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The underestimated toxic effects of nanoplastics coming from marine sources: A demonstration on oysters (Isognomon alatus)

Arini Adeline [1], Gigault Julien [2], Venel Zelie [1], Bertucci Anthony [1], Baudrimont Magalie [1, *]

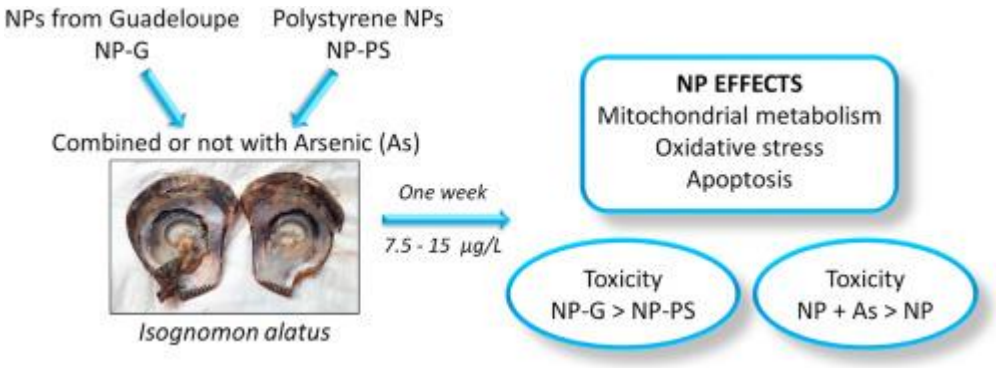
[1] University of Bordeaux, UMR EPOC 5805, équipe Ecotoxicologie Aquatique, Station Marine D'Arcachon, Place Du Dr Peyneau, 33120, Arcachon, France
[2] Université Laval, Département de BiologiePavillon Alexandre-Vachon, 1045, Av. de La Médecine, Local 2064, Québec, Québec, G1V 0A6, Canada

* Corresponding author : Magalie Baudrimont, email address : magalie.baudrimont@u-bordeaux.fr

Abstract :

This study aims to assess the potential toxicity of (1) nanoplastics (NPs) issued from the fragmentation of larger plastic particles collected on the Caribbean marine coast (NP-G), and (2) polystyrene NPs (NP-PS), commonly used in the literature, on Caribbean swamp oysters (Isognomon alatus). Oysters were exposed to 7.5 and 15 mu g.L- 1 of each type of NPs, combined or not with arsenic (As) at 1 mg.L-1 for one week before molecular analyses at gene levels. Overall, the NP-G triggered more significant changes than NP-PS, especially when combined with As. Genes involved in the mitochondrial metabolism were strongly up-regulated in most of the conditions tested (up to 11.6 fold change for the NP-PS exposure at 7.5 mu g.L-1 for the 12s). NPs in combination with As or not triggered a response against oxidative stress, with an intense repression of cat and sod1 (0.01 fold-changes for the NP-G condition at 7.5 mu g.L-1). Both NP-G and NP-PS combined or not with As led to an up-regulation of apoptotic genes p53 and bax (up to 59.3 fold-changes for bax in the NP-G condition with As). Our study reported very innovative molecular results on oysters exposed to NPs from environmental sources. Our results suggest that the composition, surface charge, size, and the adsorbed contaminants of plastics from the natural environment may have synergic effects with plastic, which are underestimated when using manufactured NPs as NP-PS in ecotoxicological studies.

Graphical abstract



Highlights

► Caribbean oysters were exposed to a mix of nanoplastics from marine sources and to nanoplastics of polystyrere ► Nanoplastics impaired mitochondrial metabolism, oxidative stress, and apoptotic response in oysters. ► Nanoplastics from marine sources are more harmful for osysters than manufactured ones. ► Arsenic causes synergic effects when combined to nanoplastics.

Keywords : Ecotoxicology, Nanoplastics, Adverse effects, Transcriptomic, Arsenic, Isognomon alatus

1. Introduction

Around 360 million tons of plastic are produced each year (Plastics Europe 2019), and nearly 10% are released in aquatic environments, having macroscopic to nanoscopic sizes (Thompson 2006, Eriksen et al. 2014). Vast areas of convergence, known as "ocean gyres," have been discovered in the Pacific, Atlantic, and Indian Oceans and concentrate tons of plastic wastes. In 2014, a study estimated over 5,000 billion plastic debris of all sizes in the oceans, representing nearly 250,000 tons (Eriksen et al. 2014). Some authors have suggested that physical (waves, temperature, and UVs) and chemical (oxidation or hydrolysis) constraints would contribute to weakening the plastic structure and reduce them to smaller pieces (Koelmans 2015) until nanoscale particles. Several definitions exist to define nanoparticles of plastics, commonly called nanoplastics (NP). According to the recent literature, they correspond to plastic particles whose size is between 1 and 1000 nm, resulting mainly from the degradation of larger plastic particles and colloidal behavior in the environment (Hartmann 2015, Nolte et al. 2017, Gigault et al. 2018b). Recently, Gigault et al. (2018a) have demonstrated the formation of nanoscale plastic debris after a photochemical degradation of microplastic waste collected from the North Atlantic Ocean. Based on this preliminary finding, these same authors have reported the presence of nanoscale plastics in seawater samples taken directly from the North Atlantic Gyre (Ter Halle et al., 2017). There still exist technical limitations to determine the real nanoplastic concentrations in hydro systems.

For this reason, NPs represent the least known part of plastic wastes present in the environment. Yet, they remain the most hazardous for aquatic life because of their nanometric properties, which

significantly differentiate them from plastic waste (Koelmans 2015). Unlike microplastics that float, NPs diffuse throughout the water column and interact with more system components such as organic matter, microbes, dissolved metals, organic pollutants, etc. (Bouwmeester et al. 2015). The NPs' hydrophobicity and surface specificity facilitate the adsorption of hydrophobic organic and inorganic compounds present in the environment, such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), or metals (Rochman et al. 2013, Velzeboer et al. 2014). In addition, it is estimated that plastic particles can contain up to 4% of their total weight in additives such as plasticizers (Andrady 2011), which can subsequently be released into the environment and then into the organs of aquatic organisms ingested or absorbed. Therefore, the question of the toxicity of NP-associated pollutants needs to be also addressed urgently. Because of their nanometric size, shape, and specific surface area, NPs can cross all biological barriers (cells, tissues) (Manfra et al. 2017) and thus be potentially ubiquitous in the environment (Bouwmeester et al. 2015). These crucial interactions with aquatic life suggest that NPs can play a key role in the functioning of ecosystems. These recent discoveries add to the well-established concerns about plastic wastes and, more specifically to their fate and their ecotoxicological effects in the environment. Studies on the microplastics and NPs ecotoxicity have principally been performed using functionalized polystyrene (PS, PS-COOH, or PS-NH2) (Haegerbaeumer et al. (2019).

Very little data exists in the literature about NP toxicity, and fewer about NPs issued from the fragmentation of larger plastic particles collected in the environment, like in the present study. In 2020, 50% of all studies had used PS in their experimental setup, making it difficult to compare and discuss our results (Kögel et al., 2019). Most other studies have focused on PE, PA, PVC, and PP (23, 23, 15, and 15%, respectively) (Haegerbaeumer et al. 2019). Because of this gap between experimental setups, many publications cited here report results on manufactured NPs, made from one type of plastic or microplastics, mainly PS. Few studies have also targeted polyethylene (PE), polyamide (PA), polyvinyl chloride (PVC), and polypropylene particles (PP) (Haegerbaeumer et al., 2019). To our knowledge, only a few studies have so far used NPs derived from the fragmentation of larger plastic particles coming from the natural environment (Baudrimont et al. 2020, Lebordais et al. 2021a, Lebordais et al. 2021b). It may have undergone degradation processes and present different physical and chemical properties,

potentially leading to adverse effects in aquatic organisms. Baudrimont et al. (2020) showed that NPs made from polyethylene collected in the North Atlantic gyre could inhibit the growth of the green algae *Scenedesmus subspicatus* and increase fecal production of the freshwater bivalve *Corbicula fluminea*. However, no investigation was made at the biomolecular level to characterize the early responses implemented towards the potential toxicity of NPs.

