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Cellular and molecular mechanisms of NiONPs toxicity on eel hepatocytes HEPA-E1: An illustration of the impact of Ni release from mining activity in New Caledonia

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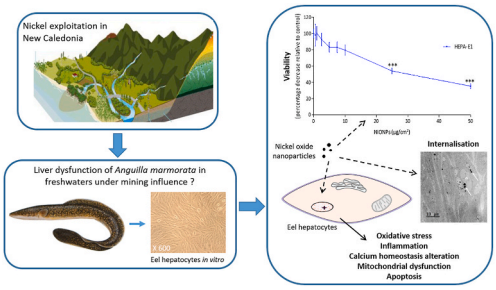
HIGHLIGHTS

- NiONPs decrease cell viability and induce a ROS overproduction in eel hepatocytes.
- Oxidative stress leads to a [Ca2+] rise and a pro-inflammatory response in HEPA-E1.
- In mitochondria, NiONPs induce ROS generation, ΨMP collapse and Ca^{2+} overload.
- NiONPs adverse effects could compromise lipid metabolism and reproduction of eels.

GRAPHICAL ABSTRACT



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ABSTRACT

Anthropic activities such as open pit mining, amplify the natural erosion of metals contained in the soils, particularly in New Caledonia, leading to atmospheric emission of nickel oxide nanoparticles (NiONPs). These particles are produced during extraction end up in aquatic ecosystems through deposition or leaching in the rivers. Despite alarming freshwater Ni concentrations, only few studies have focused on the cellular and molecular mechanisms of NiONPs toxicity on aquatic organisms and particularly on eels. Those fish are known to be sensitive to metal contamination, especially their liver, which is a key organ for lipid metabolism, detoxification and reproduction. The objective of this study was to assess *in vitro* the cytotoxic effects of NiONPs on *Anguilla japonica* hepatocytes, HEPA-E1. HEPA-E1 were exposed to NiONPs (0.5–5 μg/cm^2) for 4 or 24 h. Several

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endpoints were studied: (i) viability, (ii) ROS production, SOD activity and selected anti-oxidant genes expression, (iii) inflammation, (iv) calcium signalling, (v) mitochondrial function and (vi) apoptosis. The results evidenced that NiONPs induce a decrease of cell viability and an increase in oxidative stress with a significant superoxide anion production. An increase of mitochondrial calcium concentration and a decrease of mitochondrial membrane potential were observed, leading to apoptosis. These results underline the potential toxic impact of NiONPs on eels living in mining areas. Therefore, eel exposure to NiONPs can affect their migration and reproduction in New Caledonia.

1. Introduction

Nowadays, mining activities represent the second source of heavy metal contamination in soils (Singh et al., 2005). Mine tailings are subject to significant metal leaching due to the high concentration of heavy metals and the low capacity of soils to retain water and metals (Ashraf et al., 2011). Therefore, mining soils are an important source of contamination for watercourse and hence metals remain persistent in aquatic environment for a long time (Nouri et al., 2008). This issue is particularly important in New Caledonia, one of the largest nickel (Ni) producer in the world with resources concentrated in ultramafic formations characterized by a high concentration of metal trace elements including nickel, chromium (Cr), cobalt (Co), and manganese (Mn) that cover nearly 30% of the New Caledonian subsoils (Losfeld et al., 2015; Isnard et al., 2016). Natural erosion, due to the laterite soils fragility and instability (Ambatsian et al., 1997), is amplified by anthropic activities such as mining, and leads to atmospheric emission of ultrafine particles (UFPs), such as Ni UFPs (He et al., 2005; Pasquet et al., 2016). UFPs are called nanoparticles (NPs) once manufactured. The hydrometallurgical process that is used in New Caledonia contributes to the Ni UFPs oxidation that mostly generates nickel oxide NPs (NiONPs), used in many areas of industry (Imran Din and Rani, 2016). In coastal region with nickel industry, like in New Caledonia, the NiONPs production due to the welding fumes is a potential nano-pollution source of freshwater downstream the mine by end up in aquatic ecosystem through deposition, or through leaching, affecting living organisms (Wiesner et al., 2006). In New Caledonia, this pollution is confirmed by high nickel concentrations in rivers in mining areas (12.3–23 μg/L) compared to those away from mining (0.05–2.9 μg/L) whereas the European regulation advises to not exceed 4 μg/L (AEL, ValeNC 2013; Soproner, SLN, 2013) (Migon et al., 2007). The extended use of NiONPs in industry may contribute to an augmentation of particulate matter emission and, consequently, to an increase of bioaccumulation of Ni in fish living in freshwaters. In addition, previous studies have highlighted that exploitation of nickel in New Caledonia contributes to the natural ecosystems and lagoon degradation. Indeed, genetic alterations in the plant such as conifers *Araucaria nemorosa* are reported as well as a significant bioaccumulation of Ni and Cr on coral reef fish (moray eels and congers), sediments and freshwater bivalves (Hedouin et al., 2007; Kettle et al., 2007; Bonnet et al., 2014; Gunkel-Grillon et al., 2014). Some studies have highlighted the significant bioaccumulation of nickel in gills and livers of five fish species (*Khulia rupestris*, *Awaous guamensis* and *Sicyopterus lagocephalus, Sarotherodon occidentalis* and *Anguilla marmorata,* marbled eels) living in rivers under mining influence (DMML project, CNRT-2016). Among those species, *Anguilla marmorata* are particularly interesting. Firstly, eels are good bio-indicators of water metal contamination (Barak and Mason, 1990; van der Oost et al., 2002), especially as predators at the end of the trophic chain, potentially accumulating metals consumed by other species. Secondly, eels have a complex biological cycle with different stages of metamorphoses as previously described (Van Ginneken and Maes, 2005). During their juvenile growth stage, eels live several years in freshwaters accumulating nutrients and storing lipids before beginning several month of migration without eating and fasting reproduction in open sea, tens of thousands of miles away. During this migration, eels use the stored energy to swim and mature their gonads. Therefore, the quality of lipid storage and metabolisation during growth in freshwaters is essential to ensure the success of migration and reproduction (Boetius and Boetius, 1980; Van Ginneken and Maes, 2005). To ensure a good lipid metabolisation, the liver is particularly important. However, eel liver concentrates high levels of different metals like cadmium, mercury, or nickel (Usero et al., 2004; Turan et al., 2020), because it represents the main organ of metal detoxification by scavenging metals on metallothionein (Gonzalez et al., 2006). Nevertheless, accumulation of metals can lead to liver dysfunction, potentially affecting the completion of the fish life cycle. Indeed, it has been previously shown in the European eel that this contamination can affect the lipid storage by altering the expression level of several lipolysis and lipogenesis enzymes leading to an increase in lipid consumption (Pierron et al., 2007). Lipid storage essential to provide the energy necessary for their migration is thus altered (Larsson et al., 1990), and eels have insufficient energy reserves to perform migration and maturation of their gonads, which directly impairs their reproduction (Pierron et al., 2008).

