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Ecotoxicological read-across models for predicting acute toxicity of freshly dispersed versus medium-aged NMs to *Daphnia magna*

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ABSTRACT

Nanoinformatics models to predict the toxicity/ecotoxicity of nanomaterials (NMs) are urgently needed to support commercialization of nanotechnologies and allow grouping of NMs based on their physico-chemical and/or (eco)toxicological properties, to facilitate read-across of knowledge from data-rich NMs to data-poor ones. Here we present the first ecotoxicological read-across models for predicting NMs ecotoxicity, which were developed in accordance with ECHA's recommended strategy for grouping of NMs as a means to explore *in silico* the effects of a panel of freshly dispersed versus environmentally aged (in various media) Ag and TiO_2 NMs on the freshwater zooplankton *Daphnia magna*, a keystone species used in regulatory testing. The dataset used to develop the models consisted of dose-response data from 11 NMs (5 TiO_2 NMs of identical cores with different coatings, and 6 Ag NMs with different capping agents/coatings) each dispersed in three different media (a high hardness medium (HH Combo) and two representative river waters containing different amounts of natural organic matter (NOM) and having different ionic strengths), generated in accordance with the OECD 202 immobilization test. The experimental hypotheses being tested were (1) that the presence of NOM in the medium would reduce the toxicity of the NMs by forming an ecological corona, and (2) that environmental ageing of NMs reduces their toxicity compared to the freshly dispersed NMs irrespective of the medium composition (salt only or NOM-containing). As per the ECHA guidance, the NMs were grouped into two categories - freshly dispersed and 2-year-aged and explored *in silico* to identify the most important features driving the toxicity in each group. The final predictive models have been validated according to the OECD criteria and a QSAR model report form (QMRF) report included in the supplementary information to support adoption of the models for regulatory purposes.

1. Introduction

Nanoinformatics is an evolving field of research that includes the development of *in silico* models and tools that could be an alternative to the experimental evaluation of nanomaterials (NMs) environmental health and safety (exposure and toxicity) and thus contribute to NMs hazard and risk assessment. A wide range of methodologies and techniques have been integrated within nanoinformatics workflows, including quantum-mechanical simulations, finite element simulations, regression/classification algorithms, quantitative structure-activity relationships (QSARs), neural networks, genetic algorithms and many more (Rajan, 2018; Toropov and Toropova, 2015; Toropova et al., 2017). Specifically, predictive modelling approaches that have already contributed to the cheminformatics field can also significantly contribute in the nanoinformatics area, provided that the methods and tools proposed are adjusted to the specific needs of NMs, such as utilization of algorithms that can treat limited or sparse data, as well as methods and tools to generate computational (quantum mechanical or image based) descriptors describing the NM entity (Varsou et al., 2020). NanoQSAR or QNAR (quantitative nanostructure–activity relationship) models, developed for the prediction of biological and toxicological adverse effects, have already been proposed in the literature (Fourches et al., 2010; Gajewicz et al., 2015a; Leone et al., 2018; Manganelli et al.,

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Abbreviations

DLS	Dynamic Light Scattering
ECHA	European Chemical Agency
(E)NM	(Engineered) Nanomaterial
HH combo	High-hardness combo media
NOM	Natural organic matter
OECD	Organization for Economic Cooperation and Development
QNAR	Quantitative Nanostructure-Activity Relationship (modeling)
QSAR	Quantitative Structure-Activity Relationship (modeling)
TEM	Transmission Electron Microscopy
UPW	Ultrapure water

2016; Melagraki and Afantitis, 2014; Singh and Gupta, 2014; Toropov and Toropova, 2019). The need to build reliable nanoQSAR models is often highlighted as a significant regulatory challenge, since it is not possible to experimentally assess all variants of NMs due to time, cost and ethical restrictions, and thus computational models are needed. However, the lack of large experimental datasets and the range of different mechanisms of action for diverse NMs hinder efforts to develop robust and accurate predictive models (Gajewicz et al., 2015a; Winkler et al., 2013).

The lack of suitable datasets for development of nanoQSARs arises in part from the challenges in generating such datasets for panels of NMs. These challenges arise from a number of factors including difficulties to keep NMs dispersed in test media over they assay duration (Petersen et al., 2015), the quantity of NMs required for many (eco)toxicity tests, the relatively limited panel of commercially available NMs of systematically varied physico-chemical properties and the fact that NMs are so responsive to, and dynamic in, biological and environmental media undergoing a range of physical, chemical and biological transformations that alter their uptake and toxicity (Hjorth et al., 2017; Hund-Rinke et al., 2016). Common transformations that are likely to occur in the environment include adsorption of NOM to the NMs surface (Cupi et al., 2015), oxidation and potentially oxidative dissolution (Collin et al., 2016; Furtado et al., 2016), or sulfidation which decreases the rate of dissolution and thus, reduces NM toxicity (Levard et al., 2011). Ageing and transformations of NMs are considered to alter their surface speciation, e.g., via sulfidation, phosphidation, chloride binding or other changes to the surface chemistry depending on the salts present in the media), and/or reduce their surface reactivity as a result of adsorption of biomolecules from their surroundings (Briffa et al., 2018; Lowry et al., 2013; Nasser and Lynch, 2016). Surface coatings generally act as a stabilizing mechanism and thus reduce the dissolution process of the core material (El Badawy et al., 2012), however, some studies have identified that NM ageing removes protective coatings and stabilizers (Izak-Nau et al., 2015; Virkutyte and Al-Abed, 2012), which will affect the NM stability and ecotoxicological outcome.

