira C.G., Cardoso C., Sousa V.S., Ribau Teixeira M., Pinheiro J.P., Bebianno M.J. 2014b. Effects of silver nanoparticles exposure in the mussel *Mytilus galloprovincialis*. Marine Environmental Research 101: 208-214.

Gomes T., Albergamo A., Costa R., Mondello L., Dugo G. 2017. Potential Use of Proteomics in Shellfish Aquaculture: from Assessment of Environmental Toxicity to Evaluation of Seafood Quality and Safety. Current Organic Chemistry 21: 1-24.

Gómez-Mendikute A., Etxeberria A., Olabarrieta I., Cajaraville M.P. 2002. Oxygen radicals production and actin filament disruption in bivalve haemocytes treated with benzo(a)pyrene. Marine Environmental Research 54: 431-436.

Gómez-Mendikute A., Cajaraville M.P. 2003. Comparative effects of cadmium, copper, paraquat and benzo[a]pyrene on the actin cytoskeleton and production of reactive oxygen species (ROS) in mussel haemocytes. Toxicology In Vitro 17: 539-546.

Gooday G.W. 1999. Chitin and chitinases. Edited by: P. Jolles and R.A.A. Muzzarelli Birkhäuser Verlag. p.157.

Gottschalk F., Sonderer T., Scholz R.W., Nowack B. 2009. Modeled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag, CNT, fullerenes) for different regions. Environmental Science and Technology 43: 9216-9222.

Griffit R.J., Hyndman K., Denslow N.D., Barber D.S. 2009. Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanoparticles. Toxicological Science 107: 404-415.

Griffit R.J., Lavelle C.M., Kane A.S., Denslow N.D., Barber D.S. 2013. Chronic nanoparticulate silver exposure results in tissue accumulation and transcriptomic changes in zebrafish. Aquatic Toxicology 130-131: 192-200.

Hu W., Culloty S., Darmody G., Lynch S., Davenport J., Ramirez-Garcia S., Dawson K.A., Lynch I., Blasco J., Sheehan D. 2014. Toxicity of copper oxide nanoparticles in the blue mussel, *Mytilus edulis*: a redox proteomic investigation. Chemosphere 108: 289-299.

Jaafar S.N.T., Coelho A.V., Sheehan D. 2015. Redox proteomic analysis of *Mytilus edulis* gills: effects of the pharmaceutical diclofenac on a non-target organism. Drug Testing and Analysis 7: 957-966.

Ji C., Li F., Wang Q., Zhao J., Sun Z., Wu H. 2016. An integrated proteomic and metabolomics study on the gender specific responses of mussels *Mytilus galloprovincialis* to tetrabromobisphenol A (TBBPA). Chemosphere 144: 527-539.

Jimeno-Romero A., Bilbao E., Izagirre U., Cajaraville M.P., Marigómez I., Soto M. 2017. Digestive cell lysosomes as main targets for Ag accumulation and toxicity in marine mussels, *Mytilus galloprovincialis*, exposed to maltose- stabilized Ag nanoparticles of different sizes. Nanotoxicology 11: 168-183.

Kanehisa M., Furumichi M., Tanabe M., Sato Y., Morishima K. 2017. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Research 45: D353-D361.

Katsumiti A., Gilliland D., Arostegui I., Cajaraville M.P. 2014. Cytotoxicity and cellular mechanisms involved in the toxicity of CdS quantum dots in hemocytes and gill cells of the mussel *Mytilus galloprovincialis*. Aquatic Toxicology 153: 39-52.

Katsumiti A., Gilliland D., Arostegui I., Cajaraville M.P. 2015. Mechanisms of toxicity of Ag nanoparticles in comparison to bulk and ionic Ag on mussel hemocytes and gill cells. PLoS ONE 10 (6): e0129039. doi:10.1371/journal.pone.0129039.

Katsumiti A., Thorley A.J., Arostegui I., Reip P., Valsami-Jones E., Tetley T.D., Cajaraville M.P. 2018. Cytotoxicity and cellular mechanisms of toxicity of CuO NPs in mussel cells *in vitro* and comparative sensitivity with human cells. Toxicology In Vitro 48: 146-158.

Khan F.R., Syberg K., Shashoua Y., Bury N.R. 2015. Influence of polyethylene microplastic beads on the uptake and localization of silver in zebrafish (*Danio rerio*). Environmental Pollution 206: 73-79.

Kim Y., Ashton-Alcox K.A., Powell E.N. 2006. Gonadal analysis. NOAA histological techniques for marine bivalve mollusks: update. NOAA Technical Memories NOS NCCOS 27, Silver Spring (USA). pp. 1–18.

Lacave J.M., Fanjul A., Bilbao E., Gutierrez N., Barrio I., Arostegui I., Cajaraville M.P., Orbea A. 2017. Acute toxicity, bioaccumulation and effects of dietary transfer of silver from brine shrimp exposed to PVP/PEI coated silver nanoparticles to zebrafish. Comparative Biochemistry and Physiology, Part C 199: 69-80.

