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Research Paper

On virus and nanomaterials – Lessons learned from the innate immune system – ACE activation in the invertebrate model *Enchytraeus crypticus*

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HIGHLIGHTS

- SARS Cov-2 spike proteins attach to ACE2 human receptors.
- *Enchytraeus crypticus* innate immune system gene family is expanded (e.g. ACE).
- Nanomaterials (NMs) are often perceived as invaders, covered by a corona, like virus.
- Extensive knowledge from response to NMs can support the immuno-research.
- Assembled ACE gene expression database provides cross species exploration basis.

ABSTRACT

Current human research on COVID-19 – SARS-CoV-2 (Severe Acute Respiratory Syndrome-Corona Virus) showed that ACE2 (Angiotensin Converting Enzyme 2) is a functional receptor to which the spike proteins attach. Invertebrates have been exposed to a wide array of threats for millennia and their immune system has evolved to deal with these efficiently. The annelid *Enchytraeus crypticus*, a standard ecotoxicological species, is an invertebrate species where extensive mechanisms of response studies are available, covering all levels from gene to population responses. Nanomaterials (NMs) are often perceived as invaders (e.g. virus) and can enter the cell covered by a corona, triggering similar responses. We created a database on *E. crypticus* ACE gene expression, aiming to analyse the potential knowledge transfer between invertebrates and vertebrates. Total exposure experiments sum 87 stress conditions for 18 different nanomaterials (NMs). ACE expression following TiO₂ NM exposure was clearly different from other NMs showing a clear (6–7 fold) ACE down-regulation, not observed for any other NMs. Other NMs, notably Ag NMs, and to some extent Cu NMs, caused ACE up-regulation (up to 4 fold). The extensive knowledge from response to NMs can support the immuno-research community, especially to develop therapies for virus that trigger the innate immune system.

1. Introduction

A lot of the current human research on COVID-19 (SARS-CoV-2, Severe Acute Respiratory Syndrome-Corona Virus) focus on the observation that ACE2 (Angiotensin Converting Enzyme), a functional receptor in the airways, attach to the spike proteins of SARS-CoV-2 (Vardhana and Wolchok, 2020). A lot of research has over the year also focussed on the uptake of nanoparticles into cells (which also may...
be mediated via surface receptors followed by endocytosis and how the uptake affects cell responses. In this context, it is worth-while to remember that responses to stress are well-known to be one of the most conserved mechanisms across taxa, i.e. across species, including the ACE related mechanisms. The conservation of functionality has been the foundation for much of the research developed on surrogate human model species. Examples of such species, that also have reached considerable advanced -omic status in science, include e.g. Saccharomyces cerevisiae (Fungi), Drosophila melanogaster (Animal), Arabidopsis thaliana (Plant). In the field of ecotoxicology there are several model species, aiming to cover the environmental diversity. The aquatic compartment is probably the most well studied, e.g. where Daphnia magna (OECD, 2004a) stands out, followed by the terrestrial compartment, e.g. with the equivalent Eisenia fetida (OECD, 1984) and Enchytraeus crypticus (OECD, 2004b). Hence, there is a long history of testing environmental hazards using relevant species, with certain model ecotoxicological species reaching a highly developed status in terms of tools and methods to assess the mechanisms of response to toxins, this besides the phenotypical effects. Further, there is a large body of knowledge on how to use these endpoints to develop adverse outcome pathways (for details see the recent review (Gomes et al., 2021a)).

When it comes to human health (toxicology), in vitro cell models are the most routinely used, with few in vivo surrogate animal models available, namely mouse and rat (Morgan et al., 2013), as e.g. required for safety risk of new drugs release. The importance of moving towards non-animal alternative testing methods and models has been emphasised and has even been implemented in Europe, e.g. via legal frameworks like REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) and the Cosmetics Regulation (Scholz et al., 2013). Invertebrates have been exposed to a wide array of threats for millennia and their immune system has evolved to deal with these efficiently. In earthworms, the cells involved in the defence mechanisms are the coelomocytes, the equivalent circulating and sessile blood cells. Coelomocytes can respond via material uptake and elimination based on endocytosis, enzyme activation (e.g. lysozyme), reactive oxygen species (ROS) formation, metabolic activation, and antimicrobial protein (AMP) production (e.g. defensins). A comparison between earthworm and human in vitro response to Ag NMs (Hayashi et al., 2012) showed a similar response between the coelomocytes and differentiated (macrophage-like) THP-1 cells, and intracellular accumulation of Ag NMs in the coelomocytes occurred. Besides this immune memory is a key defensive mechanism that allows invertebrate organisms to adapt [note this is different from the adaptive immunity in vertebrates, although also present in mammals (Wendeln et al., 2018)].