Assessment of NMs hazards is currently performed using experimentally produced dose-response relationships to compare the median effect and lethal concentrations (EC/LC_{50}) with observed behavior in order to provide assessments of hazard (Fadel et al., 2015; Mueller and Nowack, 2008; Nowack and Mueller, 2008). For NMs hazard assessment, the results from an array of scientific data are difficult to compare since there are no specific guidelines on the measurement of NM physicochemical characteristics to facilitate comparison across studies (Das et al., 2013), and since the NMs undergo transformations during the exposure that are often not considered or described (Hjorth et al., 2017; Nasser et al., 2020; Svendsen et al., 2020). Given the lack of experimental validation of exposure conditions and the form of the NMs actually presented to organisms, experimental datasets are often difficult to reproduce since they focus mainly on the outcome or response caused by the NMs (El Badawy et al., 2010; Li et al., 2013; Morelli et al., 2018), and less on the environmental transformations that drive the NM response in exposure models. Until recently, the focus of acute NM testing was on freshly dispersed synthesized/engineered NMs (Hu et al., 2018; Pace et al., 2010; Shen et al., 2015), despite increasing understanding of NMs transformations in the environment (Beaudrie et al., 2013; Lowry et al., 2013; Svendsen et al., 2020). For example, silver NMs become more toxic as they decrease in size due to increased surface area for release of ions (Kim et al., 2012).

Although the OECD does advise use of specific media for acute and chronic toxicity testing (OECD, 2017), there are no specific guidance or test modifications on how to prepare NMs for ecotoxicity testing as yet (Hund-Rinke et al., 2016), although work to this end is underway via the OECD. To simplify the tests and facilitate comparison across chemical compounds, the media recommended for OECD acute tests are salt only, which is fine for soluble chemicals as it removes the potential for binding of chemicals to NOM which may impact their bioavailability, but fails to provide a means to stabilize NMs or to account for their highly reactive surfaces which means they will acquire a corona through damaging organism's membranes if there are no alternative biomolecules available (Lesniak et al., 2012; Nasser and Lynch, 2019). In real environments, NMs will acquire a coating of biomolecules almost instantly upon contact with the aquatic environment, and indeed even over the duration of the acute tests the organisms condition their medium through their filtrating action (Nasser et al., 2020; Nasser and Lynch, 2019), resulting in eco-corona formation in situ which is not currently accounted for but which can provide important mechanistic insights into how the organisms respond to the presence of the NMs (Ellis and Lynch, 2020). Thus, there is an absence of examination of NMs as environmental pollutants under environmentally representative conditions and with full consideration of the physico-chemical transformations they undergo in the environment that can reduce or enhance their toxicity over time (Virkutyte and Al-Abed, 2012). It is therefore crucial to study both "freshly dispersed" and "environmentally aged" forms of NMs using environmentally relevant dispersion conditions. Using NMs which are "aged" under realistic exposure conditions is expected to generate data that is more predictive of realistic exposure scenarios, and therefore more appropriate for regulatory and risk assessment (Guineé et al., 2017). Utilization of dose-response and ecotoxicity data generated under realistic environmentally relevant media and using environmentally aged NMs is also expected to increase the predictivity of nanoQSAR models and facilitate their adoption for regulatory testing.

Grouping and read-across have been proposed as a non-testing strategy for nanotoxicity assessment, established through the Read-Across Assessment Framework under the EU REACH regulation of the European Chemicals Agency (ECHA), and are considered as an efficient approach for data gap filling especially in the case of NMs datasets of limited size (ECHA, 2015). Two approaches for grouping and read-across have been proposed by ECHA and the OECD: the category and the analogue approach. In the first case, it is assumed that structural similarity between NMs may lead to the expression of similar (eco) toxicological and biological activity. Thus, NMs that have a regular pattern in their structural characteristics can be considered as one group and read-across predictions may refer to the whole group whereas, an analogue approach can be applied within the group for sample-specific toxicity estimation. In the analogue approach, regular patterns are not obvious in the structural characteristics of a set of NMs. In this case, the search for similar NMs through read-across prediction methodologies is restricted to a limited area of the data space and it is based either on experts' critical judgment, or on computational methods that mathematically measure NMs similarity such as hierarchical clustering, principal components analysis and linear discriminant analysis (Lamon et al., 2018; Sayes et al., 2013). In that case, a "local" interpolation methodology can be applied to the data for toxicity/property estimation.

However, the boundaries between the two approaches are still unclear and depend on the number of available samples (Gajewicz et al., 2017, 2015b; Giusti et al., 2019; Helma et al., 2017; Varsou et al., 2018b; Varsou et al., 2019a,b).

Due to the abundance of different proposed grouping approaches, for harmonization purposes, ECHA has presented a methodical NM-specific workflow comprising of seven well-defined steps for grouping and read-across (ECHA, 2017). This workflow was summarized by Lamon et al. (2018) and Aschberger et al. (2019) into four main steps that were followed for two NM-specific case studies for the read-across prediction of toxicity endpoints of TiO_2 nanoforms and multi-walled carbon nanotubes (MWCNTs) respectively. In all cases, the pivotal step for grouping is the formation of a clear hypothesis, based on the available data and the apparent trends in their properties, that is evaluated for its ability to be used for data gap filling for other materials where the biological or (eco)toxicological data is not available.