Lacave J.M., Vicario-Parés U., Bilbao E., Gilliland D., Mura F., Dini L., Cajaraville M.P., Orbea A. 2018. Waterborne exposure of adult zebrafish to silver nanoparticles and to ionic silver results in differential silver accumulation and effects at cellular and molecular levels. Science of the Total Environment 642: 1209-1220.

Lanceleur L., Schäfer J., Chiffoleau J.F., Blanc G., Auger D., Renault S., Baudrimont M., Audry S. 2011. Long-term records of cadmium and silver contamination in sediments and oysters from the Gironde fluvial–estuarine continuum – Evidence of changing silver sources. Chemosphere 85: 1299-1305.

Lapresta-Fernández A., Fernández A., Blasco J. 2012. Nanoecotoxicity effects of engineered silver and gold nanoparticles in aquatic organisms. Trends in Analytical Chemistry 32: 40-59.

Larguinho M., Correia D., Diniz M.S., Baptista P.V. 2014. Evidence of one-way flow bioaccumulation of gold nanoparticles across two trophic levels. Journal of Nanoparticle Research 16: 2549-2560.

Leiniö S., Lehntonen K. 2005. Seasonal variability in biomarkers in the bivalves *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea. Comparative Biochemistry and Physiology, Part C 140: 408-421.

Livingstone D.R., Farrar S.V. 1984. Tissue and subcellular distribution of enzyme activities of mixed function oxygenase and benzo[a]pyrene metabolism in the common mussel *Mytilus edulis*. Science of the Total Environment 39: 209-235.

Luoma S.N., Rainbow P.S. 2005. Why Is Metal Bioaccumulation So Variable? Biodynamics as a Unifying Concept. Environmental Science of Technology 39: 1921-1931.

Manzo S., Schiavo S., Oliviero M., Toscano A., Ciaravolo M., Cirino P. 2017. Immune and reproductive system impairment in adult sea urchin exposed to nanosized ZnO via food. Science of the Total Environment 599-600: 9-13.

Maria V.L., Gomes T., Barreira L., Bebianno M.J. 2013. Impact of benzo(a)pyrene, Cu and their mixture on the proteomic response of *Mytilus galloprovincialis*. Aquatic Toxicology 144-145: 284-295.

McCarthy M., Carroll D.L., Ringwood A.H. 2013. Tissue specific response of oysters, *Crassostrea virginica*, to silver nanoparticles. Aquatic Toxicology 138-139: 123-128.

McGuillicuddy E., Murray I., Kavanagh S., Morrison L., Fogarty A., Cormican M., Dockery P., Prendergast M., Rowan N., Morris D. 2017. Silver nanoparticles in the environment: Sources, detection and ecotoxicology. Science of the Total Environment 575: 231-246.

Mehennaoui K., Cambier S., Serchi T., Ziebel J., Lentzen E., Valle N., Guérold F., Thomann J.S., Giamberini L., Gutleb A.C. 2018. Do the pristine physico-chemical properties of silver and gold nanoparticles influence uptake and molecular effects on *Gammarus fossarum* (Crustacea Amphipoda)? Science of the Total Environment 643: 1200-1215.

Moore M.N. 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environment International 32: 967-976.

Mubiana V.K., Qadah D., Meys J., Blust R. 2005. Temporal and spatial trends in heavy metal concentrations in the marine mussel *Mytilus edulis* from the Western Scheldt estuary (The Netherlands). Hydrobiologia 540: 169-180.

Navarro E., Piccapietra F., Wagner B., Marconi F., Kaegi R., Odzak N., Sigg L., Behra R. 2008. Toxicity of silver nanoparticles to *Chlamydomonas reinhardtii*. Environmental Science of Technology 42: 8959-8964.

Negri A., Oliveri C., Sforzini S., Mignione F., Viarengo A., Banni M. 2013. Transcriptional Response of the Mussel *Mytilus galloprovincialis* (Lam.) following Exposure to Heat Stress and Copper. PLoS ONE 2013; 8(6): e66802.

Orbea A., González-Soto N., Lacave J.M., Barrio I., Cajaraville M.P. 2017. Developmental and reproductive toxicity of PVP/PEI-coated silver nanoparticles to zebrafish. Comparative Biochemistry and Physiology, Part C 199: 59-68.

Páez-Osuna F., Frías-Espericueta M.G., Osuna-López J.I. 1995. Trace Metal Concentrations in Relation to Season and Gonadal Maturation in the Oyster *Crassostrea iridescens.* Marine Environmental Research 40: 19-31.

Pakrashi S., Tan C., Wang W.X. 2017. Bioaccumulation-based silver nanoparticle toxicity in *Daphnia magna* and maternal impacts. Environmental Toxicology and Chemistry 36: 3359-3366.

Regoli F., Orlando E. 1994. Seasonal variation of trace metal concentrations in the digestive gland of the mediterranean mussel *Mytilus galloprovincialis*: Comparison between a polluted and a non-polluted site. Archives of Environmental Contamination and Toxicology 27: 36-43.