In humans, macrophages are activated by damage-associated molecular patterns (DAMPs) (e.g. intracellular contents released from dying cells and/or proteins released following tissue injury, such as heat-shock proteins, hyaluronan fragments, or heparin sulphate) or pathogen-associated molecular patterns (PAMPs) (e.g. viral RNA or oxidized phospholipids). Both DAMPs and PAMPs activate multiple innate immune pathways, through either TLRs (Toll Like Receptor), NLRP3 (NOD-Like Receptor)/inflammasome activation or triggering of cytoplasmic DNA sensors such as cGAS-STING and RIG-1-MAVS. The inflamatory cascade initiated by macrophages leads to both viral control and tissue damage. For instance, in plants, NOD-like intracellular receptors (NLR) can recognize some virulence factors that trigger a faster defence response (ROS production, MAPK activation), but that can also lead to a hypersensitive response resulting in cell death of the infected parts, restricting nutrition to the invading pathogen (Boraschi et al., 2020). As seen, the immune innate building blocks are ancient, some were lost in evolution but other incorporated in vertebrates adaptive immunity (Buchmann, 2014), hence there is great potential for cross-species extrapolation between invertebrate knowledge to vertebrae. Exposure to nanomaterial (NM) contamination also activates the innate immune system via different mechanisms (Gomes et al., 2018a, 2019a, 2017). Often when NMs enter a biological environment they become covered with a corona (proteins, sugars or other compounds) which masks the surface. The composition of the corona depends on the composition and charge of the primary particle, and this corona may have patches of different surface charge depending on the corona composition (Sheibani et al., 2021). Viruses in general have negative charge, at pH 7, but also have patches of different charges due to various surface compounds (Joonaki et al., 2020). In the following it may be remembered that SARS-Cov-2 probably have a size of 70–90 nm (Kim et al., 2020; Park et al., 2020), whereas the NMs from the current paper are generally smaller (10–20 nm). Recognition can first occur upon interaction with surface receptors typically innate immune pattern recognition receptors (PRRs) (Boraschi et al., 2020), similar to the surface receptors of viruses. Therefore, NMs are often handled by cells, like viruses, and can activate similar mechanisms (Hayashi et al., 2012). Still recognising viruses replicate within the cell, which eventually leads to cell death (Icard et al., 2020), it is important to transfer this knowledge, often shown to promote development in diverse areas and even inspire innovative therapeutic alternatives (Scott-Fordsmand and Amorim, 2021).

In humans ACE2 is a functional receptor in the airways, and the spike proteins of SARS-CoV attach to ACE2 – this is known to be a key initiating event for the transmission of SARS coronavirus. SARS-CoV-2 is a compelling case of innate immune hyperactivity (Vardhana and Wolchok, 2020). As to ACE, the renin enzyme cleaves angiotensinogen protein (Agt) to angiotensin I (Ang I). Ang I is then converted to Ang II by angiotensin converting enzyme 1 (ACE). There is also angiotensin converting enzyme 2 (ACE2) that promotes alternative conversion of Ang II to Ang-(1-7) or Ang I to Ang-(1-9) (Krawczyńska et al., 2015). It is likely that this system is conserved across species (Fournier et al., 2012).

The ACE gene has been identified in the soil model Enchytraeus crypticus; the genome of this species was recently sequenced (Amorim et al., 2021) and revealed important gene family expansions, among which the innate immune system e.g. via the ACE-2 receptor. As mentioned, E. crypticus is a standard ecotoxicological species (OECD, 2004b) where extensive mechanisms of response studies are available, in e. at the molecular level, covering high-throughput (HTP) gene expression using microarray studies (Gomes et al., 2018a, 2019a, 2017, 2018a, 2018b). Hence, this species may present a non-animal alternative testing model for studying ACE related expression, keeping in mind that there are various ACE enzymes (Gathiram et al., 2021).

As seen from above, viruses and nanomaterials are both taken up by endocytosis (e.g. receptor mediated), nanomaterials may affect pathways that are related to a SARS-CoV-2 receptor, and the basic stress response is highly conserved across species. Hence, based on these observations we studied the response of the expression of the ACE gene in E. crypticus, hypothesizing that the responses due to treatments (NMs) can elucidate the mechanisms and potential cross stressor relations with known responses to viruses.

To study this, we reviewed the literature and created a database of E. crypticus ACE gene expression, aiming to analyse the potential knowledge transfer between invertebrates and vertebrates and to interpret mechanisms of response to virus. Total exposure experiments sum 87 stress conditions, including 2 natural stressors (ultra-violet radiation, UVA and UVB (Gomes et al., 2018c)) and 18 different nanomaterials (of Ag (Gomes et al., 2017), Cu (Gomes et al., 2018b), TiO₂ (Gomes et al., 2018a; Rasmussen et al., 2014; Gomes et al., 2021b, 2021c), Ni (Gomes et al., 2019a), nanoencapsulated atrazine (Gomes et al., 2022a)) and Zn (Gomes et al., 2022b) and Cd (Gomes et al., 2018b). Full details are given in the methods section.
2. Materials and Methods

2.1. Test organisms

The test species *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. The cultures were kept in agar, consisting of sterile Bacti-Agar medium (Oxoid, Agar No. 1) and a mixture of four different salt solutions at the final concentrations of 2 mM CaCl$_2$·2H$_2$O, 1 mM MgSO$_4$, 0.08 mM KCl, and 0.75 mM NaHCO$_3$, under controlled conditions of temperature (19 ± 1°C) and photoperiod (16:8 h light:dark). The cultures were fed with ground autoclaved oats twice per week. Adults with well-developed clitellum, or cocoons (1–2 days) were used; for further details on animals synchronization see (Bicho et al., 2015).

2.2. Test media

Depending on the experiments, exposure was done using 4 different media, OECD artificial soil, LUFa 2.2 soil, Hygum soil (natural soil collected at the Hygum site, Jutland, Denmark) and ISO water.

OECD artificial soil was prepared according to the guidelines (OECD, 2020, 2016) containing: 75% of sea sand (VWR, technical wash with sulphuric acid), 20% of Kaolin clay (Sigma Aldrich), 5% of sphagnum peat (sieved to < 2 mm) and CaCO$_3$ (Merck) for pH adjustment to 6 (± 0.5). All the soil constituents were mixed thoroughly and allowed to stabilize (1 week) prior to use.