In vitro studies on fish hepatocytes (rainbow trout, orange-spotted grouper, catfish) have shown that metal NPs (copper-NPs; metal oxide NPs and argent-NPs) cause oxidative stress, calcium signalling alteration and mitochondrial dysfunction, which are critical events involved in hepatocytes death (Kunjiappan et al., 2015; Wang et al., 2016; Ostaszewska et al., 2018). Oxidative stress has been identified as the common mechanism of cellular damage and cytotoxicity after NPs exposure (Ivask et al., 2015). The reactive oxygen species (ROS) generation after NPs exposure could then alter the calcium homeostasis (Görlach et al., 2015) which plays in hepatocytes a major role to ensure lipid metabolisation as a co-factor for several enzymes involved in glycolysis, β-oxidation or lipogenesis. Calcium homeostasis is then controlled by intracellular stores (*i.e.*, endoplasmic reticulum, ER, or mitochondria), therefore, the impairment of mitochondrial function may lead to an alteration of calcium homeostasis, and of hepatocytes metabolism by causing histopathologically an alteration of the liver (Arruda et al., 2014; Rieusset, 2017).

The high level of ROS production after NPs exposure has already been observed on hepatocytes from the rainbow trout (Massarsky et al., 2014), such as mitochondrial dysfunction (Bermejo-Nogales et al., 2016). Furthermore, histological alterations due to hepatocyte dysfunction has been observed in spiny eels, *Mastacembelus armatus*, that have been exposed to metals like copper, zinc, or Ni leading to a significant elevation in blood glucose and a decrease of liver glycogen, suggesting glycolytic alterations (Javed and Usmani, 2013). However, although the liver dysfunction is known to play a major role in lipids metabolism's alteration, and consequently in migration and reproduction of eel, the underlying cellular and molecular mechanisms by which NiONPs alter calcium signalling and mitochondrial function in eel hepatocytes have not yet been documented.

In this respect, the objectives of the proposed *in vitro* study were to assess the NiONPs-dependent cellular and molecular mechanisms of toxicity in eel hepatocytes to understand how liver function could be affected. Nowadays there is no hepatocyte cell line of *Anguilla marmorata*, that is why this study was carried out on *Anguilla japonica* (HEPA-E1) hepatocyte cell line, the only existing hepatocyte cell line from eel. However, the two species are genetically very close and share geographical areas as both species of Pacific eel (Kuroki et al., 2009; Chen et al., 2018). In the current study, we investigated the

NiONPs-induced HEPA-E1 (i) cytotoxicity, (ii) oxidative stress with ROS production and antioxidant enzymes activity and transcription, (iii) inflammation with cytokines secretion, (iv) calcium signalling in cytoplasm, ER and mitochondria, (v) mitochondrial dysfunction with mitochondrial mass and membrane potential and (vi) apoptosis with caspase and pro-apoptotic transcription factor.

2. Materials and methods

2.1. Reagents and chemicals

Physiological salt solution (PSS) was composed of: 130 mM NaCl, 5.6 mM KCl, 8 mM HEPES, 11 mM Glucose, 1 mM Mg^{2+} and 2 mM Ca^{2+}; adjusted at pH = 7.4. Krebs-HEPES Buffer (KHB) was composed of 118.4 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 4 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2 mM $CaCl_2$, 10 mM HEPES and 6 mM D-Glucose. Fluo-4-AM green dye (1 μM), MitoSOX™ red dye (5 μM), Fluo-4FF green dye (10 μM), TMRM red dye (100 nM), and Hoechst 33342 blue dye (2 μM) were obtained from ThermoFischer Scientific, Invitrogen™, except Rhod-2 AM red dye (1 μM) from Euromedex®. All of the following chemical reagents were purchased from Sigma-Aldrich® and used as follows: superoxide dismutase–polyethylene glycol (PEG-SOD) and catalase–polyethylene glycol (PEG-CAT), as antioxidant enzymes, diluted in L15 M (300 U/mL and 600 U/mL, respectively).

2.2. Hepatocyte eel

Hepatocyte eel (HEPA-E1) isolated from japonica eel (*Anguilla japonica*) were purchased from Riken BRC Cell Bank® and were cultured in E-RDF medium (ERDF-M) (Kyokuto Pharmaceuticals®) supplemented with antibiotics 1% (Penicillin-Streptomycin, 1000 UI/100 000 UI, Sigma-Aldriech®), sodium bicarbonate $NaHCO_3$ (1130 mg/L), RD-1 supplement E-RDF (Kyokuto Pharmaceuticals®) and 5% of foetal bovine serum (FBS, Eurobio®) following manufacturer's guidelines. For all experiments cells were cultured in Leibovitz L-15 medium (L15 M) (Fischer Scientific®) supplemented with antibiotics 1%, 5% of FBS and 3% of GlutaMAX supplement (Fischer Scientific®) following manufacturer's guidelines. For all experiments, cells were seeded at the initial concentration of 20 000 cells/cm^2 per well in a 96, 24 or 12-well plate [corresponding respectively to 6600 cells/well (0.33 cm^2), 38 000 cells/well (1.9 cm^2) and 70 000 cells/well (3.5 cm^2)] and cultured for 72 h, at 28 °C before assay.