Although it remains difficult to date to know the sources of emission, the composition, fate, and responsiveness of NPs, recent studies suggest that river transport is an essential source of plastic wastes, including NPs, in the oceans (Erik van et al. 2015, Kooi et al. 2018). Marine coastal environments are particularly impacted by plastic contamination due to their direct proximity to river estuaries and oceanic currents, providing floating plastics. The characterization of plastic contents in living organisms has not been possible because of the analytical barriers described above. However, this information needs to be urgently considered for determining the environmental impact of NPs. Bivalves which are in permanent contact with the water column and the suspended matters for respiratory and nutritional purposes, are commonly used as bioindicators because of their high capacities to accumulate pollutants at sublethal levels (Baudrimont et al. 2005, Arini et al. 2014b). In the present study, we chose a tropical bivalve species: *Isognomon alatus* living in Guadeloupean mangroves. Isognomon oysters are widely present in tropical seas around the world, particularly in mangroves, making them good models for studying the effects of sea pollutants. Guadeloupean mangroves and swamps are of particular interest since NPs can be discarded punctually from local terrestrial discharges, brought by the North Atlantic oceanic gyre, and remain captured in the mangrove due to the low water flow (do Sul et al. 2014). We aimed to use this species as a first model to explore the toxicity of NPs 1) issued from the fragmentation of larger plastic particles collected on the Guadeloupean marine coast, and 2) issued from the fragmentation of manufactured polystyrene pellets. We also chose to combine the exposure of NPs with another xenobiotic commonly found in seawater: Arsenic. Arsenic is abundant in seawater (Rodney et al. 2007), and it was found to be one of the most concentrated metals in plastic wastes collected in the North Atlantic oceanic gyre (7th continent expedition in 2015, (Baudrimont et al. 2020)). We aimed to observe its potential synergic effects when associated with NPs, which can act as vectors of

112 contamination (Rochman et al. 2013, Velzeboer et al., 2014). To date, very few studies using
113 functionalized polystyrene nanoparticles (PS) have shown that NPs could generate oxidative stress
114 leading to cell damages, ad cell death in aquatic models (Della Torre et al. 2014, Canesi et al. 2015b).
115 Gene expression is a sensitive indicator of toxicant exposure, DNA damages, and cellular metabolism.
116 It represents an interesting way to characterize the toxicity of environmental pollutants and the
117 contamination levels likely to produce changes in gene expression levels (Gonzalez et al., 2006). We
118 chose to use those molecular markers to characterize early responsetoof the potential toxicity of NPs
119 and Arsenic in *Isognomon alatus.* To date, very few gene sequences are available online for *Isognomon*
120 *alatus*. The first work consisted of completing the gene collection needed to characterize the expression
121 levels of target genes involved in mitochondrial metabolism, detoxification, and oxidative stress
122 responses of *Isognomon alatus*. These approaches should bring a first global analysis of the effects of
123 NPs in association with metal in a coastal organism barely used so far in ecotoxicology to study tropical
124 areas such as mangroves, impacted by both anthropic and coastal inputs of plastics.

125 ## 2. Methods

126

127 ### 2.1. Nanoplastic preparation

128 Two types of NPs were prepared. Crushed polystyrene nanoplastics named NP-PS were
129 obtained by a mechanical degradation using a planetary ball mill (Pulverisette 7) from Fritsch GmbH
130 (Idar-Oberstein, Germany) of reference polystyrene pellets, purchased from Goodfellow (Lille, France).
131 The second batch of NPs was obtained from the mechanical degradation of already degraded
132 microplastics collected on Guadeloupe beaches (16°21'06"N 61°23'09"W) in 2016 (for details see (El
133 Hadri et al. 2020)) and named NP-G. The plastic batches were first grinded in dry conditions and then
134 in wet conditions using ethanol as a dispersant. The powder obtained was dried under vacuum to remove
135 the ethanol, dispersed in deionized water, and filtered on cellulose acetate filters (5 µm pore size,
136 VWR).to collect nanometric particles. The total organic carbon (TOC) was measured (Shimadzu,
137 Kyoto, Japan) in samples of fractioned plastics after filtration to determine the organic carbon
138 concentrations and relate them to concentrations. The plastics used to synthesize NP-G have been

characterized by ATR-Infrared (Nicolet iS 50 FTIR, Thermo Fischer, Waltham, MD, USA) and scanning electronic microscopy (SEM, SH-3000 Hirox, Limonest, France). Their size has been measured by specific AF4 methods over the entire peak range as described in El Hadri et al. (2020) NP-G is a mix of plastics, mainly polyethylene and polypropylene (El Hadri et al. 2020). The size of NPs was on average 260 nm for NP-PS and 280 ± 23 nm for NP-G and the zeta-potential measured for NP-PS was on average −44 ± 2 mV and -30 ± 2 mV for NP-G (El Hadri et al. 2020).

2.2. Oyster collection and acclimation

Around 250 flat tree oysters, *Isognomon alatus* (Gmelin, 1791), were collected in Guadeloupe by the University of the Antilles. The collection site was located in the Grand Cul-de-Sac Marin (16°18'58.1460"N 61°32'1.9379"O), away from discharges and effluent wastewaters considered as clean. Oysters were gently detached from tree roots and brushed to remove external biofilm and parasites. They are maintained three weeks in 100 L glass tanks filled with artificial seawater (Instant Ocean Salt, 30 g/L), at 25°C, oxygenated with aeration pumps, until used for experiments. Day/night alternating lighting was provided by neons (photoperiod 12:12). Tiles were disposed of at the bottom of each tank to allow them to hang on. One-third of each tank volume was changed each day for the first week until oysters had fully decanted. Then one-third of water was renewed two times a week until the end of the acclimation period. Water was prepared the day before to adjust its temperature, oxygen, and salinity before being introduced in oyster tanks. Oysters were fed with two green and brown algal suspensions: *Isochrysis galbana* and *Thalassiosira weissflogii* (around 15 000 000 cells/ml, 20 mL per tank every two days).

2.3. Oyster exposure to NPs

Two concentrations of each kind of NPs were used for both types of plastics : 7.5 and 15 µg.L^{-1}. as a final concentration in experimental units, according to TOC measurements. Oysters were exposed to NPs with or without an additional contaminant: Arsenic (As). There were three experimental

165 replicates per condition, named: NP-G 7.5, NP-G 15, NP-PS 7.5, NP-PS 15, As + NP-G 7.5, As + NP-
166 G 15, As + NP-PS 7.5, As + NP-PS 15.

167 Each condition consisted of a 1L glass tank filled with artificial seawater (Instant Ocean Salt,
168 30g/L) maintained at 25°C and oxygenated with an aeration pump. The day before the start of the
169 experiment, four oysters were introduced in each tank. To avoid contamination by the trophic route,
170 they were fed with 10 mL of *Isochrysis galbana* algae (15 000 000 cell/ml) 2h before the exposure
171 started. At Day 0, the experimental units were spiked with NP-G or NP-PS to reach the above
172 concentrations. Temperature, pH, salinity, arsenic concentrations, and water levels were checked daily
173 to be adjusted, if necessary. In case of oyster mortality, the organisms were immediately removed from
174 tanks.