Ribeiro F., Gallego-Urrea J.A., Jurkschat K., Crossley A., Hassellöv M., Taylor C., Soares A.M.V.M., Loureiro S. 2014. Silver nanoparticles and silver nitrate induce high toxicity to

Pseudokirchneriella subcapitata, *Daphnia magna* and *Danio rerio*. Science of the Total Environment 466-467: 232-241.

Ringwood A.H., McCarthy M., Bates T.C., Carrol D.L. 2010. The effects of silver nanoparticles on oyster embryos. Marine Environmental Research 69: S49-S51.

Riva C., Binelli A., Rusconi F., Colombo G., Pedriali A., Zippel R., Provini A. 2011. A proteomic study using zebra mussels (*D. polymorpha*) exposed to benzo[*a*]pyrene: The role of gender and exposure concentrations. Aquatic Toxicology 104: 14-22.

Rocha T.L., Gomes T., Sousa V.S., Mestre N.C., Bebianno M.J. 2015a. Ecotoxicological impact of engineered nanomaterials in bivalve molluscs: An overview. Marine Environmental Research111: 74-88.

Rocha T.L., Gomes T., Mestre N.C., Cardoso C., Bebianno M.J. 2015b. Tissue specific responses to cadmium-based quantum dots in the marine mussel *Mytilus galloprovincialis*. Aquatic Toxicology 169: 10-18.

Rodríguez-Ortega M.J., Grøsvik B.E., Rodríguez-Ariza A., Goksøyr A., López-Barea J. 2003. Changes in protein expression profiles in bivalve molluscs (*Chamaelea gallina*) exposed to four model environmental pollutants. Proteomics 3: 1535-1543.

Romero-Ruiz A., Carrascal M., Alhama J., Gómez-Ariza J.L., Abian J., López-Barea J. 2006. Utility of proteomics to assess pollutant response of clams from the Doñana bank of Guadalquivir Estuary (SW Spain). Proteomics 6: S245-S255.

Ruiz P., Katsumiti A., Nieto J.A., Bori J., Reip P., Orbea A. and Cajaraville M.P. 2015. Short-term effects on antioxidant enzymes and long-term genotoxic and carcinogenic

potential of CuO nanoparticles in mussels. Marine Environmental Research 111: 107-120.

Schiavo S., Duroudier N., Bilbao E., Mikolaczyk M., Schäfer J., Cajaraville M.P., Manzo S. 2017. Effects of PVP/PEI coated and uncoated silver NPs and PVP/PEI coating agent on three species of marine microalgae. Science of the Total Environment 577: 45-53.

Schmidt W., Rainville L.C., McEneff G., Sheehan D., Quinn B. 2014. A proteomic evaluation of the effects of the pharmaceuticals diclofenac and gemfibrozil on marine mussels (Mytilus spp.): evidence for chronic sublethal effects on stress-response proteins. Drug Testing and Analysis 6: 210-219.

Sendra M., Yeste M.P., Gatica J.M., Moreno-Garrido I., Blasco J. 2017a. Direct and indirect effects of silver nanoparticles on freshwater and marine microalgae (*Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum*). Chemosphere 179: 279-289.

Sendra M., Blasco J., Araújo C.V.M. 2017b. Is the cell wall of marine phytoplankton a protective barrier or a nanoparticle interaction site? Toxicological responses of *Chlorella autotrophica* and *Dunaliella salina* to Ag and CeO_2 nanoparticles. Ecological Indicators. http://dx.doi.org/10.1016/j.ecolind.2017.08.050.

Shevchenko A., Tomas H., Havlis J., Olsen J.V., Mann M. 2007. In–gel digestion for mass spectrometric characterization of proteins and proteomes. Nature Protocols 1: 2856-2860.

Solé M., Porte C., Albaigés J. 1995. Seasonal variation in the mixed-funcion oxigenase system and antioxidant enzymes of the mussel *Mytilus galloprovincialis*. Environmental Toxicology and Chemistry 14: 157-164.

Soto M., Kortabitarte M., Marigómez I. 1995. Bioavailable heavy metals in estuarine waters as assessed by metal/shell-weight indices in sentinel mussels *Mytilus galloprovincialis.* Marine Ecology Progress Series 125: 127-136.

Tangaa S.R., Selck H., Winther-Nielsen M., Khan F.R. 2016. Trophic transfer of metal-based nanoparticles in aquatic environments: a review and recommendations for future research focus. Environmental Science: Nano 3: 966-981.

Tedesco S., Doyle H., Redmond G., Sheehan D. 2008. Gold nanoparticles and oxidative stress in *Mytilus edulis*. Marine Environmental Research 66: 131-133.

Tedesco S., Doyle H., Blasco J., Redmond G., Sheehan D. 2010a. Exposure of the blue mussel, *Mytilus edulis*, to gold nanoparticles and the pro-oxidant menadione. Comparative Biochemistry and Physiology, Part C 151: 167-174.

Tedesco S., Doyle H., Blasco J., Redmond G., Sheehan D. 2010b. Oxidative stress and toxicity of gold nanoparticles in *Mytilus edulis.* Aquatic Toxicology 100: 178-186.

Tiede K., Hassellöv M., Breitbarth E., Chaudhry Q., Boxall A.B.A. 2009. Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles. Journal of Chromatography A 1216: 503-509.