2.3. NiO nanoparticles

2.3.1. NiO nanoparticles characterization

Nickel (II) oxide nanoparticles (NiONPs) (Cat. No.637130) were purchased from Sigma-Aldrich®. These particles were already described by (Di Bucchianico et al., 2018; Germande et al., 2022). Their mean aerodynamic diameter was 50 nm (as assessed by Transmission Electron Microscopy, TEM) and their characterization is presented in SI1. NPs were suspended in L15 M at a final stock solution concentration of 2 mg/mL. Prior to use, stock suspensions were vortexed, sonicated at 3 × 30 s (Vibracell 75186, 130 W, 56–60 Hz) and diluted extemporaneously in L15 M at appropriate concentrations for the different experiments (0.5–50 μg/cm^2). These concentrations were chosen on the basis of previous studies and expressed in μg/cm^2 due to the NiONPs sedimentation in wells (Germande et al., 2022), corresponding respectively to 0.82–82.5 μg/mL in a 96-well plate (0.33 cm^2); 0.9–9.5 μg/mL in a 24-well plate (1.9 cm^2) and 1.2–12 μg/mL in a 12-well plate (3.5 cm^2). Concentrations (up to 5 μg/cm^2) reflect the atmospheric concentrations found in Noumea during pollution peaks (ScalAir) before sedimentation in the freshwaters (Gunkel-Grillon et al., 2014; Pasquet et al., 2018). Two time points were performed (4 h or/and 24 h) according to kinetic preliminary studies (results not shown).

2.3.2. NiONPs internalization

Transmission electronic microscopy (TEM) was used to study NiONPs internalization. Cells were seeded on plastic Lab-Teck chambers (Dutsher®), and cultured in supplemented L15 M before been exposed or not to NiONPs at 5 μg/cm^2. After a 24 h-exposure to NiONPs, TEM process was performed as previously described (Deweirdt et al., 2017; Germande et al., 2022).

2.4. Cytotoxic assay

Cell viability was assessed by using the WST-1 (Water Soluble Tetrazolium) cell proliferation reagent (Roche®) according to the manufacturer's recommendations and as already been described by (Deweirdt et al., 2017; Germande et al., 2022). After a 24 h - exposure with NiONPs (0.5, 1, 2.5, 5, 10, 25 and 50 μg/cm^2), cells were rinsed with L15 M and incubated with WST-1 reagent. The absorbance was measured at 450 nm by spectrophotometry using a microplate spectrophotometer reader (SPECTROstarNano2.10, BMG Labtech®, Germany). The working concentrations for all further experiments in which mortality rate was less than 30% were all determined with this test (0.5–5 μg/cm^2).

2.5. Oxidative stress

Cells were exposed to NiONPs (0.5–5 μg/cm^2) for 4 or 24 h.

2.5.1. Global reactive oxygen species (ROS) production

Intracellular ROS production was assessed using the CM-H_2DCFDA probe (Fisher Scientific®), according to the manufacturer's recommendations and adapted and described from previous studies (Chen et al., 2010; Germande et al., 2022). Before a 4 h - NiONPs -exposure cells were pre-incubated with CM-H_2DCFDA probe (final concentration 20 μM). Fluorescence intensity was measured by spectrofluorimetry at 485/520 nm (excitation/emission) by using FLUOstar Omega 2.10 plate reader, and the analyses were performed with MARS Data Analysis Software 2.30 R3 (BMG Labtech®).

2.5.2. Cytoplasmic superoxide anion ($O_2^{\bullet-}$) production

Cytoplasmic $O_2^{\bullet-}$ detection was performed by electron paramagnetic resonance (EPR)-spin probe using CMH (1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine) (500 μM), diethyldithiocarbamate (5 μM) and deferoxamine (25 μM) probes in KHB solution as previously described by (Germande et al., 2022). After a 4 h NiONPs - exposure, cells were incubated with probes before EPR analysis. All the EPR spectra were recorded using Spectrometer X Miniscope MS200 (Magnettech®). Following EPR spectra readings, protein quantities were measured by a Lowry-test (Lowry reagent, Sigma-Aldrich®), according to the manufacturer's recommendations. The results were normalized to protein quantities and expressed in EPR signal amplitude in arbitrary units AU/(mg/mL) of protein after a Lowry test.

2.5.3. Mitochondrial $O_2^{\bullet-}$ production

Mitochondrial $O_2^{\bullet-}$ production was assessed using MitoSOX™ red dye (ThermoFischer®), according to the manufacturer's recommendations by confocal microscopy (TE 2000, Nikon) and as previously described by (Germande et al., 2022). After a 4 h-exposure to NiONPs, cells were incubated with MitoSOX probe in PSS and with a nucleus probe (Hoechst 33342). Fluorescence intensity was measured for MitoSOX at 543/605 nm and Hoechst at 408/450 nm (excitation/emission). MitoSOX fluorescence intensity is proportional to the rate of the probe oxidation. Analyses were performed using NIS-Elements AR software 3.0 and Microsoft Office Excel 2007.

2.5.4. Superoxide dismutase enzyme activity

The superoxide dismutase (SOD) activity was measured with a commercially available kit (19160 SED determination kit, Sigma-

Aldrich®) and determined by utilizing Dojindo's highly WST_1 as previously described by (Germande et al., 2022). After a 24 h-exposure with NiONPs supernatants and cells were used for analysis to determine both intracellular and extracellular SOD activity. Absorbance of supernatants and cells was measured at 450 nm by spectrophotometry using a microplate spectrophotometer reader (SPECTROstarNano2.10, BMG Labtech®).

2.6. Pro-inflammation effect: cytokines IL-6 production

Cells were exposed to NiONPs for 24 h and supernatants were collected, centrifuged at 10 000 g for 10 min at 4 °C and analysed using an ELISA assay kit (IL-6 DuoSet® ELISA R&D Systems) according to the manufacturer's guidelines. The absorbance was measured at 450 nm, corrected at 570 nm by spectrophotometry using a microplate spectrophotometer reader (SPECTROstarNano 2.10, BMG Labtech®).

2.7. Cytoplasmic, Endoplasmic reticulum and mitochondrial calcium measurement

Variations in intracellular calcium $[Ca^{2+}]_c$, ER calcium $[Ca^{2+}]_{ER}$ and mitochondrial calcium $[Ca^{2+}]_m$ were detected respectively using the Fluo-4-AM green dye, the Fluo-4FF AM green dye and the Rhod-2 AM red dye according to the manufacturer's guidelines. After a 4 h-exposure with NiONPs, cells were incubated with different probes in PSS and co-incubated with Hoechst 33342. The plates were observed at ×200 magnification with an oil immersion objective on a laser scanning confocal microscope (TE 2000, Nikon). The fluorescence intensity was measured for Rhod-2 at 543/605 nm, for Fluo-4-AM and Fluo-4FF at 488/515 nm and for Hoechst at 408/450 nm (excitation/emission). For each experiment 20 at 30 cells were analysed per well. The analyses were performed using NIS-Elements AR software 3.0 and Microsoft Office Excel 2007.