175 Oysters were sacrificed after 7 days of exposure to NPs and As. Gills and visceral mass were
176 dissected. For each experimental unit, organs from two individuals were collected and pooled to
177 increase the mass sample. The sample was then split for various analyses: arsenic bioaccumulation, NPs
178 bioaccumulation, and transcriptomic analyses. To determine the arsenic bioaccumulation, samples were
179 mineralized using 1 mL of nitric acid (Merck, Darmstadt, Germany, 70%) at 100°C for 3 h (hot block
180 CAL3300, Environmental Express, USA). Digests were then diluted up to 6 ml with ultrapure water
181 and stored at 4°C until analysis. Concerning the transcriptomic, samples were stored at -80°C with 500
182 µL of RNA. Finally, the plastic bioaccumulation samples were dried in an oven at 40°C for 48h before
183 analysis.

184

185 **2.4. Arsenic exposure and bioaccumulation**

186 Exposition medium (5 mL) was sampled each day and acidified with nitric acid (HNO3, 2%)
187 before being analyzed by Inductively Coupled Plasma Optical Emission Spectrometer (700 Series ICP-
188 OES, Agilent) to measure arsenic concentrations. A stock solution of 1000 $\mu g.L^{-1}$ Arsenic (As_2O_3,
189 Merck) was used to spike the experimental units. An adjustment of the Arsenic was realized to
190 compensate for the decrease by adsorption or volatilization. Additional water samples were collected
191 on day 1, day 3, and day 7 and filtrated with Amicon membranes Ultra-15 of 3 kDa (Merck) to measure
192 the dissolved part of Arsenic. They were analyzed by Atomic Absorption spectrometry 240Z AA (AAS,

193 Agilent Technologies). Arsenic concentrations in digestates of oysters were also analyzed by AAS. The
194 validity of the methods (ICP OES and AAS) were periodically checked with a laboratory-certified
195 reference material (Dolt 5, As = 34.6 $mg.kg^{-1}$ ± 2.4 $mg.kg^{-1}$).
196
197 **2.5. Design of gene primers**
198 The total RNAs of *Isognomon alatus* were sequenced by Lemer et al. *(2019)* and found on
199 NCBI (https://www.ncbi.nlm.nih.gov/sra/SRS1382830). The data were assembled with the Trinity tool
200 provided by the Galaxy server (https://usegalaxy.org/). Once the transcriptome was obtained, it was
201 downloaded on the Blast server and aligned with sequences of genes of interest from other mollusk
202 species. When good alignment was possible, primers were designed and used for PCR amplification of
203 *Isognomon alatus* genes. Electrophoresis gels were run to confirm the amplification of one DNA
204 fragment (having the same size as the expected gene). Amplificons were sequenced, and the sequences
205 were used to design primers specific to *Isognomon alatus* (with Primers 3).
206
207 **2.6. Transcriptomic analyses**
208 Differential gene expression was assessed in the gills and visceral mass (VM) of oysters.
209 According to the manufacturer's instructions, the total mRNA extraction was performed with the SV
210 Total RNA Isolation System (Promega). The concentration of extracted RNA was measured with a
211 microplate spectrometer (BioTek EPOCH) and a Take3 plate to estimate the purity of nucleic acids by
212 comparing the ratio between values measured at 260 and 280 nm (purity is ensured if the result is over
213 2). All RNA samples had a ratio over 2. According to the manufacturer's instructions, the RNA samples
214 were diluted to have the same concentration in all samples before carrying out the reverse transcription
215 (RT) using the GoScript Reverse Transcription System (Promega). RNA samples were incubated in an
216 Eppendorf Mastercycler to synthesize cDNA after the addition of the reagents. cDNA samples were
217 kept at -20°C until their use for real-time PCR amplification.
218 Thirteen genes involved in endocytosis, detoxication, respiratory chain, DNA repair, and
219 apoptosis, and two reference genes were assessed (Table 1). Real-time PCR reactions were performed
220 in a LightCycler 480 (Roche) to quantify cDNA amplification. The kit GoTaq® qPCR Master Mix

(Promega) was used for PCR reactions in 96-well plates containing the 5x buffer, the *Taq* polymerase, $MgCl_2$, dNTP, and SybrGreen. In each wells were added 2 µL of a gene-specific primer pair, at a final concentration of 5 µM for each primer (Table 1), 12.5 µL of qPCR Master Mix mixed with 6.5 µL of RNA free water, 5 µL of cDNA, and mixed to before achieving PCR amplification.

The qPCR program began with one cycle at 95°C for 10 min, followed by 40 amplification cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The amplification specificity of each gene was checked with the PCR product dissociation curve, obtained by the gradual heating of the PCR products from 60 to 95°C. The relative quantification of each gene expression level was normalized according to the gene expression of two reference genes: *β-actin* and *rpl7* (according to the GeNorm method). The $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001) was used to calculate the differential gene expression, using ΔCt, which represents the difference between the cycle threshold (Ct) of a specific gene and the Ct of the geometric mean of the Ct of the two reference genes. Induction factors of gene expression were calculated in comparison with the controls (not exposed to xenobiotics) according to the following equation:

$$IF = \frac{2^{-\Delta\Delta Ct\ exposed}}{2^{-\Delta\Delta Ct\ control}}$$

2.7. Data treatment

Significant differences between treatments were revealed using statistical tests. Due to the non-normal distribution of data, the non-parametric Kruskal-Wallis test was performed with post hoc exact permutation tests for inter-groups comparison after sequential Bonferroni correction (XL-Stat software version 2013.5.09, 1995e2013 Addinsoft). A probability of $p < 0.05$ was considered significant for all statistical tests.

3. Results

3.1. Physicochemistry of experimental units

248 The temperature was maintained at around 25 °C and the pH around 8.2 throughout the 7 days
249 of the experiment (Table 2). The salinity was comprised between 26 and 28.6 ‰, and adjusted
250 accordingly. These values are similar to those measured where the oysters were collected (pH 8, 26°C,
251 salinity 30‰).
252 Arsenic concentrations were monitored in the water of experimental units throughout the
253 experiment. Arsenic concentration was, on average, around 1002.2 ± 28.8 µg.L^{-1} between experimental
254 unis, at the beginning of the exposure (Day 0). It was checked and adjusted every day to stay as closer
255 as possible at 1000 µg.L^{-1}. Water samples were collected and filtered with Amicon membranes to
256 measure the dissolved part of Arsenic. Arsenic was mainly under its dissolved form, with a dissolved
257 percentage averaging 103.2 ± 2.1 % in the experimental units, with no significant difference between
258 conditions.
259 No over-mortality of oysters was observed throughout the 7 days of exposure, with only 5
260 oysters on 250 used died during the experiment (no relationship was observed with the exposure
261 conditions with NPs and As).
262
263 **3.2. Arsenic bioaccumulation in oysters**
264 Arsenic bioaccumulation was measured in the gills and visceral mass of oysters (Figure 1). The
265 concentrations of Arsenic in controls (no NP) and oysters exposed to NPs without As was in the average
266 of 35.5 ± 1.2 µg.g^{-1} DW in gills and 67.6 ± 0.90 µg.g^{-1} DW in visceral mass. They were significantly
267 higher (4.5 and 3.2 times in gills and visceral mass, respectively) in oysters exposed to As and to As +
268 NPs (mean of 161 ± 10.5 µg.g^{-1} DW and 214.7 ± 4.8 µg.g^{-1} DW in gills and visceral mass, respectively).
269 In both organs, the arsenic bioaccumulation was not significantly different between oysters exposed to
270 As + NPs and oysters exposed to As alone.
271
272 **3.3. Transcriptomic results**
273 Two housekeeping genes were used as control (*β actin* and *rpl7*), and their expression showed
274 no significant difference among the different treatments. The geometric mean of these two reference
275 genes was used to normalize target gene expression. Overall, both types of NPs used in the study

276 triggered significant changes in gene expression, gills, and visceral mass of oysters (Figure 2). The NPs
277 issued from the fragmentation of larger plastic particles collected on the Guadeloupean marine coast
278 (NP-G) triggered more significant changes than NPs issued from the fragmentation of manufactured
279 polystyrene pellets (NP-PS). A mean of 32% of genes was significantly modulated in both gills and
280 visceral mass after NP-G exposure. In comparison, a mean of 18 and 30% of genes were significantly
281 modulated in gills and visceral mass after NP-PS exposure, respectively.