Tomanek L., Zuzow M.J. 2010. The proteomic response of the mussel congeners *Mytilus galloprovincialis* and *M. trossulus* to acute heat stress: implications for thermal

tolerance limits and metabolic costs of thermal stress. The Journal of Experimental Biology 213: 3559-3574.

UniProt Consortium. UniProt: the universal protein knowledgebase. 2017. Nucleic Acids Research 45: D158-D169.

Vance M.E., Kuiken T., Vejerano E.P., McGinnis S.P., Hochella M. F. Jr., Rejeski D., Hull M.S. 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. Beilstein Journal of Nanotechnology 6: 1769-1780.

Wang J., Wang W.X. 2014. Low bioavailability of silver nanoparticles presents trophic toxicity to marine medaka (*Orzyias melastigma*). Environmental Science of Technology 48: 8152-8161.

Wang Z., Yin L., Zhao J., Xing B. 2016. Trophic transfer and accumulation of TiO_2 nanoparticles from clamworm (*Perinereis aibuhitensis*) to juvenile turbot (*Scophthalmus maximus*) along a marine benthic food chain. Water Research 95: 250-259.

Weiss I.M., Schönitzer V. 2006. The distribution of chitin in larval shells of the bivalve mollusk *Mytilus galloprovincialis.* Journal of Structural Biology 153: 264-277.

Woo S., Jeon H.Y., Kim S.R., Yum S. 2011. Differentially displayed genes with oxygen depletion stress and transcriptional responses in the marine mussel, *Mytilus galloprovincialis*. Comparative Biochemistry and Physiology, Part D 6: 348-356.

Yu D., Ji C., Zhao J., Wu H. 2016. Proteomic and metabolomic analysis on the toxicological effects of As (III) and As (V) in juvenile mussel *Mytilus galloprovincialis.* Chemosphere 150: 194-201.

Zhang C., Hu Z., Deng B. 2016. Silver nanoparticles in aquatic environments: Physicochemical behavior and antimicrobial mechanisms. Water Research 88: 403-427.

Table 1. Bioaccumulation of Ag in mussels soft tissues after 21 days of dietary exposure to 10 µg /L of Ag NPs. Significant differences were set based on the 2-way ANOVA followed by the Tukey multiple comparison test. Asterisks show significant differences with respect to controls ($p<0,05$). Letters represent significant differences among seasons. Values are given as means ± S.D. Mean values belong to 4 pools of 5 individuals each.

	µg Ag/g d.w.	
	AUTUMN	SPRING
Control	0.09 ± 0.02 [a]	0.04 ± 0.003 [a]
10 µg /L Ag NPs	0.73 ± 0.069 *, [b]	0.35 ± 0.044 *, [c]

Table 2. Differentially expressed protein spots in autumn identified by MALDI–TOF. Spots present only in control samples, only in exposed samples and those common to control and exposed samples are shown.

AUTUMN	SPOT	NAME	ACCESSION NUMBER	SPECIES	p- value (<0.05) [a]	FOLD CHANGE	BIOLOGICAL PROCESS [b]	KEGG identifier	KEGG PATHWAY [c]
CONTROL	6111	*putative transcription factor PML*	EKC33587.1	*Crassostrea gigas*	0.002	-	localization, transport	crg:109618835	-
	8105	*LKD-rich protein-1*	AKS48185	*Mytilus galloprovincialis*	0.0034	-	-	crg:105344531	-
EXPOSED	7524	*chitinase-like protein-3, partial*	AIK22450	*Mytilus galloprovincialis*	0.012	-	chitin catabolic process, carbohydrate metabolic process	crg:105331570	metabolic pathways, amino sugar and nucleotide sugar metabolism
	4430	*PREDICTED: vinculin-like isoform X7*	XP_011426098	*Crassostrea gigas*	0.00085	-	cell adhesion	crg:105327367	-
	4407	*nuclear receptor subfamily 1 DEF, partial*	ABU89803	*Mytilus galloprovincialis*	0.027	-	regulation of transcription, DNA-templated; intracellular receptor signaling pathway; steroid hormone mediated signaling pathway	crg:105321080	-
COMMON	6325	*glyceraldehyde-3-phosphate dehydrogenase, partial*	AIK22450	*Mytilus galloprovincialis*	0.022	11.81	glucose metabolic process, glycolytic process, oxidation-reduction process	crg:105340512	metabolic pathways, carbon metabolism, glycolysis/ gluconeogenesis, biosynthesis of aminoacids
	7304				0.005	4.52			
	8555	*chitinase-like protein-3, partial*	AIK22450	*Mytilus galloprovincialis*	0.0046	7.53	chitin catabolic process, carbohydrate metabolic process	crg:105345920	metabolic pathways, amino sugar and nucleotide sugar metabolism
	8556				0.03	4.81			
	8558				0.05	4.73			
	8403	*paramyosin*	BAA36517	*Mytilus galloprovincialis*	0.029	-6.31	metabolic process	crg:105329634	-
	5312	*cytosolic malate dehydrogenase*	AAZ79367	*Mytilus galloprovincialis*	0.022	-5.8	carbohydrate metabolic process, tricarboxylic acid cycle, malate metabolic process, carboxylic acid metabolic process, oxidation- reduction process	crg:105343888	metabolic pathways, carbon metabolism, pyruvate metabolism, citrate cycle, cysteine and methionine metabolism, glyoxylate and dicarboxylate metabolism
	5317	*matrix metalloproteinase-19*	EKC37558	*Crassostrea gigas*	0.056	-10.67	proteolysis	crg:105338896	-
	5409	*hypothetical protein CGI_10016468*	EKC33615	*Crassostrea gigas*	0.024	-7.5	uncharacterized protein	crg:105330727	-
	5415	*hypothetical protein CGI_10001770*	EKC26982	*Crassostrea gigas*	0.049	-6.87	uncharacterized protein	crg:105328562	-
	5401	PREDICTED: *inorganic pyrophosphatase-like isoform X2*	XP_011429567	*Crassostrea gigas*	0.054	-7.17	phosphate-containing compound metabolic process	crg:105329817	-