Here we present two read-across models that have been developed and proposed to explore *in silico* the effects of a panel of freshly dispersed versus environmentally aged Ag and TiO_2 NMs on the freshwater zooplankton *Daphnia magna*, which is a keystone species used in regulatory testing. The dataset used to develop the models consisted of 11 NMs (5 TiO_2 NMs with different coatings, and 6 Ag NMs with different capping agents/coatings) each dispersed in three different media (a high hardness salt-only medium widely used for *Daphnia* experiments (HH Combo medium) and two representative river waters containing different amounts of natural organic matter (NOM) and having different ionic strengths). The NMs were studied immediately upon dispersion in the HH Combo medium and following 2-years of ageing in each of the three media, leading to 44 unique conditions in the dataset (11 NMs x 4 conditions). The NMs were fully characterized under each condition and the acute immoblisation induced by the NMs within the testing periods (24 and 48 h) was determined for each NM at each tested condition in accordance with OECD Test 202 (Immobilization of *Daphnia Magna*) (OECD, 2004).

Based on ECHA's grouping framework the NMs were grouped into two categories - freshly dispersed and 2-year-aged, which have been explored *in silico* using the NovaMechanics' in house analytics platform (Enalos) to identify the most important features driving the toxicity in each group, and thus which can be used as a basis for read-across. The computational modelling procedure was performed by combining Isalos Analytics Platform (Papadiamantis et al, 2020, 2021) and Enalos Chem/Nano informatics tools (Afantitis et al., 2020b), which facilitate the manipulation of big data, modelling, validation, and the virtual screening processes. The most significant variables correlating with the endpoint of NMs acute toxicity to *D. magna* using the EC_{40} which is the effective concentration at which 40% of the organisms were immobilized. We selected this (rather than the more conventional EC_{50}) as the threshold between toxic and non-toxic samples for two reasons: (1) to ensure a reasonable balance of the datapoints in the two categories which ensures a better prediction and outcome from the models, and (2) as in several cases of the aged NM the maximum concentration tested, which was limited by dispersion concentrations supplied by the manufacturers, only led to 40% immobilization. The final predictive models have been validated according to OECD criteria and a QSAR model report form (QMRF) prepared to promote regulatory acceptance of the models.

2. Experimental methods

In this study five TiO_2-cored and six Ag-cored NMs were characterized and exposed to *D. magna* neonates (<24 h old) in order to assess their survival at 24 and 48 h. Experimental details are presented in the following paragraphs.

2.1. Medium and representative waters

Commercially available chemicals, solvents, and humic acids (HA) were purchased from Sigma-Aldrich (Dorset, UK) and were of analytical reagent grade. Ultrapure water (UPW) with a maximum resistivity of 18.2 M Ω cm^{-1} was used throughout the experiments. Experiments were performed in *Daphnia* high hardness combo medium (HH combo) (Kilham et al., 1998) and in two synthetic waters - Class I water which represents waters typically found in Norway and the Alpine regions and lowland Class V artificial water which is representative of typical of waters found in the southern UK, Poland, Greece, France, the Balearic countries and the Iberian Peninsula (Hammes et al., 2013a). The HH combo medium represents an average hard water standard without any NOM and is commonly used for the culturing of *Daphnia*. The Class I water has low dissolved NOM concentration (1.84 mg L^{-1}) while the Class V water has high alkalinity and high NOM concentration (4.6 mg L^{-1}). A description of the water combinations is given in Table S1 in the supplemental information (SI). All three test media had comparable pH values between 7.6 and 7.8.

2.2. Nanomaterials and characterization

The Ag NMs used in this study were 60 nm uncoated Ag (from App Nano Ltd, Spain), 80 nm uncoated Ag (from Promethean Particles, Ltd.), 50 nm PVP coated Ag (Amepox Ltd., Poland), 50 nm PEG-Thiol coated Ag (from PROM), 3–8 nm paraffin coated Ag (Amepox Ltd., Poland), and 50 nm Ag_2S-PVP coated (AppNano Ltd, Spain). The TiO_2 NMs used in this study were all anatase TiO_2 with a core size of 10–12 nm, and either uncoated TiO_2, polyvinylpyrrolidone (PVP) coated TiO_2, Dispex A4040 coated TiO_2, Solplus D540 coated TiO_2 or Pluronic F127 coated TiO_2 NMs, all provided by Promethean Particles Ltd., UK. Details of the coating molecules are given in Table S2 in the SI. All NMs were obtained from the EU H2020 NanoFASE project. Ageing of the NMs was achieved by preparing stock solutions (1000 mg L^{-1}) in the HH combo, Class I and Class V synthetic waters and storing the stock solutions for two years in a dark at 4 °C in a refrigerator prior to the *Daphnia* exposures.

Dynamic light scattering (DLS) was used to measure both the "freshly dispersed" and "aged" (two years in the various media) NMs' hydrodynamic diameters. Zeta potential was used to assess the stability of the NMs in the media and to monitor the changes in the NMs' electrostatic charge and the solution conductivity using a Malvern Nanosizer 5000.

Transmission electron microscopy (TEM) analysis of the freshly dispersed and 2-year aged NMs was performed using JEOL 1200EX 80 kV (pristine) and JEOL 1400EX 80 kV (aged) microscopes. NMs were prepared by the drop casting method by depositing a 20 μL drop of the NM suspension onto a 300 mesh carbon-coated copper TEM grid (Agar Scientific, UK). TEM primary particle sizes were determined by counting at least 100 NMs.

2.3. Test organisms

Initial stocks of *Daphnia* were maintained using pools of genetically identical 3rd brood Bham2 strain. *Daphnia* were kept in a 20 °C temperature controlled environment with 16:8 h light:dark cycles and cultured in HH Combo media and in synthetic Class I and Class V river lowland waters (as described in (Hammes et al., 2013b)) which were refreshed twice weekly to ensure healthy culture maintenance. *Daphnia* cultures were fed 0.5 mg carbon daily between days 0–7 (750 μL) as *Chollera vulgaris* algae.