282 An inverse dose-response effect could be observed for most of the genes tested. Overall, there
283 were 25% fewer genes showing significant differences in their expression in oysters exposed to NP-G
284 15, compared to those exposed to NP-G 7.5, and 38% fewer in NP-PS 15 than NP-PS 7.5 (averages for
285 gills and visceral mass). A synergic effect was observed when oysters were exposed to As combined to
286 NPs. It is worth noting that we observed a strong behavioral difference in oysters throughout the
287 experiment, depending on the treatment.

288

289 The endocytosis genes, *cav,* and *cltc* were assayed to assess two different potential entry routes
290 of NPs into the organisms (clathrin-independent and clathrin-dependent endocytosis, respectively.
291 Surprisingly their modulations were mainly observed in oysters exposed to NPs combined to As. The
292 clathrin gene (*cltc*) was mainly induced in gills (2 to 14 fold changes in As + NP-G and As + NP-PS
293 for both concentrations), whereas the clathrin-independent endocytosis controlled by *cav*, primarily
294 occurred in visceral mass (5.9 fold change in As + NP-G 7.5).

295 The mdr gene can implement cell detoxication. However, the exposure to NPs did not show
296 any modulation of the *mdr* gene, except for the As + NP-G 15.

297 The mitochondrial *12s* ribosomal gene is a suitable marker for quantifying mitochondria in
298 cells, and *cox1* (subunit I of the cytochrome C oxidase) regulates the electron transfer through the
299 mitochondrial chain. The *12s* gene was over-expressed in most conditions tested in this study (NP-G,
300 NP-PS, with or without As) for both concentrations tested (up to 11.3 and 11.6 fold-change in gills and
301 visceral mass, respectively). The *cox1* gene was induced in gills of oysters exposed to As + NP-G 15
302 and to As alone (6.8 and 4.3 fold-change, respectively). The *cox1* gene was suppressed in the visceral
303 mass of oysters exposed to NP-G 7.5 and NP-PS 15 (with and without As) and to As alone.

The oxidative stress was evaluated through the expression of *sod1* and *cat* and was balanced between induction and repression of genes. The reactive oxygen species are mainly generated by a dysfunction of the mitochondrial respiratory chain. The modulation of the *cat* gene expression in organs followed overall the opposite pattern compared to the mitochondrial gene *12s. Cat* was repressed (0.01 to 0.4 fold-change) when *12s* was induced (except for As + NP-G 15). The *cat* gene was up-regulated in gills and suppressed in the visceral mass of oysters exposed to As (11.4 and 0.001 fold-change, respectively). Like *cat*, *sod1* was repressed in gills and visceral mass of oysters exposed to NP-PS 7.5 (0.47, 0.46, respectively) and in gills of the NP-PS 15 condition (0.34). But it was induced in the As conditions and the As + NP-PS 15 conditions in visceral mass.

The expression of *gadd45*, involved in DNA repair and growth arrest, was up-regulated in gills of oysters exposed to the As + NP-G 15 and the As treatments (3.4 and 2.5, respectively). At the same time, it was down-regulated in the visceral mass of oysters exposed to NP-G 7.5, As + NP-G 7.5, As + NP-G 15, and As (0.5 to 0.6 fold-change).

The cell cycle and apoptosis promotor, *p53*, was up-regulated in gills of oysters exposed to NP-G 15 and As + NP-G 15 (4.7 and 52.5 fold-change, respectively), but not in the As condition. The apoptotic response was followed through the expression of *bax* and *gapdh*. The expression of *bax* was up-regulated in the visceral mass of oysters exposed to NP-PS 7.5 (2.2 fold-change). It was also strongly induced in the As + NP-G 15 treatment (59.3 fold-change), coinciding with the strong induction of *p53*. The expression of *gapdh* was not induced in any treatment. Arsenic down-regulated *gapdh* expression in the visceral mass of oysters exposed to As + NP-PS 15 and As (0.5 and 0.3 fold-change, respectively).

4. Discussion

4.1. NP and associated chemicals

In the present study, we tested different nanoparticles of plastics: 1) issued from the fragmentation of larger plastic particles collected on the Guadeloupean marine coast (NP-G), and 2) issued from the fragmentation of manufactured polystyrene pellets (NP-PS). We aimed to see if they could generate toxic effects on swamp oysters and act as vectors of other pollutants present in their

surrounding environment and adsorbed onto their surface. NPs are made of plastics but can also be composed of additional xenobiotics, adsorbed onto their surface, or part of their composition (Andrady 2011, Rochman et al. 2013, Velzeboer et al. 2014). Arsenic is abundant in seawater and is released from natural and anthropogenic sources (Rodney et al. 20,07). It has been quantified among other metals in plastic wastes collected in the North Atlantic oceanic gyre (7th continent expedition in 2015, (Baudrimont et al. 2020)). Its concentration in NPs was 0.084 $\mu g.g^{-1}$, more than 40 times higher than that measured in manufactured polystyrene NPs (0.002 $\mu g.g^{-1}$) (Baudrimont et al. 2020). These results motivated our choice to use Arsenic with NPs, to a potential synergic effect of both pollutants in oysters. Arsenic used in this study was under its dissolved form, commonly described as the most bioavailable and the most toxic fraction (de Paiva Magalhães et al. 2015). Arsenic bioaccumulated in gills and visceral mass of oysters, with no significant difference between concentrations measured in oysters exposed to As and the ones exposed to As combined to NPs, and no significant difference between organs. We could have expected higher As concentrations in the conditions with NPs, which could have acted as vectors of contamination. Still, our results suggest that NPs had a minor role in transferring Arsenic into oysters tissues. We could also have expected higher concentrations in the visceral mass as a storage organ (Arini et al. 2014a), as described in a similar study using the trophic route to expose *Isognomon alatus* to NPs combined to As (1 $mg.L^{-1}$ for 7 days). The authors showed significant differences between the bioaccumulation of gills and visceral mass, which was nearly 2-fold higher than in gills (Lebordais et al. 2021b).

4.2. Gene expression

Overall, our results showed that NP-G triggered more differential expression of target genes than NP-PS (31% and 24% of gene expression significantly modulated, respectively, for conditions with and without As), mainly for *cox1, 12s, cat,* and *p53* (Figure 3). It suggests that the composition, surface charge, size, and the adsorbed contaminants of plastics from the natural environment may have synergic effects with plastic. It has been reported that the sorption of other chemicals to NPs may affect their biological impact (Canesi et al. 2015a). Not only the NP composition and behavior can influence NPs' toxicity on aquatic organisms. Indeed, in two recent studies, Lebordais et al. (2021a, 2021b)

compared toxicity of similar NP solutions, namely NP-G and NP-PS, on dietary-exposed oysters. Interestingly, the authors showed that NP-PS triggered more effects at the gene level than NP-G when oysters were exposed to NP-contaminated algae, which is in opposition with our results obtained after waterborne exposure.