[a] Significance value of the matches obtained using MASCOT software for the protein identification using *M. galloprovincialis* or Bivalvia databases.

[b] Biological process in which proteins are involved according to Uniprot database.

[c] Metabolic pathways in which proteins are involved based on KEGG analysis.

Table 3. Differentially expressed protein spots in spring identified by MALDI–TOF. Spots present only in control samples, only in exposed samples and those common to control and exposed samples are shown.

SPRING	SPOT	NAME	ACCESSION NUMBER	SPECIES	p- value (<0.05) [a]	FOLD CHANGE	BIOLOGICAL PROCESS [b]	KEGG identifier	KEGG PATHWAY [c]
CONTROL	8602	*PREDICTED: centrosomal protein of 152 kDa-like*	XP_011436998	*Crassostrea gigas*	0.0063	-	centriole replication, de novo centriole assembly	crg:105335033	-
	5413	*PREDICTED: lipoxygenase homology domain-containing protein 1-like isoform X3*	XP_011448787	*Crassostrea gigas*	0.065	-	-	crg:105343219	-
EXPOSED	1410	*putative C1q domain containing protein MgC1q52*	CBX41701	*Mytilus galloprovincialis*	0.026	-	immune response	-	-
	3420	*actin*	AAD40314	*Mytilus galloprovincialis*	$1.7\ e^{-5}$	-	-	crg:105326698	formation of phagosomes
	2419				0.0056	-		crg:105335713	
	1506	*PREDICTED: centrosomal protein of 152 kDa-like*	XP_011436998	*Crassostrea gigas*	0.026	-	centriole replication, de novo centriole assembly	crg:105335033	-
	7301	*Selenoprotein H*	EKC30285	*Crassostrea gigas*	0.045	-	-	crg:105317517	-
COMMON	8206	*putative transcription factor PML*	EKC33587	*Crassostrea gigas*	0.021	7.18	-	crg:109618835	-
	5201				0.0085	5.17			
	5423	*connector enhancer of kinase suppressor of ras 2*	XP_011420299	*Crassostrea gigas*	0.044	24.85	phosphorylation	crg:105323047	-
	5601	*chitinase-like protein-3, partial*	AKS48199	*Mytilus galloprovincialis*	0.029	-5.57	chitin catabolic process, carbohydrate metabolic process	crg:105345920	metabolic pathways, amino sugar and nucleotide sugar metabolism
	5110	*superoxide dismutase*	CAQ68509	*Mytilus galloprovincialis*	0.0045	-5.3	superoxide metabolic process, removal of superoxide radicals, oxidation- reduction process	crg:105327946	hydrogen peroxide metabolism in peroxisomes
	7202	*glyceraldehyde-3-phosphate dehydrogenase, partial*	AIK22450	*Mytilus galloprovincialis*	0.015	-5.3	glucose metabolic process, glycolytic process, oxidation-reduction process	crg:105340512	metabolic pathways, carbon metabolism, glycolysis/ gluconeogenesis, biosynthesis of aminoacids
	5307	*PREDICTED: GRIP and coiled-coil domain-containing protein 2-like*	XP_011448370	*Crassostrea gigas*	0.0076	-12.12	protein targeting to Golgi	crg:105342939	-
	8220	*3-hydroxybutyryl-CoA dehydrogenase*	EKC26490	*Crassostrea gigas*	0.063	-6.05	fatty acid metabolic process, oxidation- reduction process	crg:105329039	-

[a] Significance value of the matches obtained using MASCOT software for the protein identification using *M. galloprovincialis* or Bivalvia databases.

[b] Biological process in which proteins are involved according to Uniprot database.

[c] Metabolic pathways in which proteins are involved based on KEGG analysis.

LEGEND OF FIGURES

Figure 1. Hyperspectral images showing the presence of PVP/PEI coated 5 nm Ag NPs in the digestive gland tubule cells (A, B) and gills (C, D) of mussels dietarily exposed to 10 µg Ag/L of Ag NPs for 21 days in autumn (A, C) and spring (B, D). Micrographs in the left correspond to tissue sections observed under CytoViva® hyperspectral imaging system and micrographs in the right show the localization of Ag NPs. Red and white arrows indicate the presence of Ag NPs.