LUFa 2.2 soil (Speyer, Germany) main characteristics can be described as follows: pH (0.01 M CaCl$_2$, ratio 1:5 w/v) = 5.5, organic matter = 1.77 mg/100 g, cation exchange capacity (CEC) = 10.1%, water holding capacity (WHC) = 41.8%, grain size distribution of 7.3% clay, 13.8% silt, and 78.9% sand.

Hygum soil has been historically exposed to contamination with CuSO$_4$ (due to activities of timber preservation, ceased more than 80 years ago), originating a well-known Cu gradient along the field, ranging from the natural background levels of 30 up to 2900 mg Cu/kg soil, further referred as Cu-field. For further details on materials characterization and soil [Cu-field] spiking see Gomes et al (Gomes et al., 2021). and Gomes et al (Gomes et al., 2021c) and the three reference TiO$_2$ NMs from the JRC Repository (Rasmussen et al., 2014) plus bulk TiO$_2$ (micro-sized, >99.9%, 14027 Sigma-Aldrich). All materials are fully characterised (Gomes et al., 2018a, 2021c; George et al., 2011).

Details are summarised in Table 1.

Nickel (Ni) NM (99.9%, 20 nm, American Elements) was tested together with nickel nitrate (Ni (NO$_3$)$_2$·6H$_2$O, ≥ 98.5%). For further details on materials characterization and soil [LUFA 2.2] spiking see Gomes et al (Gomes et al., 2019a).

Atrazine (ATZ)-based pesticide exposure included a nano-encapsulated ATZ (nano_ATZ, 230–250 nm, synthesized as described in Grillo et al. (2012).), pure atrazine (ATZ, Pestanal, analytical grade, UVB 220), and Gomes et al (Gomes et al., 2021c).

Table 1

| Test materials | Test concentrations | Units | Exposure time (days) | Control | Reference |
|----------------|---------------------|-------|---------------------|---------|-----------|
| AgNO$_3$      | 45, 60              | mg    | 3, 7                | unspike | (Gomes et al., 2017) |
| PVP-Ag NM     | non-coated           | Ag/ kg|                     |         |           |
| Cu-NM300K     | 500, 1400           | mg    | 3, 7                | unspike | (Gomes et al., 2018a) |
| Cu-NPs        | 980, 1760           | Cu/ kg|                     |         |           |
| CuO/NPs       | 290, 360            | soil  |                     |         |           |
| Ni NM         | 980, 1760           | Ni/kg | 3, 7                | unspike | (Gomes et al., 2019a) |
| NIN03         | 40, 60              | soil  |                     |         |           |
| 2%Fe-TiO$_2$  | 10                  | mg/L  | 5                   | Non-UV  | (Gomes et al., 2021b) |
| 6%Fe-TiO$_2$  |                     |       |                     |         |           |
| 10%Fe-TiO$_2$|                     |       |                     |         |           |
| TiO$_2$       | 10, 100             | 1     |                     |         |           |
| TiO$_2$, 12nm | TiO$_2$, 27nm       |       |                     |         |           |
| TiO$_2$, 10nm | 1                   |       | UVA                 |         |           |
| UVA           | 15,934              |       |                     |         |           |
| Bulk-TiO$_2$  | 1                   | mg/L  | 5                   | Non-UV  | (Gomes et al., 2018a) |
| TiO$_2$       | NM103               |       |                     |         |           |
| TiO$_2$       | NM104               |       |                     |         |           |
| TiO$_2$       | NM105               |       |                     |         |           |
| Bulk-TiO$_2$  | TiO$_2$, 10nm       |       |                     |         |           |
| TiO$_2$       | NM103               |       |                     |         |           |
| TiO$_2$       | TiO$_2$, 12nm       |       |                     |         |           |
| TiO$_2$, 27nm | 10                  |       | UVA                 |         |           |
| UVB           | 204, 220            |       |                     |         |           |
| ATZ           | 100, 200            | mg    | 3, 7                | acetone | (Gomes et al., 2022a) |
| Gesaprim      | 200, 400            | ATZ/kg|                     |         |           |
| Cd            | 5, 16               | Cd/kg | 1                   | unspike | (Gomes et al., 2018b) |
| Zn            | 93, 145             | Zn/kg | 1, 2, 3, 4          | unspike | (Gomes et al., 2022b) |

2.3. Test materials and background

Silver (Ag) materials tested included silver nitrate (AgNO$_3$ >99%, Sigma Aldrich) and 3 Ag NMs, i.e. non-coated Ag NM (99%, 20–30 nm, American Elements), polyvinylpyrrolidone (PVP)-coated Ag NM (99%, 20–30 nm, American Elements, further referred as PVP-Ag NM) and Ag NM300K (10.2% w/w Ag, 15 nm; JRC Repository (Klein et al., 2011)). For details on characterization and soil [LUFa 2.2] spiking see Gomes et al (Gomes et al., 2017).