The impacts of the freshly dispersed and aged NMs were assessed on neonates (<24 h old) over 48 h to establish the dose response relationships to both the freshly dispersed and the 2 years aged forms to each of the NMs. A total of 30 (10 × 3) *Daphnia* were exposed to each of a range of NM concentrations (both freshly dispersed and 2-year aged NMs) spanning 1–1600 μL for the Ag NMs and 10–1000 mg L^{-1} for the TiO_2 NMs. These ranges were selected based on our previous studies

where Ag NMs were found to be toxic at lower concentrations than TiO_2 NMs(Ellis et al., 2020, 2021) . Neonates were assessed at 24 and 48 h for survival as per the OECD 202 test. Control daphnids were not exposed to any NMs and were kept in only medium (HH Combo, Class I or Class V water) under the same conditions as the exposed neonates over the 48-h period and assessments of survival were obtained.

3. Modelling methods

3.1. Dataset

The available experimental data points were organized, pre-processed and harmonized prior to any modelling activities. The experimental data for each one of the 11 tested NMs were organized according to the NMs' age (freshly dispersed or 2 years aged in the various media), the medium (HH Combo, Class I or V river water) and the concentrations used to interact with *Daphnia*. In the experimental study the effective concentrations (ECs) of NMs on *Daphnia* were explored at 24 and 48 h (OECD, 2004). The concentrations of the NMs stock solutions (which were received as aqueous dispersions) were a limiting factor in the concentration range that could be tested. The much lower toxicity of the NMs following ageing in the media compared to the pristine NMs meant that it was not possible to experimentally determine the EC_{50} values for all the NMs at 48 h, meaning that we could only indicate that EC_{50} was above the highest tested concentration in some cases, as shown in Table 1. The experimental data for all treatments (pristine and aged NMs) did include a concentration where 40% of the test organisms were immobilized, and thus we chose to utilize these experimental values (in which we were confident) rather than extrapolating to an EC_{50} value based on experimental data covering only the bottom part of a dose-response curve. Thus, a separation threshold of 40% decrease of the initial population at 48 h (EC_{40}) was used to produce two classes with relatively balanced distributions ("toxic", population decreased more than 40% relative to the untreated controls/"non-toxic", population decreased less than 40% relative to the untreated controls). In total, considering the different samples in terms of NM age, testing medium and concentrations, 353 values were made available for input modelling data (150 characterized as "toxic" and 203 characterized as "non-toxic" based on whether the concentration was above or below the EC_{40} threshold).

Table 1
Summary of the acute 48-h EC_{50} values (mg/L) determined for the NMs freshly dispersed in HH Combo medium and aged for 2-years in the 3 different media (HH Combo, and Class I and Class V artificial waters).

NM	EC_{50} of pristine NMs in HH combo (mg/L)	EC_{50} of aged NMs in HH combo (mg/L)	EC_{50} of aged NMs in Class I water (mg/L)	EC_{50} of aged NMs in Class V water (mg/L)
Uncoated Ag (AppNano)	0.037	0.125	0.23	>1
PVP Ag (Amepox)	0.029	0.170	0.365	0.87
Ag_2S (AppNano)	0.184	0.250	>1	>1
Paraffin Ag (Amepox)	0.077	0.537	0.453	>1
PEG Thiol Ag (Prom)	0.269	>1	0.542	>1
Uncoated Ag (Prom)	0.190	>1	0.800	>1
TiO_2 uncoated (Prom)	75	>1000	570	≪400
TiO_2 PVP (Prom)	45	>1000	548	≪400
TiO_2 Dispex (Prom)	70	>1000	285	430
TiO_2 Solplus (Prom)	715	>1000	360	>1000
TiO_2 Pluronic (Prom)	120	150	409	847

The available numerical descriptors (TEM size, DLS size, electrophoretic mobility, zeta potential at pH = 7.6–7.8, medium conductivity, tested concentration) were normalized (by applying the Gaussian normalization method) in order to guarantee their equal contribution to the analysis (Leach and Gillet, 2007). The most significant variables were identified by the *BestFirst* variable selection method and *CfsSubsetEval (CFS)* evaluator using the Isalos Analytics Platform. CFS subset evaluator identifies a subset of uncorrelated variables that are highly correlated to the endpoint (EC_{40} in this case). The method searches the possible combinations of variables and selects the best one using the *BestFirst* search method, which performs greedy hill climbing with backtracking (Witten et al., 2011).

3.2. Modelling methodology

Grouping and read-across for data gap filling, have been proposed as alternative techniques to experimental hazard assessment of chemicals and NMs. Even though the relevant concepts are well-defined for small molecules, when NMs are concerned, due to challenges and uncertainties in the distinction between the different nanoforms, grouping and consequently read-across strategies and approaches are still under investigation. ECHA provided systematic guidance regarding NMs grouping, read-across of their (eco)toxicological properties and reporting in an effort to harmonize the different proposed methods and guide future efforts. In this guidance a stepwise workflow is presented consisting of seven steps, including adequate collection of data, grouping under a robust hypothesis and justification (ECHA, 2017).

In the present work, the grouping hypothesis is that environmental ageing of NMs will reduce their ecotoxicity to *Daphnia magna* compared to the freshly dispersed NMs, and that this will be more pronounced for the media containing natural organic matter than for the salt-only medium. In order to evaluate the adequacy of this hypothesis, we used the *k* nearest neighbors (*k*NN) methodology by dividing the initial dataset into subgroups of NMs of the same age (i.e., freshly dispersed versus 2-year aged) and developing two separate models in each subgroup. All details are presented in Table S4.