Our results also showed a synergic effect when oysters were exposed to As combined with NPs. There were more significant changes in gene expression (38% averaged in gills and visceral mass for all treatments) than the conditions without As (22%). The oysters exposed to As alone had 59% of their genes significantly modulated. The main differences were found for the *cat*, *cltc, cav, 12S,* and *gadd45* genes, with As or As + NPs treatments triggering more significant gene-level effects than those without NPs. Overall, more genes were modulated in case of As contamination compared to As + NPs, which suggests that NPs could decrease the bioavailability of Arsenic and then have a protective role against its toxicity. The different treatments also led to different responses in the two organs tested, mainly depending on the gene function. For instance, the endocytosis *cltc* gene was induced in gills and not in visceral mass (for conditions with As + NPs) since gills are the first biological barrier with the surrounding medium. The mitochondrial gene *cox1* and the gene *gadd45* were repressed in visceral mass and not in gills. Experimental evidence shows that once nanoparticles gain systemic access, they mainly accumulate in the liver and kidneys of animals which are the main storage organs of xenobiotics (van der Zande et al. 2012, Kögel et al., 2019).

In our study, we used two concentrations of each type of NPs tested to see a potential dose-response effect. However, a reverse dose-response effect could be observed for most of the genes tested. Throughout the experiment, we observed a substantial behavioral difference in oysters, depending on the treatment and the concentrations tested, which could explain, at least partly, these differences. When exposed to the highest concentrations of NPs (NP-G 15 and NP-PS 15), oysters kept their valves closed most of the time and secreted a white substance, forming filaments excreted from their valves and floating into the water tanks. We hypothesized that it could be mucus. There is no data available to our knowledge in the literature to date about such phenomenon described after NP exposure. However, a study on mussels exposed to microplastic fibers (polyethylene terephthalate, 30 MPF/mL) showed that filtration rates decreased, and 71% of microplastic fibers were rejected in pseudofeces. In contrast, only

388 9% were ingested (Woods et al. 2018). The feeding rate of the blue mussel *Mytilus edulis* was also
389 affected after exposure to NP PS with the observation of valve closures, concomitant with increased
390 production of pseudofeces (Wegner et al. 2012). This corroborates our statement of essential quantities
391 of mucus excreted by oysters in contact with NPs. It could be hypothesized that this acts as a protective
392 mechanism against NPs, by rejecting them before reaching the digestive tract. Indeed, many species
393 implement selective mechanisms by eliminating unwanted particles as pseudofeces before ingestion
394 (Ward and Shumway 2004). For instance, Zhao et al. (2018) found that the length of microplastics was
395 significantly longer in pseudofeces produced by wild mussels than in their digestive gland and feces. In
396 our study, we can hypothesize that similar phenomena occurred with higher concentrations of NPs, that
397 had potentially aggregated. Indeed, a recent study using microfluidic devices to mimic salinity gradient
398 showed that NP aggregation occurred through the salinity gradient (Venel et al., 2021) since high ionic
399 force tends to aggregate nanoparticles (Chen and Elimelech 2006, Venel et al. 2021).

400 The NP-G obtained from plastics collected in the ocean underwent different degradation
401 processes (Andrady et al. 2011, Koelmans 2015). As a consequence, NP-G may be more oxidized than
402 reference pellets of PS used to get NP-PS and then probably more negatively charged than NP-PS
403 (Blancho et al. 2021). Some authors suggest that the mechanism of the internalization of negatively
404 charged NPs occurs via clathrin pathways (Kögel et al. 2019). Surprisingly, our results showed that
405 neither clathrin nor caveolin genes was regulated after NP exposures, while they were up-regulated in
406 case of combined exposure to As + NPs. It suggests that the genes of endocytosis were predominately
407 triggered after exposure to As and not NPs themselves and that the clathrin gene (*cltc*) was mainly
408 induced in gills, whereas the clathrin-independent endocytosis controlled by *cav*, primarily occurred in
409 visceral mass. Our results correspond to two similar studies conducted on *Isognomon alatus*, exposed
410 via the trophic route to either NP-G or NP-PS (Lebordais et al. 2021a, Lebordais et al. 2021b). Neither
411 clathrin nor caveolin was up-regulated after NP trophic exposures. A study on endocytosis pathways
412 showed that clathrin and caveolin endocytosis occurred mainly for particles up to 100 nm and 60-80
413 nm, respectively (Benmerah and Lamaze 2007). In our study, particles sizes were comprised between
414 260 and 280 nm, which could explain why we observed low responses of the *cav* and *cltc* genes. There
415 could also exist a different entry route for NPs. In a recent study, Sendra et al. (2019) inhibited caveolin

and clathrin endocytosis pathways in *Mytilus galloprovincialis*. They showed that the internalization of PS NP at 50 nm was lower than controls (not inhibited), suggesting that these pathways are used for NP internalization in mussels. They also showed that larger particles were instead internalized through phagocytosis than endocytosis.

It looks like if NPs can enter cells, it does not trigger a detoxication response through the *mdr* pathway, as no regulation of this gene was observed in any of the conditions tested. Detoxication might involve other genes and pathways of the ABC transporter family. For example, Della Torre et al. (2014) showed that the exposure of sea urchins to carboxylated NP PS resulted in an upregulation of the *abcb1* gene–efflux pump. Similar results were observed with a significant up-regulation of the *abcb* gene in *Mytilus galloprovincialis* larvae (24 hpf) exposed to PS-NH2 (Balbi et al. 2017).

Mitochondria are the primary source of reactive oxygen species (ROS) production in cells. There are very little data available on literature about the potential of NPs to generate oxidative stress. In our study, the exposure to NPs and NPs + As (not As alone) induced an increase in the number of mitochondria in oysters, revealed by a global over-expression of the *12s* gene in most of the tested conditions, both the gills and the visceral mass. The impact on the respiratory chain, pointed out by the *cox1* repression in visceral mass, may explain the increase in *12s* expression, suggesting a compensatory mechanism by the organism to maintain its mitochondrial metabolism. Interestingly, oysters exposed to NP-G via the trophic route did not show similar inductions on the mitochondrial metabolism (Lebordais et al. 2,021b) suggesting that the NP effects are mainly triggered by water contamination.

Despite implementing compensatory mechanisms, we observed that NP exposure might have triggered a response against oxidative stress. There was intense repression of *cat* and *sod1* in several conditions tested with NPs in combination with As or not. The modulation of the *cat* gene expression in organs followed overall the opposite pattern compared to the mitochondrial gene *12s.* The down-regulation of these genes may have occurred after a possible over-expression to return to a basal level. We hypothesize that the *cat* repression underlines a response against oxidative stress that may have happened before *12s* was induced to counteract a dysfunction of the mitochondrial chain. To corroborate our hypothesis, we found some studies that reported oxidative stress in response to NP exposure. For

example, Canesi et al. (2015a) showed that the exposure to PS-NH2 (1 to 50 mg.L^{-1}) led to the reduction of the Cytochrome C, increasing ROS production in *Mytilus galloprovincialis* hemocytes. Balbi et al. (2017) also tested PS-NH2 exposure (0.150 mg.L^{-1}) on fertilized eggs of *Mytilus galloprovincialis* for 24 to 48h. The authors looked at different gene expressions, including *cat* and *sod1,* and did not report any significant modulation of those genes in response to NP exposure.