Copper (Cu) materials tested included 2 NMs, i.e., Cu-NPs (99.8%, 20–30 nm, American elements) and Cu-nanowires (further referred as Cu-Nwires, synthesized following the procedure described in Chang et al. (2005)), and 2 ionic forms, i.e., copper nitrate (Cu(NO$_3$)$_2$·3H$_2$O, 99%, Sigma Aldrich), and CuSO$_4$ field historical contamination [soil collected at Hygum site, at measured Cu concentrations of 500 and 1400 mg Cu/kg soil, further referred as Cu-field]. For further details on materials characterization and soil [Cu-field] spiking see Gomes et al (Gomes et al., 2018a).

Titanium dioxide nanomaterials (TiO$_2$ NMs) were tested [exposure in ISO water, plus post-exposure in clean soil] as described in (Gomes et al., 2018a, 2021c, 2015). NMs included pure and iron doped TiO$_2$ NMs library [synthesised as described in George et al (George et al., 2011), and Gomes et al (Gomes et al., 2021c).] and the three reference TiO$_2$ NMs from the JRC Repository (Rasmussen et al., 2014) plus bulk TiO$_2$ (micro-sized, >99.9%, 14027 Sigma-Aldrich). All materials are fully characterised (Gomes et al., 2018a, 2021c; George et al., 2011). Details are summarised in Table 1.
>98%, Sigma-Aldrich) and the commercial non-nanof ormulation Gesaprim® 500 CG (50% m/v atrazine, Syngenta). For further details on materials characterization and soil [LUFA 2.2] spiking see Gomes et al. (2019b).

Zinc (ZnCl₂, Merck) was tested [in LUFA 2.2 soil] at the reproduction effect concentrations EC10 (93 mg Zn/kg soil) and EC50 (145 mg Zn/kg soil), as determined based on the standard Enchytraeid Reproduction Test (OECD 220, 2016) results. For further details see Gomes et al. (2022b).

Cadmium (CdCl₂·2 H₂O, 98%, Fluka) was tested [in LUFA 2.2 soil] as described in Gomes et al. (2018b), and 1 UV-A dose, as described in Gomes et al. (2021c).

2.4. Test procedures

For the materials tested in soil (i.e., Ag, Cu, Ni, and ATZ), exposure followed the standard guidelines (OECD 220, 2016; ISO 16387, 2005), with adaptations as follows: 20–30 adults with well-developed clitellum were introduced in each test vessel (glass vessels, ø 4 cm) containing 20 g of moist soil (control or spiked). The organisms were exposed for 3 and 7 days under controlled conditions of photoperiod (16:8 h light:dark) and temperature (20 ± 1 °C) without food. After the exposure period, the organisms were carefully removed from the soil, rinsed in deionised water and frozen in liquid nitrogen. The samples were stored at –80 °C until analysis. Further details can be found in Gomes et al. (2018a, 2019a, 2017). For Zn, the procedures were similar, but the exposure lasted 1, 2, 3 and 4 days (Gomes et al., 2022b).

For Cd, 10 cocoons of 1–2 days old (synchronized age (Bicho et al., 2015)) were introduced in each well [6-well plates, 35 mm ø] containing 5 g of moistened soil (control or spiked), in a random design. Exposure lasted 1 day under controlled conditions of photoperiod (16:8 h light:dark) and temperature (20 ± 1 °C). After that, the cocoons were carefully removed from the soil to a Petri dish with reconstituted ISO water to remove soil particles, snap frozen and stored at –80 °C until further analysis. Replicates per test condition consisted of a pool of 30 well-plates per condition. For further details see Gomes et al. (2018b).

For the tests performed in ISO water (i.e., TiO₂ NMs and UV radiation alone), 5 adults with well-developed clitellum were introduced in each well [24-well plates, 15.6 mm ø] containing 1 ml of media (control: 0.5 ml ISO water + 0.5 ml of ultra-pure water; or treatments: 0.5 ml ISO water + 0.5 ml test suspension prepared in ultra-pure water). Exposure lasted 5 days under controlled conditions of photoperiod (16:8 h light:dark) and temperature (20 ± 1 °C). The combined exposure to UV radiation was performed during the light period. For the Fe doped TiO₂ NMs, combined exposure to UV was done under UV-A radiation [UVP XX-15 L Longwave UV lamp (UVP LLC, CA, USA) peak emission at 365 nm, at 800 μW/cm² of UV, respectively. After the exposure period, the organisms were collected, washed in ISO water, snap frozen and stored at –80 °C until further analysis. Replicates per test condition consisted of a pool of 30 organisms (corresponding to 32 well-plates per test condition). For further details see Gomes et al. (2018a). For the UV-B-alone exposure, the lamp and time of exposure were similar to the JRC reference TiO₂ NMs exposure. The two doses were obtained by varying the distance of the UV lamp to the test vessels, i.e., the lamp was placed at 43 and 46 cm above the test vessels to obtain 220 and 204 J/m² of UV, respectively. After the exposure period, the organisms were collected, washed in ISO water, snap frozen and stored at –80 °C until further analysis. Replicates per test condition consisted of a pool of 30 organisms (corresponding to 32 well-plates per test condition). For further details see Gomes et al. (2018c).