3.2.1. kNN

k nearest neighbors (*k*NN) is an instance-based ("lazy") classifier that does not need to develop a predetermined model for classification of new instances, as each new instance is assigned to the class indicated by the closest training examples (neighbors) in the feature space. Majority vote, in this case weighted by neighbors' distance values, determines the class assigned to each new instance. This simple technique produces reliable predictions and performs well (in terms of computational cost) when relatively-small sets of data are treated and thus, it can be considered as an ideal classifier for the analogue read-across approach (OECD, 2007; Papadiamantis et al., 2021; Triguero et al., 2019). The EnaloskNN algorithm was used in order to apply the *k*NN method in our workflow which allowed us to observe the neighbors (analogues) from the training set of each test sample (NovaMechanics Ltd, 2019).

3.3. Validation

According to ECHA, predictive models should meet and be accompanied by the following information for their use in regulatory purposes: a defined endpoint, an unambiguous algorithm, a defined domain of applicability, appropriate measures of goodness-of-fit, robustness and predictivity and a mechanistic interpretation, if possible (European Chemicals Agency, 2016; OECD, 2007). The validation measures used in this workflow are presented as follows and the compliance of the models with the entire set of OECD principles are summarized in the Supporting Information file, presented as a QSAR model report form (QMRF) report.

In order to ensure the robustness of the produced models, in terms of accuracy and predictive power, an external and an internal validation scheme was applied. In the external validation approach the full dataset was divided randomly into training and test subsets in a ratio of 75:25. The training set was used to determine the subset of optimal descriptors for toxicity prediction initially and was then used to define the modelling parameters. In an iterative process, for each model developed using the training set, the performance was evaluated using the test set, until the optimal modeling parameters were met, determined by calculating the following parameters (eq. [1]-[3]): sensitivity (Sn), specificity (Sp) and accuracy (Ac) that describe the proportion of actual toxic NMs that are correctly classified as "toxic", the proportion of actual non-toxic NMs that are correctly classified as "non-toxic" and the overall success rate of the model, respectively (OECD, 2007). The Mathew's correlation coefficient (MCC, eq. (4)) was also calculated to estimate the performance of the models in relation to a purely random prediction.

$$\mathrm{Sn} = \frac{\mathrm{TP}}{\mathrm{TP} + \mathrm{FN}} \tag{1}$$

$$\mathrm{Sp} = \frac{\mathrm{TN}}{\mathrm{TN} + \mathrm{FP}} \tag{2}$$

$$\mathrm{Ac} = \frac{\mathrm{TP} + \mathrm{TN}}{\mathrm{TP} + \mathrm{FP} + \mathrm{TN} + \mathrm{FN}} \tag{3}$$

$$\mathrm{MCC} = \frac{\mathrm{TP} \times \mathrm{TN} - \mathrm{FP} \times \mathrm{FN}}{\sqrt{(\mathrm{TP} + \mathrm{FP})(\mathrm{TP} + \mathrm{FN})(\mathrm{TN} + \mathrm{FP})(\mathrm{TN} + \mathrm{FN})}} \tag{4}$$

where, *TP* are true positives (toxic samples correctly classified as "toxic"), *TN* are true negatives (non-toxic samples correctly classified as "non-toxic"), *FP* are false positives (non-toxic samples incorrectly classified as "toxic") and *FN* are false negatives (toxic samples incorrectly classified as "non-toxic").

Y-scrambling testing was also performed in order to ensure that the accuracy of the produced models is not a coincidental outcome. In this test, the modelling calculations are repeated using the initial matrix of independent variables and shuffled values for the dependent variable. In each Y-scrambling test, a model is developed using the scrambled training set and validated with the original test set. If the statistical metrics (eq. [1]-[3]) of the produced model are reduced, comparing to the model built with the actual endpoints, then the original model is considered reliable. If the scrambled data produce higher statistical metrics, an acceptable predictive model cannot be produced for both modeling methodologies and original training set (Melagraki and Afantitis, 2015; Tropsha, 2010; Varsou et al., 2019a,b). The Y-scrambling test was performed 5 times for each model using Isalos Y Randomization function.

Finally, the produced models were validated internally to reduce the bias produced from a possible unbalanced separation of the two categories between the training and the test set. Thus, for the training set leave-one-out (LOO) and leave-ten-out (L10O) cross-validation (CV) methods were employed.

3.4. Applicability domain

A fully validated and robust model must not be expected to provide reliable predictions for all possible NMs with known properties and unknown (adverse) outcomes on *Daphnia*. For example, we should not expect reliable predictions for gold NMs or multi-walled carbon nanotubes using the model developed for Ag and TiO_2-cored NMs. Therefore, it is important, especially within a safety-by-design framework, to denote whether a property estimation should be considered reliable or not, through a well-defined applicability domain (AD). The AD reinforces the confidence of experimentalists and regulators, who wish to make use of the model, concerning the reliability of their predictions and thus, the theoretical model can be accepted in real-life applications (Gadaleta et al., 2016).

In the present work the AD was defined via Euclidean distance method among all training and test NMs. The distance of each test NM to each nearest neighbor of the training NMs was compared to a predefined AD threshold (see eq. [5]); if this distance is lower than the threshold then its endpoint prediction can be considered reliable.

$$thr = d + Z\sigma \tag{5}$$

Initially, all Euclidean distances between all training NMs are calculated, as well as the mean value of these distances. In a next step the new average value d and standard deviation σ of the distances included in the subset of training NMs which have lower distance than the average distance of all training NMs, are calculated. Z, is an empirical cut-off value and in this study was set equal to 0.5 (Melagraki and Afantitis, 2013).