2.8. Mitochondrial activity

Cells were exposed to NiONPs (0.5–5 μg/cm^2) for 4 h.

2.8.1. Mitochondrial membrane potential measurement

The mitochondrial membrane potential (ΨMP) was measured using confocal microscopy (TE 2000, Nikon) by TMRM red dye (Tetramethylrhodamine, Methyl Ester, Perchlorate) (ThermoFischer®) according to the manufacturer's guidelines. After a 4 h-exposure with NiONPs cells were incubated with TMRM probe in PSS co-incubated with Hoechst 33342. The plates were observed at ×200 magnification with an oil immersion objective on a laser scanning confocal microscope (TE 2000, Nikon). Fluorescence intensity was measured at 543/605 nm (excitation/emission). The analyses were performed using NIS-Elements AR software 3.0 and Microsoft Office Excel 2007.

2.8.2. Mitochondrial mass

The mitochondrial mass was measured using confocal microscopy by Mitotracker green dye (ThermoFischer®) according to the manufacturer's guidelines. After a 4 h - exposure with NiONPs cells were incubated with Mitotracker probe in PSS co-incubated with Hoechst 33342. The fluorescence intensity was measured at 408/450 nm (excitation/emission). The analyses were performed using NIS-Elements AR software 3.0 and Microsoft Office Excel.

2.9. Apoptosis induction

The apoptosis was measured using confocal microscopy (TE 2000, Nikon) by caspase 9 green dye (ImmunoChemistry Technologies®) according to the manufacturer's guidelines. After a 24 h-exposure with NiONPs, cells were incubated with caspase 9 fluorescent probe in PSS co-incubated with Hoechst 33342. The plates were observed at ×200 magnification with an oil immersion objective on a laser scanning confocal microscope (TE 2000, Nikon). The fluorescence intensity was measured at 488/515 nm (excitation/emission). The analyses were performed using NIS-Elements AR software 3.0 and Microsoft Office Excel 2007.

2.10. Gene expression

A real time quantitative polymerase chain reaction (qPCR) was performed to evaluate mRNA expression of *cav2*, *cltb*, $sod_{Zn/Cu}$, *cat*, *mt*, *cox1*, *atp6-8*, *acc* and *bax* (details on SI2). Cells were exposed to NiONPs for 24 h then, mRNA extraction and purification were performed using a commercially available kit (SV Total RNA Isolation System, Promega), according to the manufacturer's recommendations and as previously described by (Pierron et al., 2009). The elongation factor-1 α (*ef1-α*), ribosomal protein L7 (*rpl7*) and β-actin (*β-act*) genes were used as internal controls. The relative quantification of each gene expression level was normalized against the mean housekeeping genes *ef1α*, *β-act* and *rpl7*, and changes in Ct values were recorded. From this comparison, fold-change factors were obtained according to $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primer sequences used are described in SI2.

2.11. Statistical analysis

Each experimental condition was independently repeated several times (n indicates the number of experiments) and for each independent experiment, 3 to 8 wells per condition were assessed. Data are expressed as mean ± Standard Error of the Mean (SEM) for n independent experiments. Assumptions necessary for using the parametric test were checked (normality, independence and homoscedasticity of the error term). Statistical analyses were performed using either one-way ANOVA followed by Tukey's post-test for multiple comparisons ($n > 30$), or Kruskal Wallis test followed by Dunn's post-test ($n < 30$). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$). All data were analysed using GraphPad statistics software. P values < 0.05 were considered statistically significant.

3. Results

After internalization, NiONPs were mainly clustered in vesicles in the cytoplasm (Fig. 1B), compared to their absence in controls (Fig. 1A). Internalized NPs were mostly organized in aggregates of various sizes. Single or isolated NPs were rarely observed. NiONPs were also localized near the nucleus and near the mitochondria. These results were confirmed by the qPCR assay which showed a significant increase of the expression of two membrane proteins involved in the internalization (i.e.,caveolin-2 and clathrin-b), (*$P < 0.05$) as compared to untreated cells (Fig. 1C and D). The WST-1 cytotoxic assay evaluated on HEPA-E1 after a 24-h treatment with NiONPs showed a significant and concentration – dependent decrease of cell viability from 25 μg/cm^2 as compared to untreated cells (Fig. 1E).

After a 4 h-exposure of HEPA-E1 to NiONPs we observed a significant and concentration-dependent decrease in intracellular global ROS production as compared to untreated cells (Fig. 2A). Nevertheless, a significant increase in intracellular and mitochondrial $O_2^{\bullet-}$ production as compared to untreated cells is observed (Fig. 2B and C). After a 24 h-exposure to NiONPs IL-6 secretion was observed from 2.5 μg/cm^2 (Fig. 2D). After a 24 h-exposure of HEPA-E1 to NiONPs a significant increase in $sod_{cu/zn}$, *cat* and *mt* expressions was observed at the highest concentration as compared to the untreated cells (Fig. 2E, F and 2G) but a significant decrease in SOD activity was observed at the three concentrations compared to the control (Fig. 2H).

After a 4 h-exposure of HEPA-E1 to NiONPs a significant concentration-dependent decrease of the basal cytoplasmic and ER calcium level as compared to untreated cells was observed (Fig. 3A and B). Nevertheless, the results showed a significant concentration-