For an overview of the exposure details please see Table 1. For Ag materials, 3 Ag NMs (Ag NM300K, PVP-coated Ag NM, and non-coated Ag NM) and AgNO₃ (ionic) were tested, in soil, at reproduction effect concentrations EC20 and EC50 (Gomes et al., 2017). For Cu materials, 2 Cu NMs (nanoparticles and nanowires) and 2 ionic forms (freshly spiked CuNO₃ and Cu-salt field historical contamination) were tested, at the EC20 and EC50 (Gomes et al., 2018a). For TiO₂ NMs, the 3 JRC repository (Rasmussen et al., 2014) reference materials, NM103, NM104 and NM105, in addition to bulk TiO₂, at 1 mg/L, via aqueous exposure, were tested with and without UVB radiation (Gomes et al., 2018a). For Fe-doped TiO₂ NMs library (which includes a list of 8 different TiO₂ NMs: 3 sizes of Pure TiO₂, plus 1%, 2%, 6%, 8%, and 10% Fe-TiO₂ NMs) were investigated at selected concentrations (ranging from 1 to 1000 mg/L), with and without UV(A) radiation, via water exposure (Gomes et al., 2021b). The combinations of NM-concentration-radiation tested were selected to cover different levels of effects on reproduction (Gomes et al., 2021c). For Ni materials, Ni NM was compared to NiNO₃, via soil exposure, to the EC20 and EC50 (Gomes et al., 2019a). For atrazine based pesticides, exposure included nano-encapsulated atrazine (nano-formulation) compared to the pure atrazine and a commercial (non-nano) formulation (Gesaprim), via soil exposure, to the EC10 and EC50 (Gomes et al., 2022a). For non-nanomaterials, the effects of Zn were investigated at the reproduction EC10 and EC50 (Gomes et al., 2022b) the effects of Cd were investigated in E. crypticus cocoons, exposed in soil, for 1 day, to two concentrations (5 and 16 mg Cd/kg soil, causing effects and totally disrupt hatching, respectively) (Gomes et al., 2018b). For the natural stressors, besides the mentioned UV(A), UV(B) radiation effects were investigated at two doses, that although realistic (equivalent to winter days in northern Europe), inhibit reproduction in 80%, via water exposure, for 5 days (Gomes et al., 2018c).

2.5. ACE gene expression quantification

2.5.1. Selected treatments

A list of test treatments from the microarray (MA) exposure was selected (Table 2) based on significantly different ACE gene expression (for full list see Table S1 – Supplementary Material).

2.5.2. RNA extraction

RNA was extracted from a pool of animals (40 adults/ replicate). Three biological replicates per treatment were done. The SV Total RNA Isolation System (Promega) (according to manufacturer’s protocol) was used. The quantity and purity of the isolated RNA were measured with nanodrop (Nanodrop ND-1000 Spectrophotometer), and its integrity was checked on a denaturing formaldehyde agarose gel electrophoresis.

2.5.3. Primers

Primers were designed (Oligo Explorer™ v.1.1.2), based on E. crypticus gene sequences (Gomes et al., 2018a, 2017; Castro-Ferreira et al., 2014) coding for the gene target Angiotensin-converting enzyme (ACE), and the gene references, Zgc:174506 protein (Gdc6) and Insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1) (Tab. S2).

Reference genes were selected based on no expression variation (i.e. less than 2 cycles) in all treatments, as from data in microarray studies (Gomes et al., 2018a, 2017).

Determination of PCR efficiency and specificity was done by observing the obtained standard and melting curves, respectively, with four dilutions of cDNA from control samples, for all primer sets.

2.5.4. Quantitative real-time PCR (qPCR)

Total RNA (2 μg) from samples was converted into cDNA through a reverse transcription reaction using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was diluted 4X and 2 μl were used in 20 μl PCR reaction volumes containing 2 μl of forward and reverse primers (5 μM), 10 ml of SsoFast EvaGreen Supermix (Bio-Rad) and 4 ml of nuclease free water. Each replicate was applied in triplicate. For each qPCR plate non-template control (NTC) was added in duplicate, where nuclease free water was used instead of cDNA.
Table 2
List of tested TiO2 materials for the selected treatments for real time quantitative PCR (qPCR) gene target confirmation, including exposure conditions (light source: No-UV: standard fluorescent light, UVA, and UVB), size (nm), tested concentration (mg TiO2/L), EC: % effect concentration (reproduction), MA: Microarray results (FC: Fold Change, as log2 ratio), reference.

| # | Control | TiO2 NM | Size (nm) | Concentration (mg/L) | EC | MA | qPCR | Source |
|---|---------|---------|-----------|----------------------|----|----|------|--------|
| 41 | No-UV  | 2%Fe-TiO2 | 10        | 10                   | 0  | -6.8 | -8.4 ± 4.4e-5 | (Gomes et al., 2021c) |
| 42 | No-UV  | 6%Fe-TiO2 | 5         | 10                   | 50 | -6.1 | -8.3 ± 8.9e-5 | Gomes et al. (2021b) |
| 43 | No-UV  | 8%Fe-TiO2 | 5         | 10                   | 0  | -7.7 | -8.7 ± 3.2e-5 |                |
| 44 | No-UV  | 10%Fe-TiO2 | 10       | 100                  | 40 | -6.9 | -8.1 ± 1.0e-4 |                |
| 46 | No-UV  | TiO2     | 10        | 100                  | 80 | -7.8 | -8.3 ± 8.9e-5 |                |
| 48 | No-UV  | TiO2     | 12        | 10                   | 50 | -4.0 | -2.4 ± 0.1    |                |
| 49 | No-UV  | TiO2     | 27        | 10                   | 50 | -6.6 | -4.9 ± 2.3e-3 |                |
| 51 | UVA    | 1%Fe-TiO2 | 11        | 10                   | 50 | -4.2 | -5.3 ± 1.7e-3 |                |
| 59 | UVB    | Bulk-TiO2 | 400       | 1                    | 60 | 1.5  | 0.6 ± 0.6     | Gomes et al. (2015) |
| 60 | UVB    | NM103    | 26        | 1                    | 35 | 2.6  | -0.8 ± 0.4    |                |
| 61 | UVB    | NM104    | 26        | 1                    | 40 | 3.5  | 1.6 ± 2.1     |                |
| 62 | UVB    | NM105    | 21        | 1                    | 25 | 3.5  | 1.9 ± 1.9     |                |

* Based on effects on reproduction, determined after 5 days’ water exposure plus 3 weeks in clean soil.