The assessment of the AD of the proposed models was elaborated directly in Isalos Analytics Platform, using the Domain–APD function that executes the aforementioned procedure (Melagraki and Afantitis, 2013; NovaMechanics Ltd, 2019).

4. Experimental results and discussion

The TEM characterization of the NMs for both the pristine and 2-year aged NMs in each of the three waters tested are presented in Figs. 1 and 2, and Tables S2 and S3 in the supplemental information. The medium type and effect of long-term ageing on the particles affected the zeta potential and size, both TEM and hydrodynamic size as determined by DLS (see Table S2).

The hypotheses being tested experimentally were that: (1) the presence of NOM in the medium reduces the toxicity of the NMs (by forming an ecological corona); and (2) that environmental ageing of NMs reduces their toxicity compared to the freshly dispersed NMs irrespective of the medium composition (salt only or containing biomolecules). For the models, the grouping hypotheses were (1) that freshly dispersed NMs had a higher toxicity than environmentally aged NMs and thus constituted distinct groups, and (2) that the NM properties most correlated with toxicity would be different for the freshly dispersed NMs than those for the 2-year aged NMs, with ageing core compositions would correlate with different physico-chemical characteristics of the NMs (see Fig. 3).

Table 1 summarizes the determined EC_{50} values at 48 h based on the % daphnids immobilized at each tested concentration across a concentration range. In some cases, we were not able to determine an EC_{50} as it exceeded the NM concentrations of the stock solutions used to age the NMs, in which case we indicated it as being above the highest tested concentration. In a couple of the TiO_2 NMs cases, the EC_{50} was below the lowest tested concentration which is also indicated using $\ll$, and unfortunately, we ran out of aged NMs and thus could not repeat the study at lower concentrations. Based on the data in Table 1, it is clear that both of the hypotheses are experimentally confirmed.

According to the Classification and Labelling of Chemicals legislation (Karjalainen et al., 2014), a chemical is considered to be toxic Category Acute 1 if the 48 h EC_{50} value is ≤ 1 mg L^{-1}, toxic Category Acute 2 if the 48 h EC_{50} value is between 1 and 10 mg L^{-1} and toxic Category Acute 3 if the 48 h EC_{50} value is $\leq$ 100 mg L^{-1}. Above this, chemicals are considered to be non-toxic. Thus, there is quite a spread in terms of the NMs in terms of the toxicity classifications overall from Category Acute 1 (all the Ag NMs freshly dispersed in HH Combo medium) moving to Acute Category 2 for most of the Ag NMs aged in the Class V water, while some of the pristine titania were Category Acute 3 initially, and in all cases the aged TiO_2 NMs would be classified as non-toxic. Due to the unique physicochemical properties of NMs and the effects and adverse outcome displayed in the daphnids at these concentrations, it is not yet clear how such information will feed into the regulatory risk assessment. However, it would be particularly useful to understand, for example, how long a NM may remain as a Category Acute 1 material or how

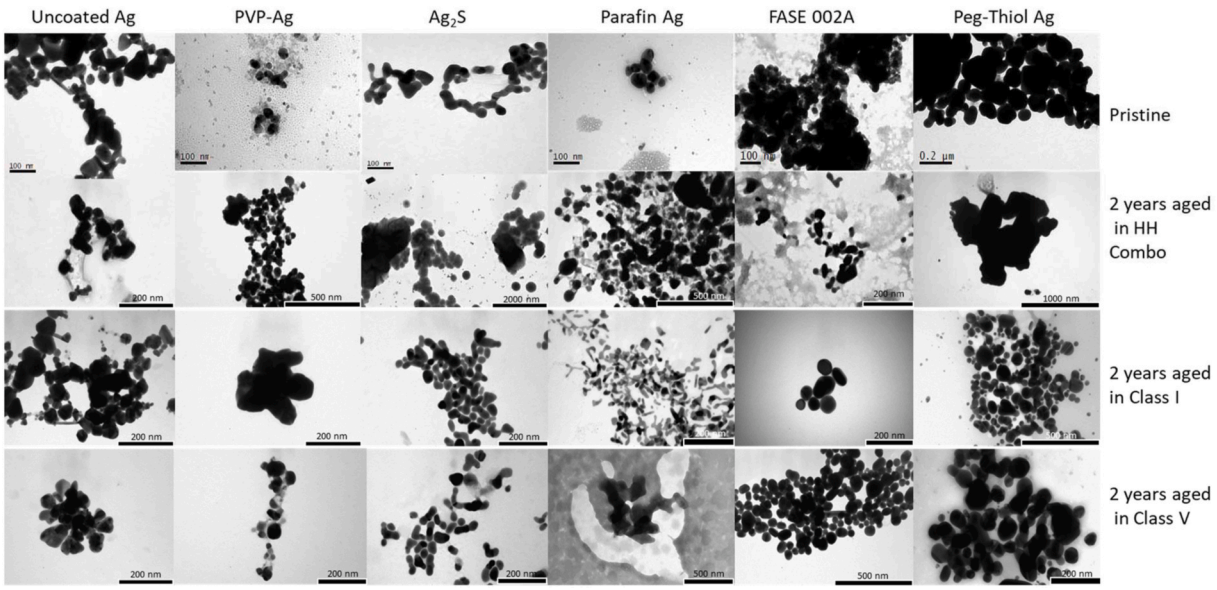

Fig. 1. TEM images of the Ag NMs showing their morphology in each media conditions versus timescale to reflect the ageing process.