Figure 2. Representative micrographs showing the developmental stage of gametes of mussels belonging to A) non-exposed females in autumn, B) exposed females in autumn, C) non-exposed females in spring and D) exposed females in spring.

Figure 3. Representative 2-DE gels showing the expression profile of proteins observed in the digestive gland of mussels *M. galloprovincialis* in A) autumn and in B) spring. Gels on the left belong to non-exposed mussels and gels on the right correspond to mussels dietarily exposed to 10 µg /L of Ag NPs for 21 days. pH gradient is represented in the x-axis and molecular weight (Mw) in kDa in the y-axis. Blue circles point out protein spots specific for each treatment that were picked for identification. Over and underexpressed protein spots picked for identification are pointed out by green and red rhombus, respectively.

Figure 4. Venn diagrams showing the number of common and differentially expressed protein spots in A) autumn, B) spring C) controls and D) dietarily exposed mussels.

Figure 5. PCA plot based on the significantly expressed protein spots in all the treatments. PC1 was selected to be shown in both plots as it explained the highest

percentage of variability. PC1= 36,8%; PC2= 34,6%; PC3= 28,4%. Each symbol point represents one gel (n=4 gels per treatment) and legend at the bottom of the graph shows symbols corresponding to each experimental group.

Fig. 1

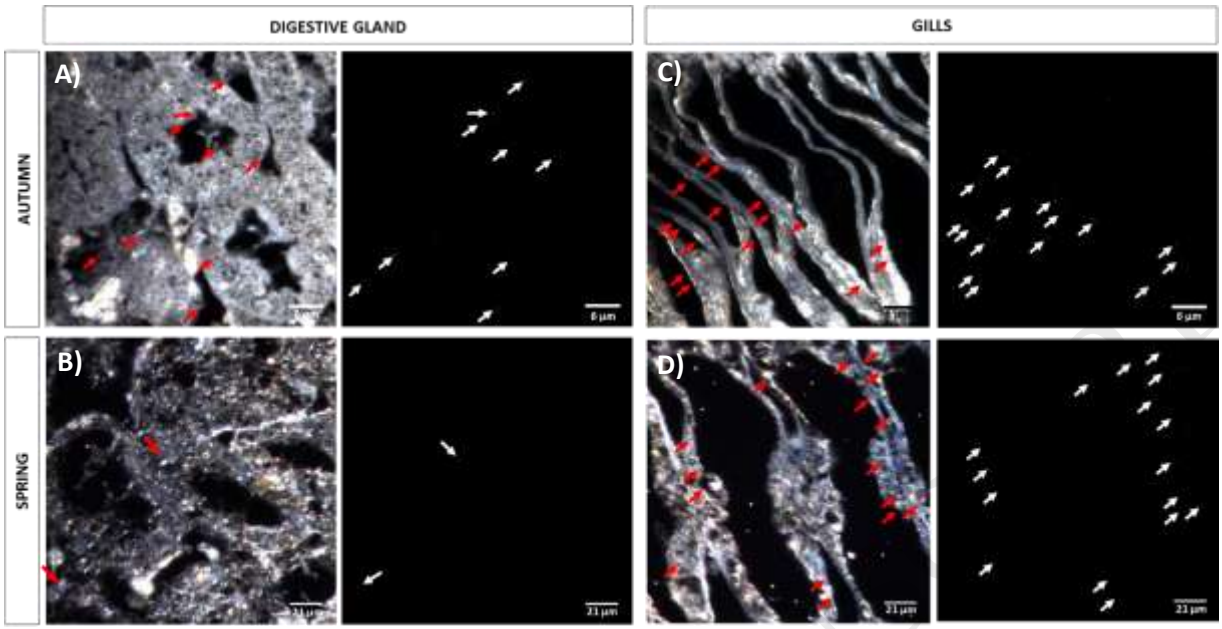

Fig. 2

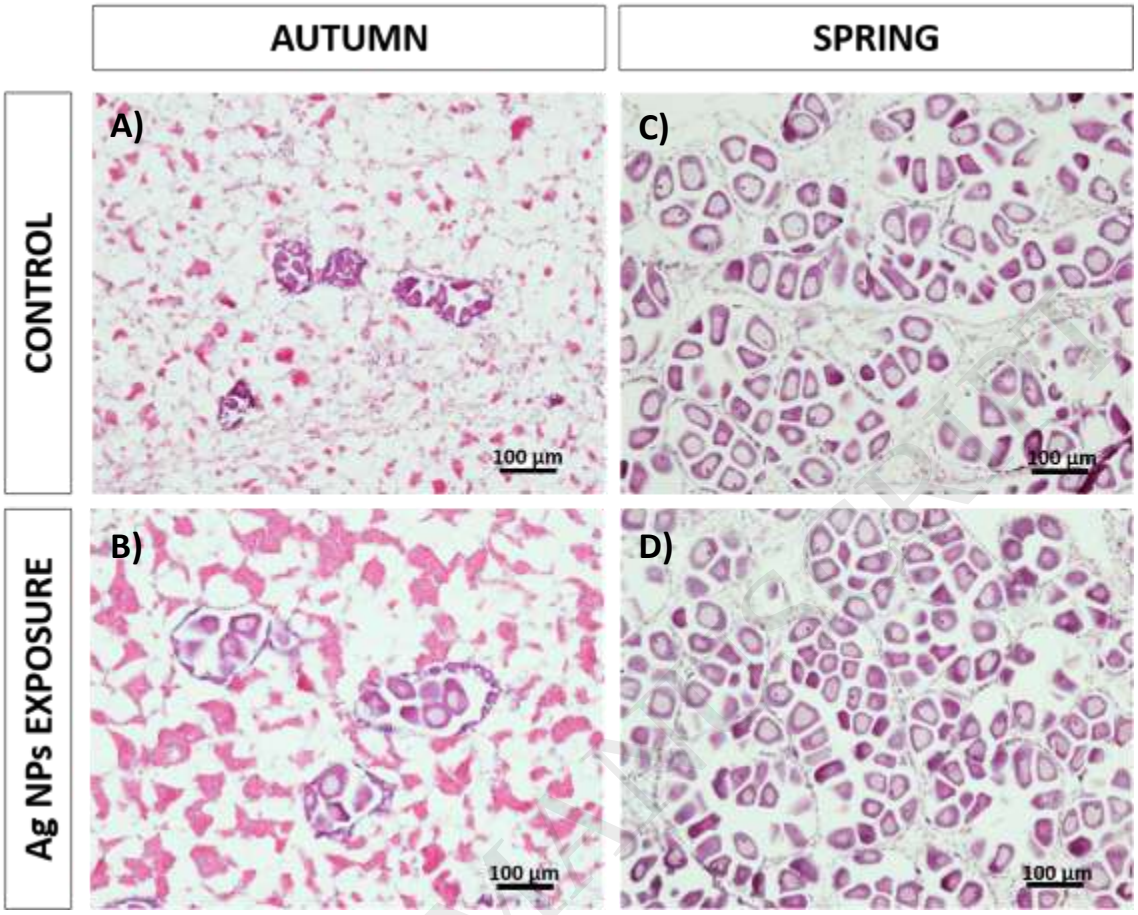

Fig. 3

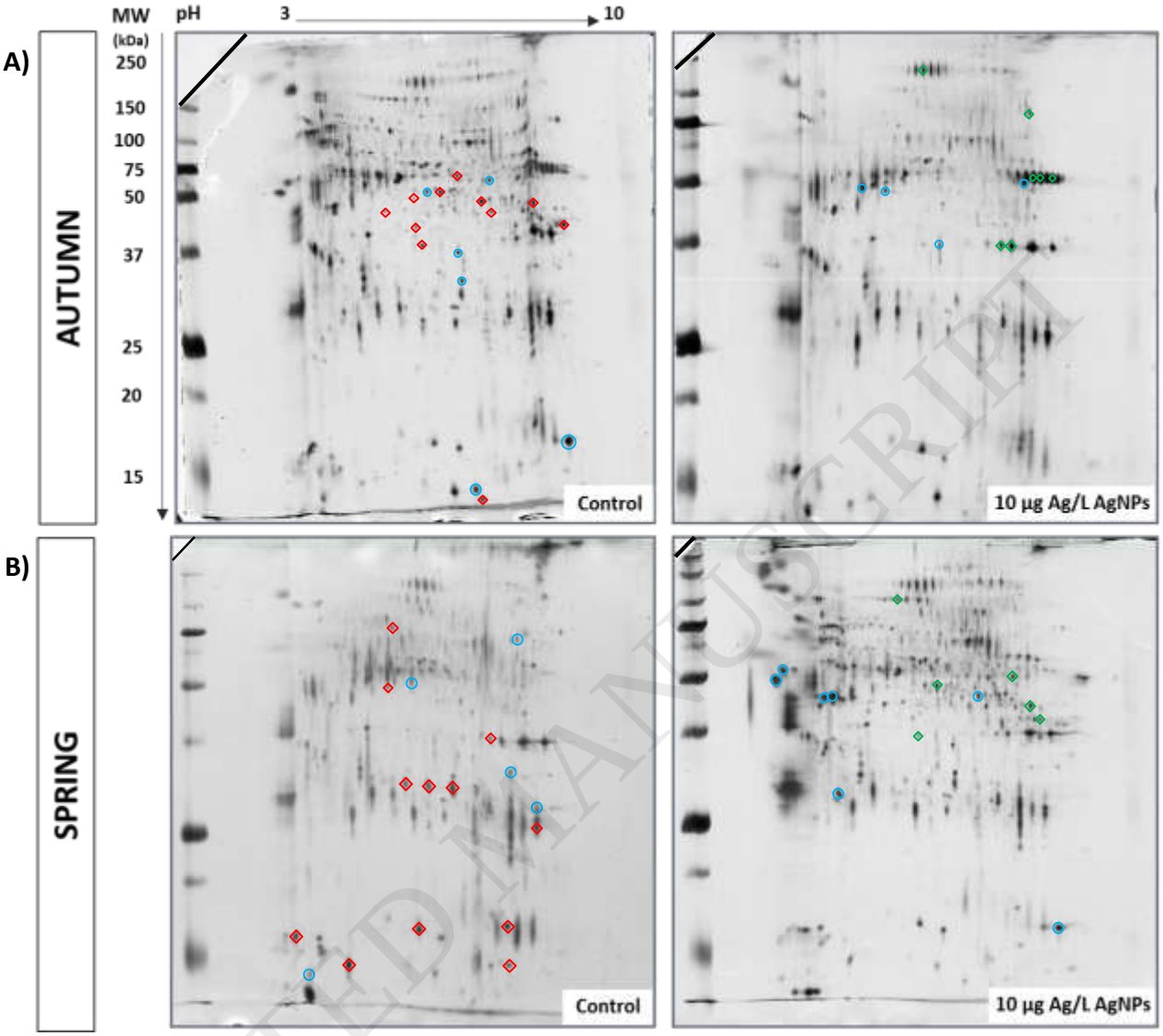

Fig. 4

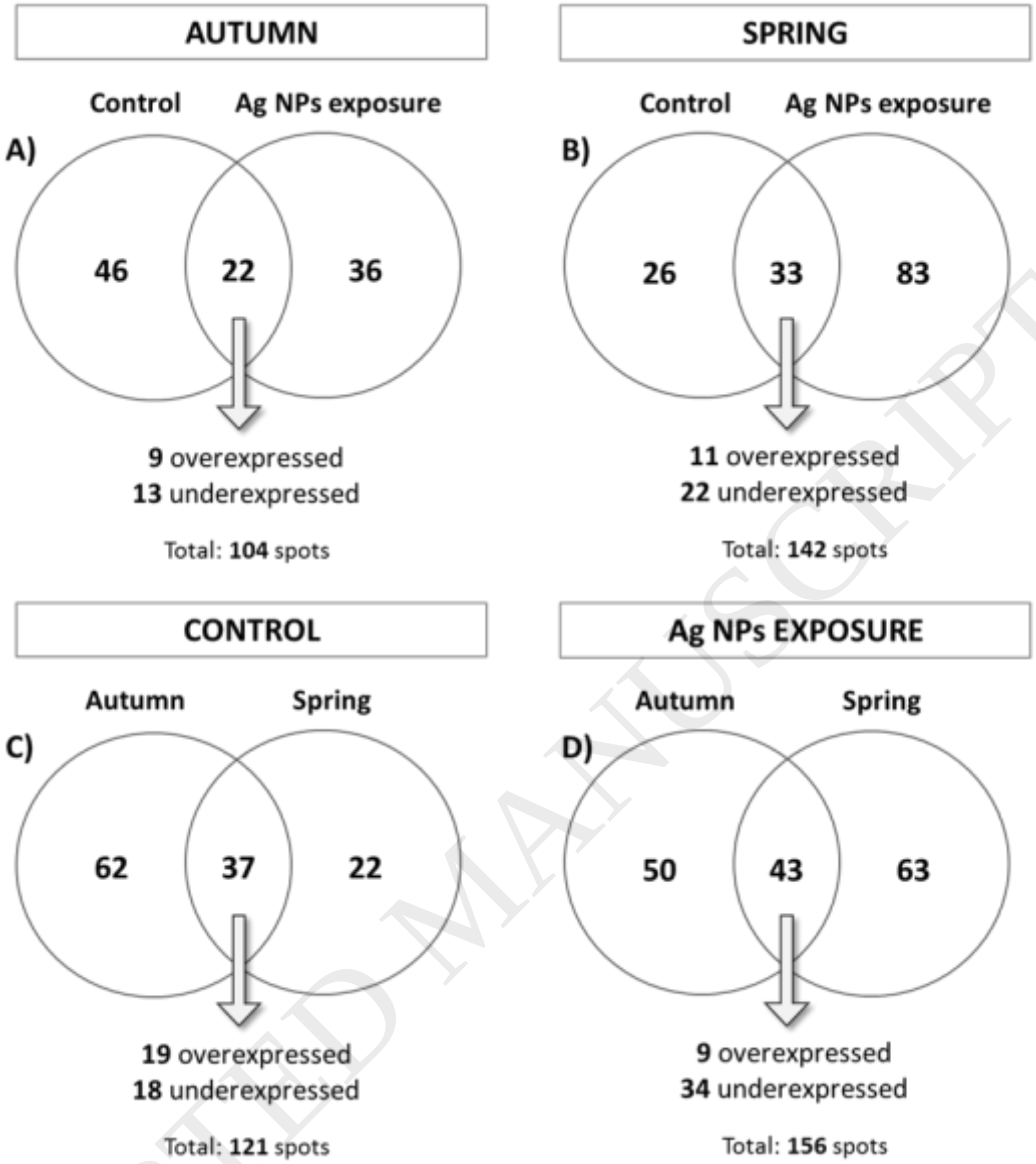

Fig. 5