Amplification was performed on the CFX Connect Real-Time PCR Detection System (Bio-Rad). Reaction conditions consisted of one denaturation step at 95°C for 30 s and 40 cycles at 95°C for 5 s and 60°C for 10 s.

2.6. Data analysis

To quantify gene expression, the mean normalized expression value was calculated from the obtained cycle threshold (Ct) values. Statistical differences were assessed using the Relative Expression Software Tool (REST-MSC).

3. Results

The inspection from HTP microarray results revealed changes in ACE regulation, i.e. down- and up-regulation (Fig. 1). The full list of the HTP ACE coding gene results, per treatment, is summarised on Table S1.

The results overview show that certain treatments caused much larger changes (Fold Change, FC) than other, e.g. treatments like UVB or Cd did not affect ACE, while there was down-regulation to TiO2, several of the Fe doped TiO2 NMs library and the JRC TiO2 materials and up-regulation to Ag and Cu. On a more detailed inspection (Fig. 2), it is possible to see the relation to exposure time and how e.g. PVP-coated and non-coated Ag NM caused higher up-regulation after 3 days of exposure. Overall, ACE was up-regulated shortly after exposure (3 days) to Ag, Cu and Ni, followed by a down-regulation after 7 days, except for atrazine materials, in which ACE was upregulated at day 7, after a down-regulation at day 3. The wide range of tested TiO2 NPs showed that combination with UV caused an ACE up-regulation compared to non-UV exposure. Concentration can have a role, e.g. exposure to 1 mg/L of TiO2,12nm_UVA caused ACE down-regulation whereas exposure to 10 mg/L of TiO2,12nm_UVA caused no effect. The comparison of Fe doping % did not show an effect pattern.

Gene expression results of selected treatments (Fig. 3, Table 2) showed good correlation between microarray (MA) and qPCR data. A significant ACE down-regulation was observed from exposure to Fe-TiO2 NMs library. The exposure to JRC-NMs (103, 104, 105) was not confirmed significantly affecting ACE expression. It may be noted that JRC-NMs were rutile (103–104) and rutile anatase (105) containing Aluminium. The other TiO2 were primarily anatase form, although the higher the Fe doping the more rutile was present.

4. Discussion

The correlation between microarray and qPCR measurements was confirmed and was higher for the larger expression (Log2 fold change). This is common, and due to both technical and biological factors (Roca et al., 2017).

The regulation of ACE, which occurred as a response to many of the tested materials, was occurring via both up and down-regulation mechanism, e.g., clearly the TiO2 (various %Fe-TiO2 library, TiO2 NM103, NM104, NM105) caused ACE down-regulation while the exposure to TiO2 NM103, NM104, NM105 combined with UVB caused its up-regulation. The difference observed, i.e. down versus up-regulation, may be because the JRC materials have Aluminium contamination.

TiO2 is photoactive, and the combined exposure with UVB causes the release of ROS and is toxic to E. crypticus (Gomes et al., 2015). Hence it seems that the shift from ACE down-regulation to up-regulation when exposed to TiO2+UVB could be related to the cascade response to ROS of TiO2. It is of interest to note that UVB alone, although also toxic to E. crypticus (Gomes et al., 2015), did not regulate ACE (Gomes et al., 2018a), hence the response to UV toxicity is via different mechanisms. Analysis of exposure to UVB alone (equivalent dose to UV during the winter months in northern Europe) showed 80% decrease in reproduction and approximately 5% of the genes were differentially expressed, including activation of the DNA repair mechanisms, nucleotide excision repair and repressing of apoptosis (Gomes et al., 2018c). The mechanisms activated by UV were similar to those activated in humans, again showing conservation across species in the response to stress mechanisms.

Clearly there was a relevant interaction in the exposure TiO2+UVB, and the overall analysis could discriminate pathways, namely, without UV: bulk TiO2 caused DNA damage response, NM103 affected transcription/translation, NM104 affected sensory perception, NM105 affected developmental processes; with UV: NM104 negatively affected the reproductive system/organs, NM105 activated superoxide anion

Fig. 1. Results on ACE gene expression (Fold Change (FC)), based on microarray analysis, from Enchytraeus crypticus when exposed to 67 different treatments (see details in materials section); for the number treatment code please check Table S1.
Fig. 2. Results on ACE gene expression (Fold Change (FC)), based on microarray analysis from *Enchytraeus crypticus* when exposed to various materials (see details in Section 2). PVP: polyvinylpyrrolidone; ATZ: Atrazine; [X]: concentration X; [X]*10: concentration X*10.
response (Gomes et al., 2018a). On the other hand, all TiO$_2$ materials (UV and No-UV) caused an increase in the cell adhesion molecule (CAM) response, proinflammatory mediators. This is also observed in human cell lines following exposure to several different TiO$_2$ NMs, where up-regulation of CAMs activates human monocytes.