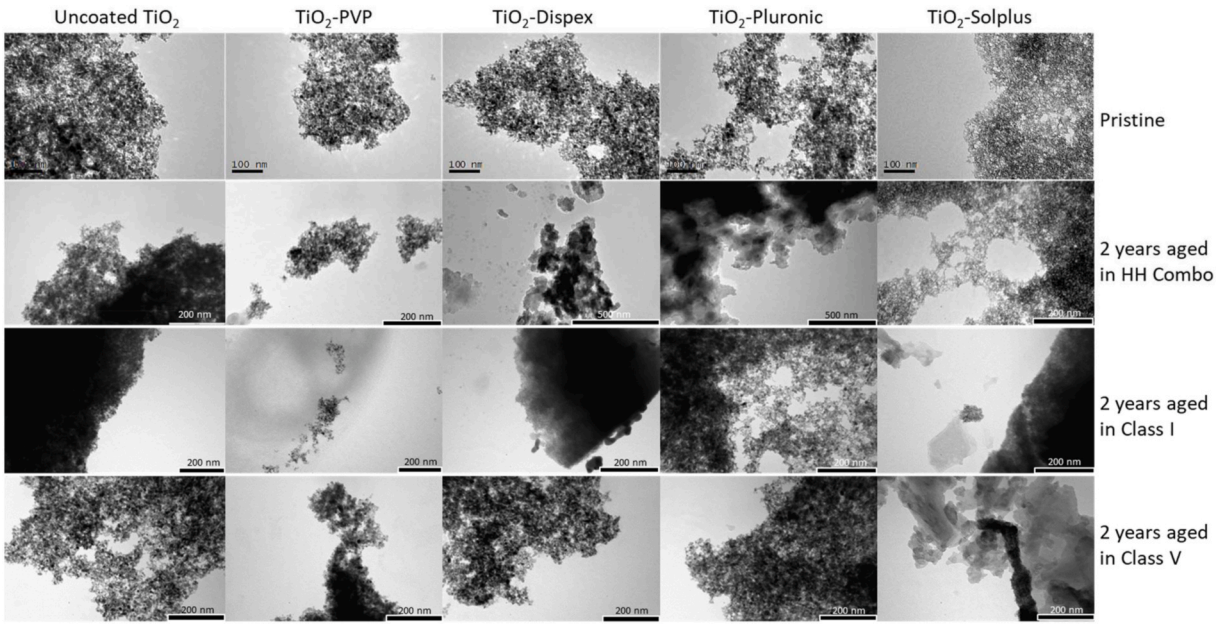

Fig. 2. TEM images of the TiO_2 NMs showing their morphology in each media conditions versus timescale to reflect the ageing process.

quickly its' toxicity might be reduced once released into the environment.

5. Modelling results and discussion

The implementation of the predictive model for the NMs toxicity assessment was performed within the Isalos Analytics Platform. All analysis steps including normalization, variable selection, modelling and validation were performed in Isalos Analytics Platform using the available functions, as well as the proprietary Enalos + nodes (Afantitis et al., 2020b; Varsou et al., 2018a), developed by NovaMechanics Ltd (http://enalosplus.novamechanics.com/). A summary of the modelling results can be found in the publication's supplementary file, presented as a QMRF.

A dataset of 11 NMs tested in different media (HH Combo, Class I and V water) under different ageing conditions (freshly dispersed, 2 years aged) was exploited *in silico*. The available dataset variables were: core, surface coating, surface charge, hydrophobicity, age condition, testing medium, NOM concentration in the testing medium, alkalinity of the medium (in equivalents of $CaCO_3$), TEM size (fresh or aged), DLS size (fresh or aged), zeta potential (fresh or aged), NM surface conductivity (fresh or aged), NM electrophoretic mobility (fresh or aged) and tested concentration. As previously stated, to test our read-across hypothesis, the dataset was divided into two subsets based on the age of the NMs (freshly dispersed, 2-year aged). In both cases the same normalization and variable selection processes were applied to the data: For the development of our predictive models, each subset of NMs was divided into training and test sets in a proportion of 75:25 following a stratified sampling technique. The descriptor values of the training sets were normalized, and the applied normalization parameters were used for the normalization of the test set during external validation.

The *BestFirst* variable selection along with the *CfsSubsetEval*

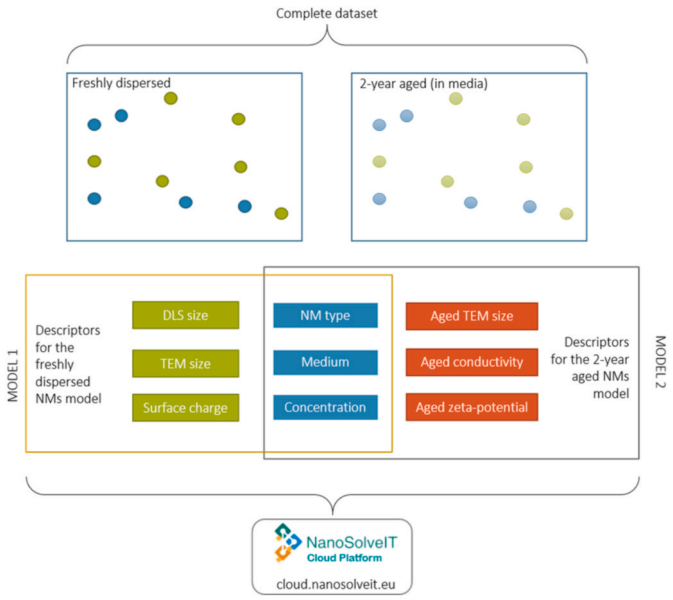

Fig. 3. Based on the Grouping hypotheses: (i) NMs ageing reduces their toxicity to *D. magna* and (ii) Different NMs physico-chemical descriptors explain the toxicity of freshly dispersed versus aged NMs, two models were built each based on a set of six descriptors most predictive of the NMs' toxicity to *D. magna*. Both models are included in the NanoSolveIT Cloud Platform developed by the H2020 Nanoinformatics Project NanoSolveIT (www.nanosolveit.eu) (Afantitis et al., 2020a) to reach all interested stakeholders including industry and regulators.