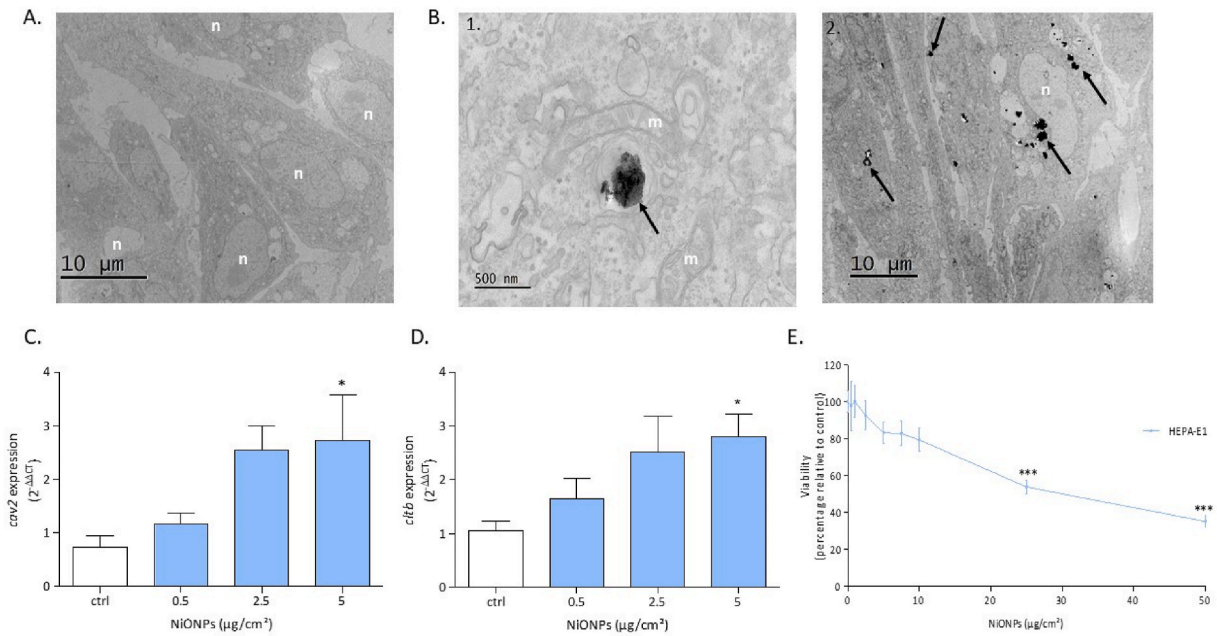

Fig. 1. (A) HEPA-E1 observed by TEM, control (X2500) and (B1 and 2) NiONPs (5 µg/cm^2) (X30000). Blacks arrows show intracellular NiONPs aggregate, n: nucleus, m: mitochondria. (C) cav2 mRNA expression and (D) cltb mRNA expression. Data were mean ± SEM of five independent experiments, n = 5 (three wells/concentration). (E) Cell viability after a 24 h-exposure with NiONPs (1–50 µg/cm^2). Results are expressed as the percentage of cell viability in treated cells, as compared to control cells (100%). Data are mean ± SEM of four independent experiments, n = 4) (eight wells/concentration). Statistically significant at $P < 0.05$(*), $P < 0.01$(**) and $P < 0.001$(***) as compared to untreated control cells.

dependent increase of the basal mitochondrial calcium level (Fig. 3C). A pre-treatment of 1-h with PEG-SOD and PEG-CAT, prior to a 4 h-exposure of HEPA-E1 to NiONPs didn't induce significant alteration in cytoplasmic or mitochondrial calcium levels compared to untreated cells but significantly decreased the ER calcium level at the highest concentration 5 µg/cm^2. These results indicate that ROS production is involved in Ca^{2+} signalling impairment. As compared to controls, after a 4 h-exposure of HEPA-E1 to NiONPs, the ΨMP significantly decreased from 0.5 µg/cm^2 as compared to untreated cells (Fig. 3D), effect reversed by a pre-treatment of 1-h with PEG-SOD and PEG-CAT. The mitochondrial mass significantly increased from 2.5 µg/cm^2 (Fig. 3E).

Finally after 24-h exposure, qPCR results showed a significant increase of mitochondrial metabolism genes expression for *cox1*, *atp6-8* and *acc* as compared to control cells at 5 µg/cm^2 (Fig. 4A, B and 4C) and a significant concentration-dependent increase of apoptotic cells with a caspase 9 increased (Fig. 4D), confirmed by a significant increase of *bax* mRNA expression in HEPA-E1 with NiONPs (Fig. 4E).

4. Discussion

In New Caledonia among species affected by Ni mining activities, Pacific eels such as marbled eels, *Anguilla marmorata*, are endemic fishes known to be particularly sensitive to water metal contamination (Durrieu et al., 2005). The liver is the main organ for lipids metabolism, essential for growth, migration and reproduction of eels, but liver is also the main site of metal bioaccumulation. So, it appears important to study the mechanisms of NiONPs toxicity on eel hepatocytes, which to our knowledge has never described despite only a few *in vitro* studies that investigated the direct effect of some metal NPs on fishes hepatocytes but the NiONPs toxicity on eel hepatocytes remains unknown. Note that our study had to be done with another species of Pacific eel, *Anguilla japonica*, the only existing hepatocyte cell line from eel nowadays, however the two species are genetically and geographically very close (Arai et al., 2002; Kuroki et al., 2009; Chen et al., 2018).

It is well - known that oxidative stress and calcium signalling have a key role in hepatocyte regulation, and that the calcium homeostasis alteration may be involved in liver dysfunctions (Amaya and Nathanson, 2013; Madreiter-Sokolowski et al., 2020). Indeed, *in vitro* studies using primary hepatocytes from *Epinephelus coioides* or *Oncorhynchus mykiss*, have demonstrated the cytotoxicity of metallic NPs leading to cytotoxicity and to ROS production generation (Massarsky et al., 2014; Wang et al., 2016). However, the underlying cellular and molecular mechanisms involving the oxidative stress and mitochondrial dysfunction remain unclear.

When HEPA-E1 were exposed to NiONPs, these particles were internalized into cells, confirmed by the interaction with the membrane proteins involved in internalization (*cav2* and *cltb*). Similar to other metallic NPs, the internalization of NiONPs appears to be a key factor for their toxicity (Munoz and Costa, 2012). The NiONPs cytotoxicity in HEPA-E1 was evaluated to select the concentrations (mortality rate <20%) used for further mechanistic experiments. Interestingly, the concentrations ranged from 0.5 to 5 µg/cm^2, reflecting the nickel concentrations in freshwater downstream mines in New Caledonia and are realistic concentrations close to the ones found from NiONPs exposure (AEL, ValeNC 2013; Soproner, SLN, 2013). Another important mechanism leading to metal oxide NPs cytotoxicity, is probably an overproduction of ROS (Ivask et al., 2015), due to the ion reactivity. In fact, NiONPs generate dissolved Ni in the HEPA-E1 culture medium. Moreover, Ni is proposed to cycle between Ni^{2+} and Ni^{3+}, catalysing the reduction of H_2O_2 leading to free radical generation like hydroxyl radical ($OH^{\cdot}$) and $O_2^{\cdot-}$ (Torreilles and Guerin, 1990). Our present study demonstrates that in HEPA-E1, NiONPs decreased the global ROS production and increased both cytoplasmic and mitochondrial $O_2^{\bullet-}$. The fluorimetric method used to assess global ROS production is a powerful assay, easy to carry out, and it can detect different ROS like H_2O_2, however, this assay does not allow for determining free radicals like $O_2^{\bullet-}$ due to their very short half-life (Wang and Joseph, 1999; Oparka et al., 2016). Therefore, we hypothesized that NiONPs might decrease H_2O_2 production. As regards as the antioxidant enzymes after ROS overproduction, the antioxidant system induced the production of antioxidant enzymes to maintain the redox balance (Sies et al., 2017). However, some authors have shown a decrease in antioxidant enzyme