Regarding the UVA alone, results showed both toxicity at phenotypical level (reproduction) (Gomes et al., 2021c) and down-regulation of ACE (Log2FC = -3.4). Similarly to UVB, the comparison between TiO$_2$ (%Fe-TiO$_2$ library) exposure with and without UVA showed a shift from down-regulation to up (in this case translated as a less down-regulation).

Despite the various tested treatments (materials, concentrations, time, UV) and sample size (87 in total), a clear pattern is not immediately apparent for why the mechanism would activate or deactivate ACE. The high-throughput data analysis output, i.e. after gene set enrichment and pathway analysis, considers the significantly differently expressed genes, but not the level or if regulation is up or down – this is a well-known caveat but not simple to integrate. Hence, this level of detail that we present here is seldom captured and discussed in high-throughput literature of varied species.

Most of the Ag materials caused ACE up-regulation, the highest Log2 FC (FC=5) was after 3 days exposure to PVP coated Ag NM (550 mg/kg, EC$_{50}$), showing an increase with dose (FC=3.7 for exposure to 380 mg/kg, EC$_{50}$). The expression variation is also apparent with time, showing that the response seems to peak immediately after exposure, and from day 3-7 there is already a decrease, or a turning off, probably a loop regulation. The same pattern occurred for exposure to all other Ag materials but with lower FC. AgNO$_3$, non-coated-Ag NM, and Ag NM300K. Hence, the level of ACE response seems related to the exposure dose, the higher the Ag dose the higher the ACE regulation. On the other hand, after an immediate up-regulation peak, the response decreased with increased exposure time, which could indicate a stabilization or other mechanisms taking over. Several of the other tested materials - Ni, Cu and ATZ - caused ACE up-regulation. Although non-significant (p > 0.05), these results showed the same dose-response and time dynamic, as described for Ag.

In humans ACE2 is a functional receptor in the lungs, and the spike proteins of SARS-CoV-2 attach to ACE2 – likely the first key initiating event (KIE) for the transmission of SARS-CoV-2 – and employs the cellular serine protease TMPRSS2 (transmembrane serine protease 2) for S protein priming, the KIE2: binding of the S protein to the ACE2 receptor, and now the virus can enter the cell. Internally pathways are also activated although the full mechanisms is not understood, for an in depth review see (Moolamalla et al., 2020). The similarities between exposure to certain NMs and acute SARS-CoV-2 infection have been pointed out by Kinaret et al. (2020). For instance, cationic polyamidoamine (PAMAM) dendrimer NMs - PAMAM bind ACE2, decrease its activity and down-regulate its expression in lung tissue. Other examples include rigid multi-walled carbon nanotubes (rMWCNT), which induce innate immune response (NF-kB, STAT3, HIF-1/2) and consequent cytokine cascade. Hence, the ACE up-regulation in both air pollution, and pulmonary fibrosis may explain the spread and aggravation of COVID-19 by these two conditions (Li et al., 2021). In line with this, if Ag exposure upregulate ACE expression this would aggravate COVID-19, whereas the TiO$_2$ exposure would minimise this. The stronger expression of ACE under TiO$_2$ compared to Ag is in agreement with Krawczynska et al. (2015), who also reported that although both Ag NMs and TiO$_2$NMs (stronger response) modulate gene expression, only TiO$_2$NMs caused a similar change in protein levels. Besides being important for SARS-CoV-2 it is well known that the Angiotensin modulates the insulin levels (Dominici et al., 2014; Mishra and Dey, 2021; Govender et al., 2021). This may lead to insulin resistance, which is in line with the result from Hu et al. (2020), who showed that TiO$_2$ impact the glucose homeostasis via inflammation leading to insulin resistance in mice (probably via lysosomal rupture resulting in ROS production) (Hu et al., 2020). Insulin resistance is not only the precursor of Type 2 diabetes and related to many other diseases, e.g. Alzheimer, but may also be a concern with COVID-19 (Finucane and Davenport, 2020).

The recognition of pathogens by pattern recognition receptors (PRRs) triggers type I interferons (a central event of the immune response against viral infection) and signalling pro-inflammatory cytokines cascades (García-Sastre and Miorin, 2016). TRIM proteins, expanded in E. crypticus (Amorim et al., 2021), are essential and act as restriction factors or by modulating PRR signalling. Curiously, SARS-CoV-2 has been observed to disable interferons – "strikingly depressed interferon activity and elevated chemokines in individuals whose disease became severe and critical" (Wadman, 2020). Certain NMs (carbon and metal) were also reported to alter the expression of interferons signalling pathways (Kinaret et al., 2020), again, this highlights the usefulness of transferring knowledge from both nanotoxicology and invertebrate model species, e.g. E. crypticus.