Overall, our results showed a weak apoptotic response (via the gene expressions of *gadd45* and *p53*), probably due to the implementation of effective systems to maintain the mitochondrial chain electron equilibrium and lower damages resulting from oxidative stress. The expression of the apoptotic gene *bax* was strongly induced in the As + NP-G 15 treatment gills, coinciding with the strong induction of *p53*. It suggests a response of the organism towards cell apoptosis, especially in gills, which are the first biological barrier in contact with the xenobiotics present in water. To date, to our knowledge, no study reports the effects of NPs on apoptotic pathways on bivalves. For instance, Lebordais et al. (2021b) showed few effects on apoptotic genes, with down-regulation of *bax* and *gadd45* after the dietary exposure of *Isognomon alatus* to NP-PS and no effects after NP-G exposure. Another study on sea urchin embryos exposed to 50 nm PS-NH2 (1 to 50 mg.L^{-1}) showed an induction of the caspase gene (*cas8*) involved in the apoptotic pathway. The authors suggested that the apoptotic response could result from cell membrane damage (Della Torre et al. 2014). Canesi et al. (2015a) also reported a decrease of mitochondrial membrane potential in *Mytilus galloprovincialis* hemocytes exposed to 50 nm PS-NH2, which was assumed to be a pre-apoptotic sign. However, these studies report results on cells or larvae and use manufactured NPs, which are not issued from the fragmentation of plastics collected in the natural environment, and are not potentially loaded with other xenobiotics, such as the ones we used. It makes comparisons with our results difficult. This underlines the lack of data available to date in the literature on the effect of NPs on cell viability and apoptotic responses. More data is available on microplastic toxicity towards aquatic organisms, especially bivalves. However, many studies have pointed out the main differences between microplastics and NPs, but not much between NP types (manufactured *vs.* environmental). Not only their size can influence their toxicity. Their surface charge, shape (pellets vs. fractioned plastics), oxidation status, and volume/surface ratio make them possibly more hazardous for wildlife than microplastics and manufactured nanoplastics (Andrady

2011, Bouwmeester et al. 2015, Manfra et al. 2017). However, most studies still use manufactured NPs namely NP-PS, with concentrations in the range of the $mg.L^{-1}$, to assess the ecotoxicological impacts of NPs, which may greatly vary the results. Our study pointed out the relevance of using NPs from environmental sources and considering their behavior in changing physicochemical conditions, which may influence their aggregation state (Venel et al. 2021), their bioaccumulation and thus their toxic effects.

5. Conclusion

In the present study we chose to use NPs issued from the fragmentation of larger plastic particles collected on the Caribbean marine coast to get closer to the one's wildlife is exposed to. We chose relatively low concentrations, even though concentrations were so far unknown in the natural environment (rivers or oceans): 7.5 to 15 $\mu g.L^{-1}$. At the same time, several studies published results using concentrations in the range of the $mg.L^{-1}$. Our study reported the first results on NPs toxicity on oysters exposed to NPs from environmental sources about mitochondrial metabolism, oxidative stress, and to a lesser extent, about the apoptotic response. We aimed to compare them to NP-PS to see potential differences in their effects on organisms and showed that NP-G triggered more effects on gene expression than NP-PS in a reverse dose-dependent manner. We also established a synergic effect of toxicity when oysters are exposed to NPs and a commonly found metalloid in seawater Arsenic. Our results suggest that NPs issued from the environment may be more toxic than commonly used NP-PS, especially when combined to xenobiotics. Further studies would be needed to explore the influence of salinity on NPs behavior and toxicity, especially when considering species from transition mediums like swamp oysters from Caribbean marine coasts.

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Figure 1

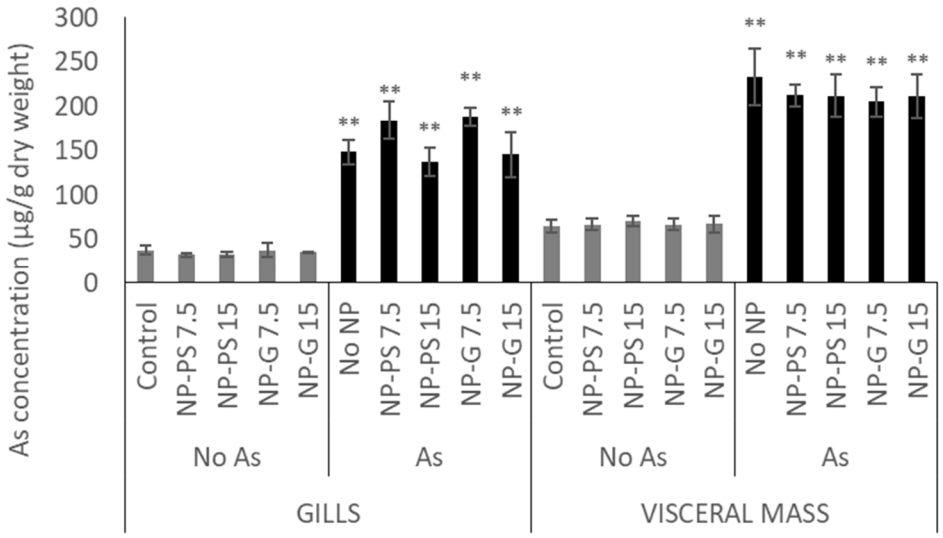
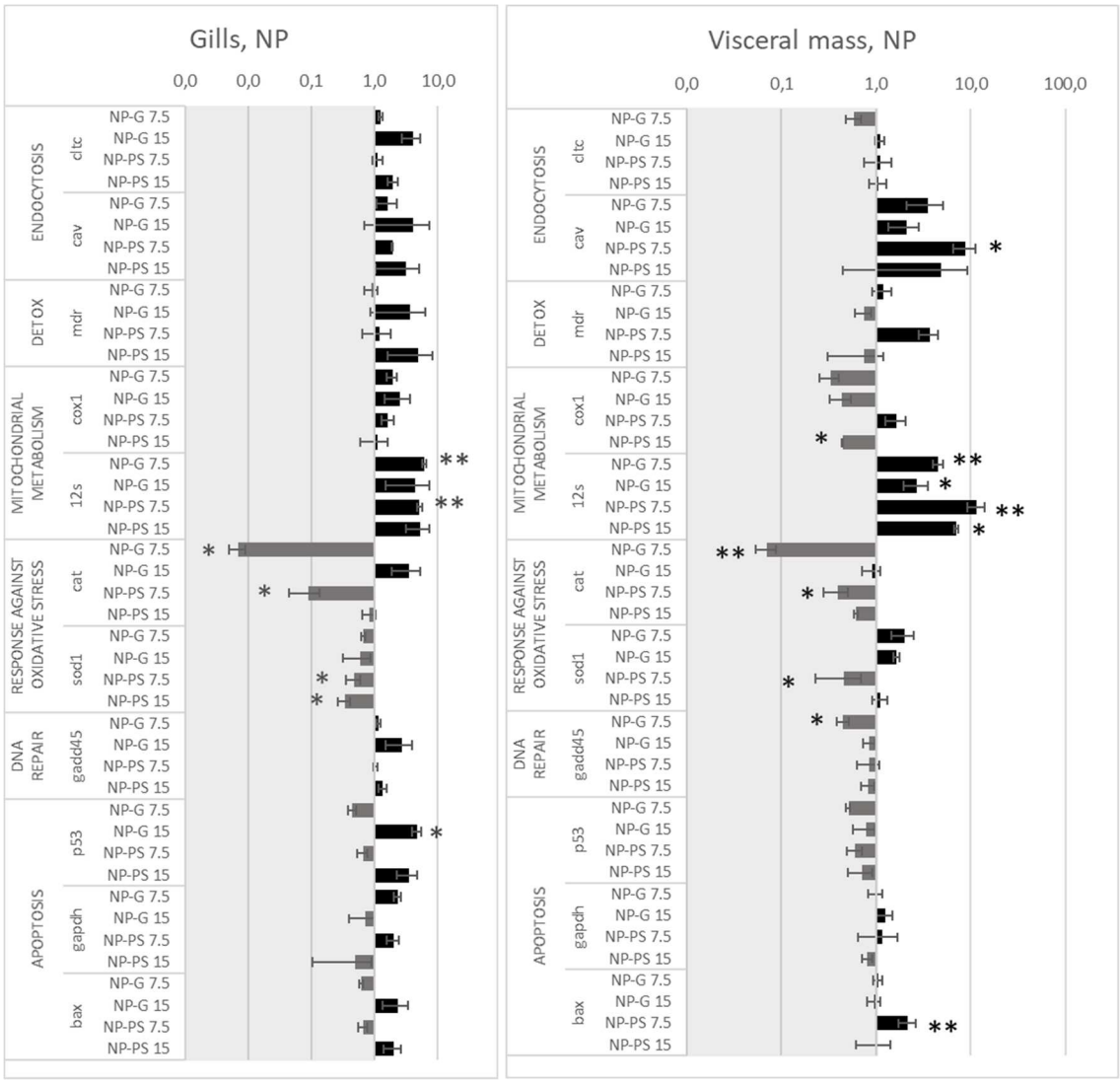