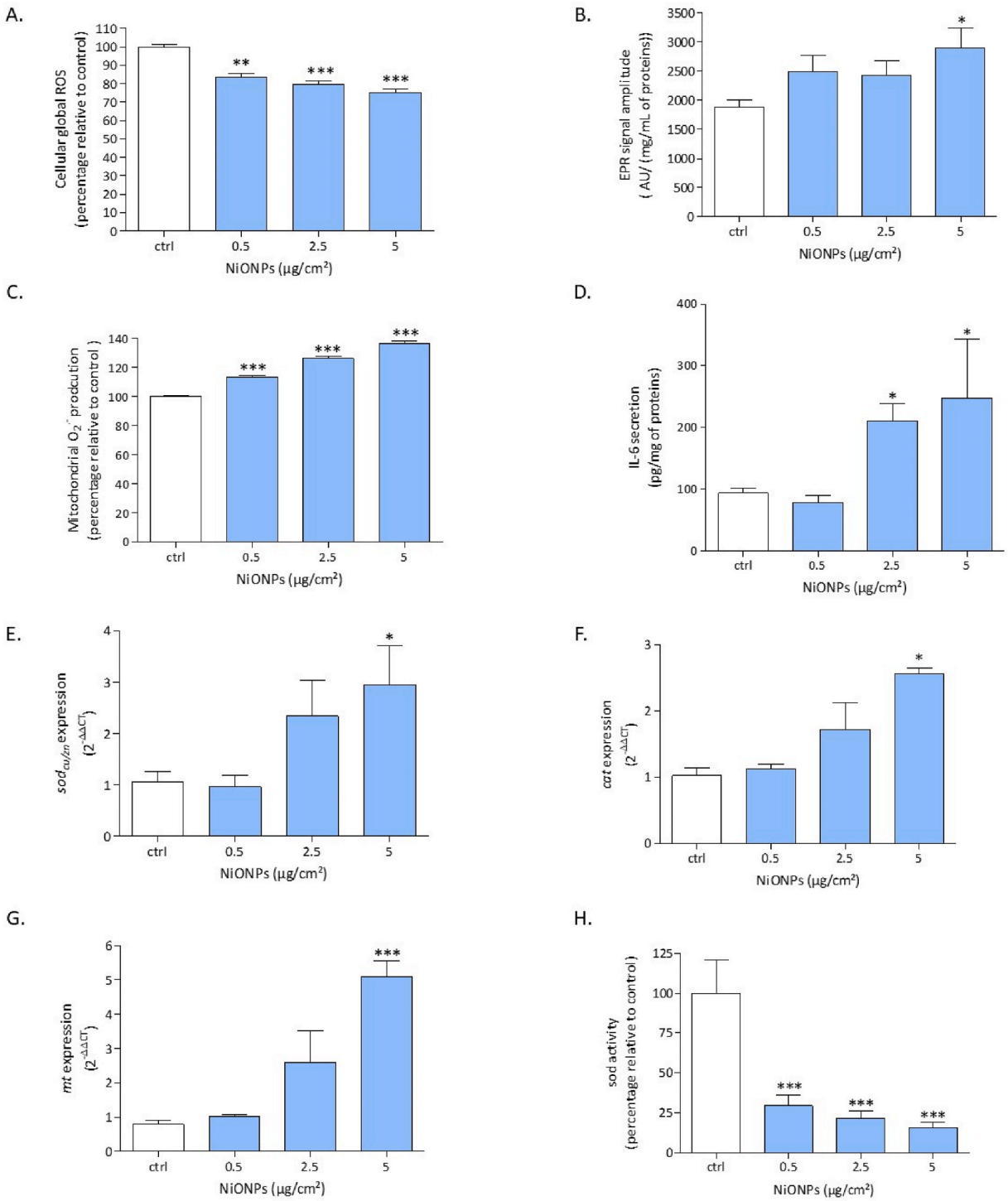

Fig. 2. (A) Global ROS production after 4 h-exposure with NiONPs (percentage of ROS production relative to control 100%, mean ± SEM of four independent experiments, n = 4 (three wells/concentration)). (B) Intracellular O2•⁻ production after 4 h-exposure with NiONPs (EPR signal amplitude in arbitrary units (AU)/mg/mL of proteins). (C) Mitochondrial O2•⁻ production after 4 h-exposure with NiONPs (percentage of MitoSOX probe fluorescence intensity relative to control cells 100%). (D) IL-6 secretion after 24 h-exposure with NiONPs (pg/mg of proteins, mean ± SEM of six independent experiments, n = 6 (three wells/concentration)). (E) sodcu/zn mRNA expression (F) cat mRNA expression (G) mt mRNA expression (mean ± SEM of five independent experiments, n = 5 (three wells/concentration)). (H) SOD activity in HEPA-E1 after NiONPs-exposure (values normalized to the untreated cells and expressed as the SOD activity inhibition rate relative to the control cells, mean ± SEM of three independent experiments, n = 3 (three wells/concentration)). Statistically significant at $P < 0.05$ (*), $P < 0.01$(**), and $P < 0.001$(***), as compared to untreated control cells.

activity, like *sod* and *cat*, in liver tissue of juvenile carp (*Cyprinus carpio*) after TiO_2NPs exposure (Hao et al., 2009), that could be explained by a direct interaction between NPs and antioxidant enzymes, which undergoes a functional change (Zhang et al., 2019). In our study, similar results were observed with an increase in $sod_{cu/zn}$ and *cat* transcriptions and a decrease in SOD activity. The NiONPs seemed then to interact with SOD at the post-transcriptional level by inhibiting its activity. This decrease of activity may have explained the NiONPs-induced $O_2^{\bullet-}$