The innate memory or immune priming in invertebrates, based on epigenetic changes, can be transferred to the next generations, enhancing the fitness of the offspring when in the same environmental challenges as the parents. This mechanism has been shown, i.e. epigenetics, in E. crypticus (Noordhoek et al., 2017) via DNA methylation. Multigenerational (MG) exposure to Cu materials causes increased global DNA methylation (Bicho et al., 2020), with associated phenotypic effects (reproduction) (Bicho et al., 2017). Specific gene expression showed a clear regulation of epigenetic related genes, e.g. DNMT1 (DNA (cytosine-5)-methyltransferase 1), MBD2 (Methyl-CpG-Binding Domain protein 2), DMAP1 (DNA Methyltransferase 1-Associated Protein 1), PARP1 (PARP1 Poly (ADP-Ribose) Polymerase 1), H3 (Histone 3), further confirmed via immunohistochemistry and with important adverse outcome pathways (AOP) (Bicho et al., 2021). For CuCl$_2$ MG exposure there was increased tolerance, explained by the activation of general stress response mechanisms (metallothionein, heat shock proteins and elongation factor) but, the transgenerational increased toxicity seemed to be the result of a higher Cu immune homeostasis, an immune memory adaptation type, and hence a deficiency if transferred to Cu absence. For CuO NMs MG exposure, the maintained toxicity is eliminated when transferred to clean media, and developmental mechanisms impaired. Hence, the apparent good fitness of the population after MG exposure masks active stress mechanisms at gene level. A follow up proteome study (Maria et al., 2018) showed that whereas CuCl$_2$
activated conserved mechanisms (constitutive genes) promoting Cu detoxification, CuO NM induced regulation of facultative genes with a differentiated cascade of events.

There are many evidences that indicate size (nano versus bulk) specific mechanisms in enchytraeids, e.g. TiO\(_2\) NMs No-UV impaired DNA repair while TiO\(_2\) bulk No-UV activated DNA repair mechanisms (Gomes et al., 2018a). Comparison between the different pesticide atrazine (ATZ) forms (Gomes et al., 2022a), indicate uptake via endocytosis of the nanoformulation, as opposed to passive diffusion for its conventional agrochemical. Nevertheless, it is also apparent that nano size is not the only source for observed differences, rather the specific size range, element, structure, shapes, etc. and which of these will dominate specific responses (Gomes et al., 2021a). For instance, in the study of TiO\(_2\) (Gomes et al., 2018a) specific mechanisms of response were differentiated between UV and No-UV, but no clear discrimination between anatase and the rutile forms could be understood for the different TiO\(_2\) forms. Machine learning analysis from exposure to Fe doped TiO\(_2\) NM library, combining 122 descriptors (both measured and atomistic modelled) (Gomes et al., 2021c), showed that without UV the hydrodynamic diameter correlated best with biological impact, whereas with UV, the zeta potential had a significant impact. Further, also the normal surface force vector of Ti/Fc atoms in the shell (modelled data) correlated with the biological impact. This descriptor reflects the stability of TiO\(_2\) on NM surface, which is linked to oxidative stress and its correlation with band gap. The results from the high-throughput gene expression microarray array (Gomes et al., 2021b) identified size dependent effects, i.e., TiO\(_2\) NMs smaller than 12 nm seem to interact with embryos to induce reproductive effects, while bigger NMs (27 nm) caused reproductive effects via different mechanisms; phagocytosis was affected by 12 and 27 nm NMs, but not by < 11 nm. A key unique mechanism included e.g. impairment in RNA processing for TiO\(_2\) 10nm, or deregulated apoptosis for 2%FeTiO\(_2\) 10nm. ACE was down-regulated to a significant level in nearly all TiO\(_2\) materials and for increases in dose, within the same TiO\(_2\) caused an increase in the |FC|= . Variation of ACE levels compared between the various %Fe-TiO\(_2\) NMs was less pronounced.

Why certain materials induce down-regulation of ACE (e.g. TiO\(_2\) NMs), while other induce up-regulation (Ag NM), is not fully clear. It seems to be linked to additional factors, for instance for TiO\(_2\) NMs the combination with UV exposure shifted the regulation from down to up in most of the treatments. The concentration and exposure time also played an important role, which may be important if Ag NMs are to be used against COVID-19. The comparisons between EC\(_{50}\) and EC\(_{50}\), and 3- and 7-days exposure, showed both increase and decrease of ACE, and this would make sense as it all depends on stress threshold levels and the efficiency of the activated mechanisms. Whether ACE can be used as a general biomarker of NMs depends on the purpose of the marker, however it is clear that the effect on ACE (and on RAS - Renin Angiotensin System) can have wide consequences, e.g. in relation to COVID-19, Type 2 diabetes, Alzheimer and others.

5. Conclusions

This database and analysis of the ACE regulation in *Enchytraeus crypticus*, showed that important lessons can be learned from invertebrates in terms of mechanisms of response to invaders, such as viruses and nanomaterials. The extensive knowledge from response to NMs (invaders) can support the immuno-research community, especially to develop therapies for virus that trigger the innate immune system as a primary mechanism. Because *Enchytraeus crypticus* genome showed the immune system to be among their expanded gene families, there is a potential for developing studies that explore this mechanism.

CRediT authorship contribution statement

M.J.B. Amorim: Conceptualization, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. S.I.L. Gomes: Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. R.C.S. Bicho: Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. J.J. Scott-Fordmand: Conceptualization, Data curation, Writing – review & editing, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of interest

The authors declare no conflict of interest.

Environmental implication

Invertebrates evolved their immune system to deal with threats efficiently. The ACE gene has been identified in *Enchytraeus crypticus*, a standard ecotoxicological species, with full genome sequenced. ACE2 – a functional receptor in the lungs of humans, the target for COVID-19 – is likely the key initiating event (KIE) for transmission. Nanomaterials (NMs) can often invade cells like virus. Results from 18 NMs showed e. g., TiO\(_2\)NM causes ACE downregulation (7 fold), whereas AgNM caused upregulation (4 fold). This extensive knowledge from response to NMs in invertebrates can support the immuno-research, especially for virus that trigger the innate immune system in vertebrates.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.129173.

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