Figure 1: Arsenic (As) concentrations in gills and visceral mass of oysters, according to the different treatments. NP: nanoplastic, NP-G: NPs from plastics collected in Guadeloupe, NP-PS: NPs from PS pellets (mean ± standard error, n=6), for the 7.5 and 15 µg/L conditions. Significant differences were tested between controls (No NP) and other treatments (Kruskal-Wallis, P<0.01: **).

Figure 2


Gills, NP
0,0
0,0
0,1
1,0
10,0
Visceral mass, NP
0,0
0,1
1,0
10,0
100,0
ENDOCYTOSIS
DETOX
MITOCHONDRIAL METABOLISM
RESPONSE AGAINST OXIDATIVE STRESS
DNA REPAIR
APOPTOSIS
cltc
cav
mdr
cox1
12s
cat
sod1
gadd45
p53
gapdh
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NP-G 15
NP-PS 7.5
NP-PS 15

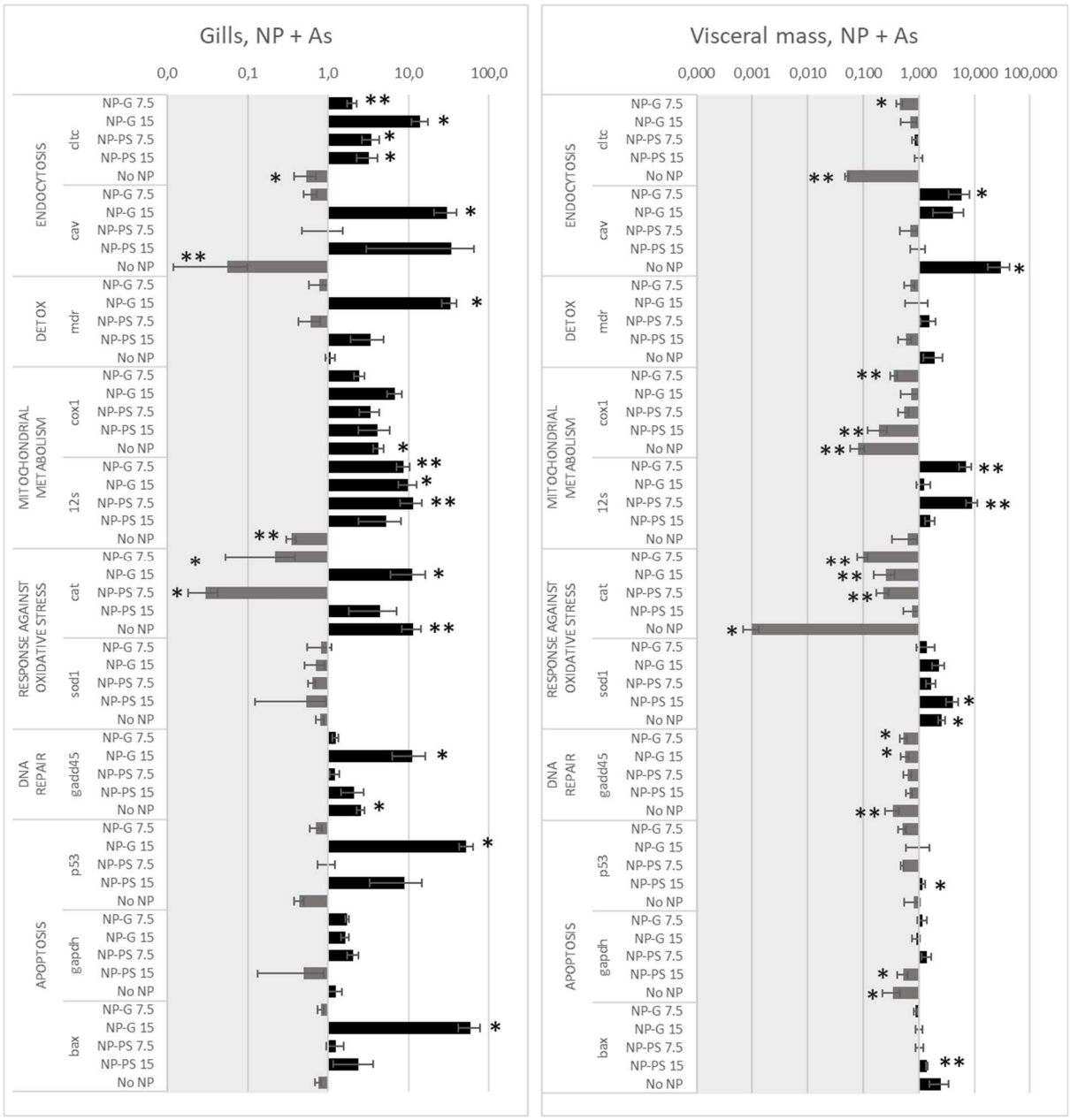


Figure 2: Differential gene expression observed in gills and visceral mass of *Isognomon alatus*, according to the different treatments. NP nanoplastic, NP-G NPs from plastics collected in Guadeloupe, NP-PS NPs from PS pellets, As Arsenic, for the 7.5 and 15 µg/L conditions. Results are presented as fold-change factors between gene expression in controls and gene expression in exposed bivalves (>1: induction; <1: repression, mean ± standard error, n=6). Significant differences were tested between controls (No NP) and other treatments (Kruskal-Wallis, P<0.01: **), and only factors <0.5 and >1.5 were considered as significant.