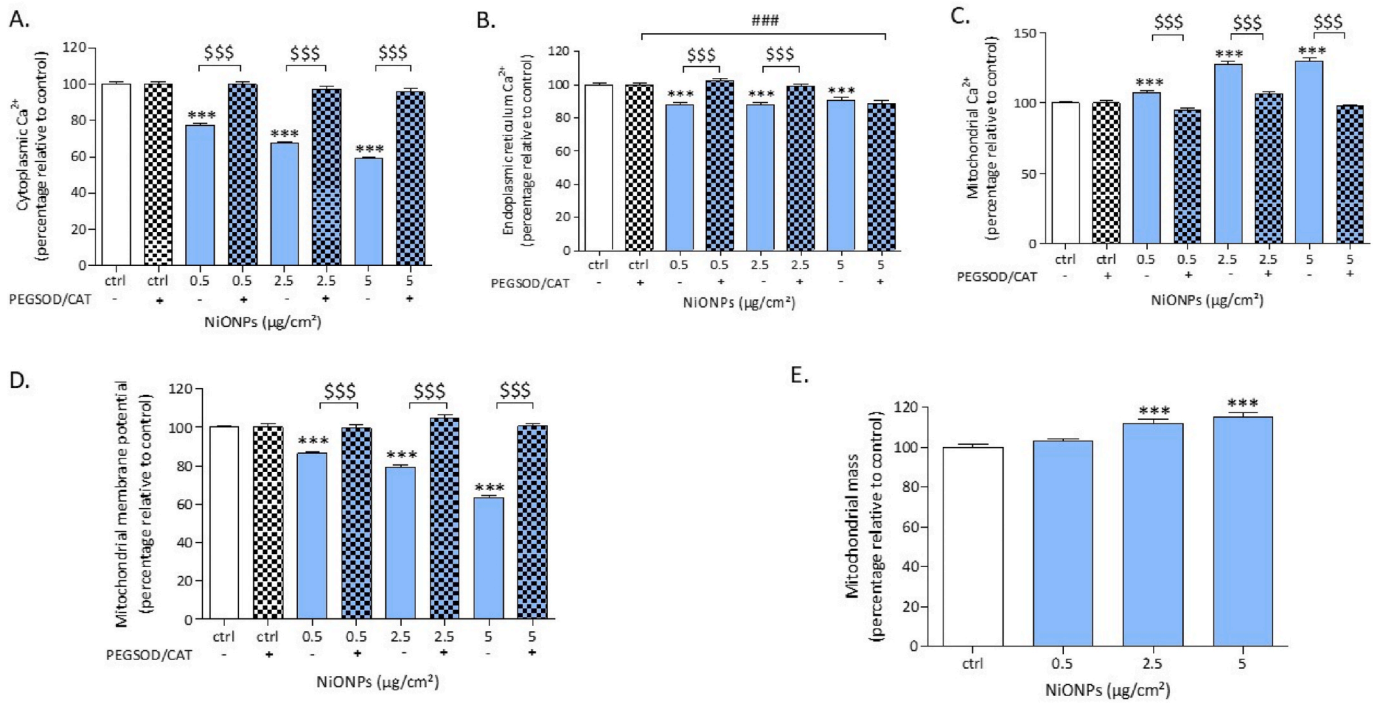

Fig. 3. Cytoplasmic ([Ca2+]i), ER ([Ca2+]ER), mitochondrial calcium ([Ca2+]m), ΨMP and mitochondrial mass. Cells are treated or not with antioxidant enzymes (PEGSOD/CAT) 1 h before 4 h-exposure with NiONPs. Results are expressed as the percentage of the probes fluorescence intensity relative to the control cells (100%). (A) [Ca2+]i (B) [Ca2+]ER (C) [Ca2+]m. (D) ΨMP (E) Mitochondrial mass. Data are mean ± SEM of three independent experiments, n = 3 (six wells/concentration). Statistically significant at P < 0,05(*) and P < 0.001(***), as compared to untreated cells, statistically significant at P < 0.001($$$), as compared to cells pre-treated with antioxidants enzymes.

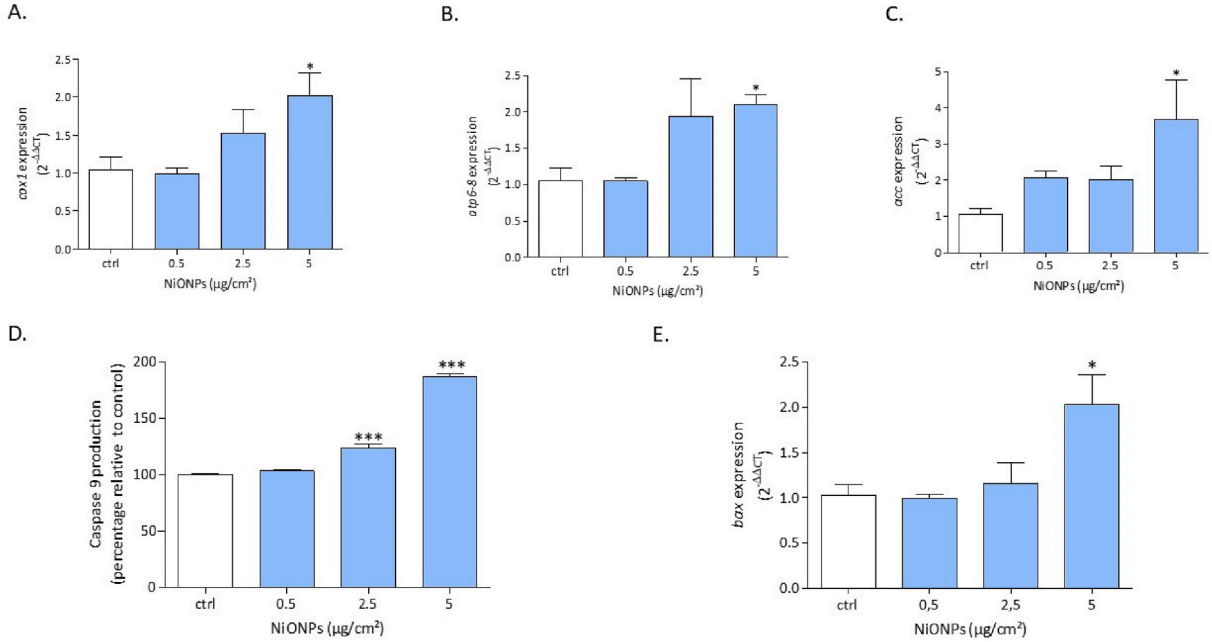

Fig. 4. Cells were exposed 24 h with NiONPs (0.5–5 µg/cm^2). qPCR results are expressed as the fold change of gene mRNA expression relative to the control cells (mean ± SEM of five independent experiments, n = 5 (three wells/concentration)). (A) cox1 mRNA expression (B) atp6-8 mRNA expression (C) acc mRNA expression. (D) Caspase 9 production (percentage of the caspase 9 probe fluorescence intensity relative to control 100%). (E) bax mRNA expression. Statistically significant at P < 0.05 (*), P < 0.01(**) and P < 0.001(***) as compared to untreated control cells.

production in HEPA-E1, and suggested that the generated $O_2^{\bullet -}$ is not or less dismutated in H_2O_2 by the SOD. The $O_2^{\bullet -}$ is therefore permanently overproduced without being detoxified, leading to a decrease in H_2O_2 production. Our observation showed good agreement with previous published data on human hepatocytes after carbon and copper oxide NPs exposure (Adeyemi et al., 2020). However, detoxification proteins such as metallothionein appear to be activated, probably in response to dissolved nickel release.

It has been demonstrated that ROS generated by metallic oxide NPs might activate the inflammatory pathways via the secretion of cytokines (Zhang et al., 2012) like IL-6, an important cytokine to control the immune systems in fish. The IL-6 expression in eel has already been described (Castellana et al., 2008; Birhanu et al., 2016; Zhu et al., 2016). Our results are in accordance with those previous studies, with a pro-inflammatory response characterized by an increase in IL-6 secretion after NiONPs exposure, which may induce impairment of liver function.

Oxidative stress is also known to play a key role in the physiopathology of liver diseases by altering the calcium homeostasis and the mitochondrial function in hepatocytes (Gordeeva et al., 2003; Madreiter-Sokolowski et al., 2020). Our study shows that the alteration of calcium homeostasis may originate from oxidative stress. Indeed, the [Ca2+] alterations were reduced by a pre-treatment with antioxidant enzymes and such pre-treatment fully restored calcium homeostasis. Due to decrease of basal cytoplasmic and ER calcium levels and the increase of basal mitochondrial calcium level, we hypothesize that the mitochondria can pumping calcium from the cytoplasm and ER, as previously observed in some liver dysfunctions (Amaya and Nathanson, 2013). Some authors have also suggested the important role of calcium homeostasis in hepatic regulation in eels (Fabbri et al., 1998). This excess of calcium could then activate the mitochondrial biogenesis, confirmed by the increase in mitochondrial enzyme transcription *atp6-8* and mitochondrial mass in our study. Nevertheless, the increase in *cox1* shows an alteration of the mitochondrial respiratory chain probably in connection with altered mitochondria activity as suggested by the decrease of ΨMP maintained over the time (results after 24 h-exposure with NiONPs are not shown). It is well known that ROS, like $O_2^{\bullet-}$, can induce a peroxidation of mitochondrial membrane lipids, leading to a ΨMP impairment (Cadenas and Davies, 2000). This later mechanism was confirmed in our current study because of the unchanged ΨMP as compared to control cells when cells are pretreated with antioxidant enzymes. All these alterations can then dysregulate the lipid metabolism (Kroemer et al., 2007; Prakash et al., 2017). The modification of ΨMP could activate mechanism of cell death like apoptosis to offset the production of altered mitochondria (Kroemer et al., 2007). Indeed, apoptosis after NiONPs exposure is observed with an increase of pro-apoptotic factor transcription *bax* and caspase 9 production.

Overall, our results show that NiONPs alter the expression of many genes involved in different functions that demonstrating the toxic potential of these NPs. The integration of transcriptomic method in our study provides strong evidence of molecular and potentially functional alterations in fish livers. Our results demonstrate with different approaches, that the main mechanism of cell death after NiONPs exposure involves apoptosis via mitochondria alteration, that might contribute to liver dysfunction leading to faulty lipid metabolism. NiONPs exposure might contribute to alter the quality of migration and maturation of eel and potentially their reproduction.

5. Conclusion

The current study describes the mechanisms of action of NiONPs on eel hepatocytes. Indeed, NiONPs induce an intracellular ROS overproduction in HEPA-E1, then leading to a pro-inflammatory response and a [Ca^{2+}] rise. Disruption of intracellular calcium homeostasis might be explained by oxidative stress. In addition, NiONPs trigger a liver toxicity through the induction of mitochondrial dysfunction characterized by mitochondrial ROS generation, ΨMP collapse and mitochondrial Ca^{2+} overload. Furthermore, we have demonstrated an apoptosis of hepatocytes, NiONPs-induced as a consequence of these toxic effects.

This study also suggests that marbled eels living in mining-influenced freshwaters could decline in population due to the high liver toxicity caused by Ni particles emitted by mining activities in New Caledonia. Indeed, owing to the fact that liver is known to play a major role in lipid metabolism and by consequence in the success of reproduction of eels, exposure of *Anguilla marmorata* to NiONPs and in a broader extend of freshwater organisms leaving in these rivers, may put them at risk to reproduce and then could impact all the aquatic ecosystem.

Statement of contributions

Germande Ophélie : Conceptualization, Formal Analysis, Methodology, Visualization, Writing – original draft.

Beaufils Fabien : Methodology, Writing – review & editing.

Daffe Guillemine : Methodology, Writing – review & editing.

Gonzalez Patrice : Methodology, Writing – review & editing.

Mornet Stéphane : Resources, Methodology, Writing – review & editing.

Bejko Megi : Resources, Formal Analysis, Writing – review & editing.

Errera Marie-Hélène : Writing – review & editing.

Lacomme Sabrina : Resources, Writing – review & editing.

Gontier Etienne : Resources, Writing – review & editing.

Guibert Christelle : Writing – review & editing.

Baudrimont Isabelle : Project administration, Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing – review & editing.

Baudrimont Magalie : Project administration, Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.135158.

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