Figure 3

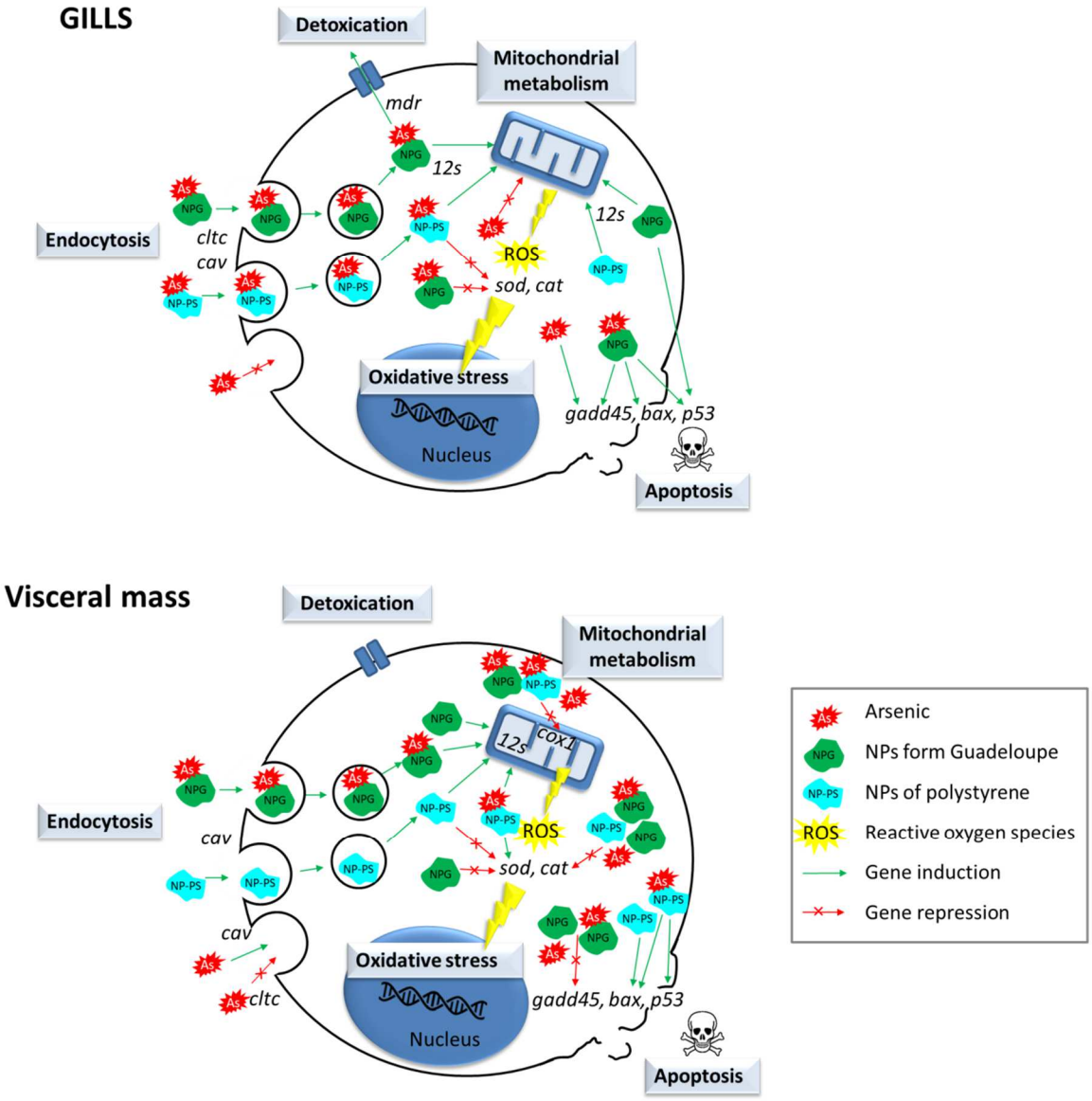


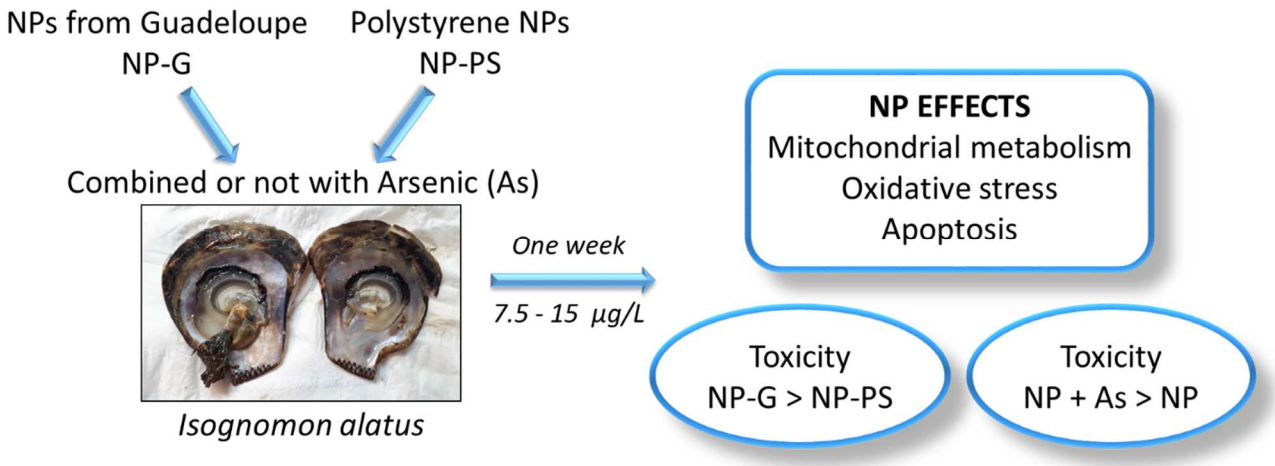
Figure 3: Schematics of significant gene expression modulation in gills and visceral mass of *Isognomon alatus* after one week of exposure to NP-G, NP-PS, and As.

Table 1: Functions and nucleotide sequences of specific primer pairs of genes used in this study. [a] Forward primer, [b] Reverse primer.

Gene	Name	Function	Sequence 5'-3'
B actin	Beta Actine	Housekeeping gene	AACGAGCGATTCAGATGTCC[a] CGATTCCTGGGTACATGGTT[b]
RPL7	Ribosomal Protein L7	Housekeeping gene	CCCAGGAAGGTCATGCAGTT[a] TCCCAGAGCCTTCTCGATGA[b]
cltc	Clathrin heavy chain	Endocytosis	AGACTCAGGACCCAGAGGAC[a] ATCACACGGGTTCTATCGGC[b]
cav	Cavolin	Endocytosis	CGTCGAGATCCAGACCTGTT[a] ACAGCATTGACTGCGTATGC[b]
mdr	ATP Binding cassette subfamily	Detoxication	GCATGTTGCAAGCCTGTCAA[a] CAGTCAACTCAAGCAACCGC[b]
cox1	Cytochrome C oxidase	Mitochondrial metabolism	GTTGCCTTGGTCGCTAGACT[a] GAGCGTCTTGGGCTTAGTCA[b]
12s	Mitochondrial 12s rRNA	Mitochondrial metabolism	TCAGGTGTTACACAGCCGTC[a] GCAGGCGTTTTAATCCCGTC[b]
cat	catalase	Oxidative stress	CGAGGCTAGCCCAGACAAAA[a] TTGGGGAAATAGTTGGGGGC[b]
sod1	Superoxide dismutase 1	Oxidative stress	AGACTGCGTCACATGCTTCA[a] GCGTCATGTAGGGGATCTGG[b]
gadd45	Growth arrest and DNA damage inducible	DNA repair	TTGGCTTGACAAAAGTGCCG[a] CTGACAACCTGCATCTCGGT[b]
p53	Tumor Protein P53	Apoptosis	CGATGATCGGGTTCAGCAGA[a] GAGCTCTCTCAACACAGCCA[b]
gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Apoptosis	CACGGCAACACAGAAGGTTG[a] CCCTTCTGAAGTCGGCAAGT[b]
bax	BCL2 associated X	Apoptosis	AACTGGGGCAGAGTTGGATG[a] AATTGCTTCCCAGCCTCCTC[b]

Table 2: Physicochemical parameters measured in experimental units.

	Day 1	Day 3	Day 6
pH	8.15 ± 0.7	8.20 ± 0.4	8.23 ± 0.6
Temperature (°C)	24.8 ± 0.3	24.9 ± 0.1	25.0 ± 0.2
Salinity (‰)	28.6 ± 0.4	26.0 ± 0.5	27.4 ± 0.5


NPs from Guadeloupe
NP-G
Polystyrene NPs
NP-PS
Combined or not with Arsenic (As)
Isognomon alatus
One week
7.5 - 15 µg/L
NP EFFECTS
Mitochondrial metabolism
Oxidative stress
Apoptosis
Toxicity
NP-G > NP-PS
Toxicity
NP + As > NP