evaluator were applied to each training set, with the purpose to select the most significant among the 13 available descriptors. Considering that the type of core (Ag, Ag_2S, TiO_2), the tested media (HH Combo, Class I water, Class V water) and the concentration [ppb] were elemental parameters during experimental evaluation (they outlined the experimental conditions), these descriptors were excluded from variable selection. The variables (apart from the three essential variables described above) that were selected and used for modelling purposes are presented in Table 2.

Modelling via the Isalos Analytics Platform allowed us to experiment with different machine learning algorithms and test their performance (*k*NN, J48 and random forests). Among the applied methodologies, the *k*NN modelling technique appeared to outperform the others and best correlate the toxicity endpoint to the selected variables, with an optimized value of $k = 3$ neighbors in both cases (freshly dispersed and 2-year aged models). Credibility assessment of the produced predictions was performed according to OECD guidelines (OECD, 2007) including determination of the accuracy of the predictions, implementation of LOO and L10O-CV for internal validation and Y-scrambling test. Tables 3 and 4 present the accuracy statistics of the models for test sets (freshly dispersed and aged NMs, respectively) and the accuracy values of CV for both models, which are higher than 0.7 thus, both models can be considered stable.

When models were trained with a training set of shuffled endpoint values but the same parameters as the proposed models, they presented statistically lower predictive power when applied to the test set in comparison to the models using the original training values, thus the possibility of chance correlation is reduced.

Table 2
Selected variables for each set of NMs.

Set of samples	Selected variables
Freshly dispersed	TEM size, surface coating charge, DLS size
Aged (2 years)	TEM size, zeta potential, conductivity

Table 3
Accuracy statistics of the kNN predictive model for the freshly dispersed NMs.

Statistics	Values
Accuracy on test samples	0.906
Sensitivity on test samples	0.941
Specificity on test samples	0.867
MCC on test samples	0.813
Accuracy in LOO CV (training samples)	0.787
Accuracy in L10O CV (training samples)	0.777
Accuracy in Y-scrambling (test samples)	0.406–0.625

Table 4
Accuracy statistics of the kNN predictive model for the aged NMs.

Statistics	Values
Accuracy on test samples	0.895
Sensitivity on test samples	0.857
Specificity on test samples	0.917
MCC on test samples	0.774
Accuracy in LOO CV (training samples)	0.765
Accuracy in L10O CV (training samples)	0.765
Accuracy in Y-scrambling (test samples)	0.474–0.614

Finally, in both cases by observing the *k* selected neighbors of the training set for each test sample, *k*NN method proved to have a selectivity in the selection of analogues, as in most cases, the selected analogues had the same core thus, further investigation on the data can reveal more subgroups of structurally similar samples.

5.1. Freshly dispersed subset

The subset including only pristine NMs consists of 94 training and 32 test samples. The accuracy statistics encoding the performance of the *k*NN model on test set are presented in Table 3. The accuracy of internal CV on training set is also presented in this table.

The AD has been determined in order to define the area of reliable predictions. The AD threshold was calculated, based on the training set, equal to 1.073. All samples in the test set had values in the range of $4.5{\cdot}10^{-5}$–0.903 thus the predictions for the test set can be considered reliable.

5.2. Aged subset

The subset including only aged NMs consists of 170 training and 57 test samples. The accuracy statistics summarizing the performance of the *k*NN model on test set are presented in Table 4, as well as the accuracy of internal CV on training set.

The AD threshold calculated based on the training set was equal to 1.687. All samples in the test set had values in the range of $7.7{\cdot}10^{-5}$–0.456 thus the predictions for the test set are all considered reliable.

5.3. Discussion on selected descriptors

A crucial demand in nanotoxicology is the correlation of properties and physicochemical characteristics of NMs to their biological or toxicity behavior. After developing and validating the predictive models, interpretation and clear definition of the variables that emerged as important for modelling the toxicity endpoint are provided. The information included in these descriptors are investigated in order to understand how they affect NMs toxicity outcome and how they are specific to the age of the NMs, i.e., considering how the NMs properties change during ageing due to interaction with the medium constituents including the salts and NOM where present.

NMs size is a crucial parameter for the assessment of their toxic effects to living organisms due to their ability to penetrate epithelial

tissues and be carried by the bloodstream to different organs, cells and organelles, causing in this way cell damage or altering cell metabolism (Sukhanova et al., 2018). NMs size is directly correlated with surface area and consequently with surface reactivity, and greatly affects the dissolution behavior for (partially) soluble materials. The extent of agglomeration phenomena of NMs in a medium is thus and important factor in determining NMs bioavailability to organisms – for example, in the case of daphnids they exist in the water column and thus if particles are highly agglomerated, they may settle out over time and thus become less bioavailable to the daphnids. Similarly, very small NMs may not be recognised by the *Daphnia* and may just be filtered with the water as part of the passive water filtration behavior. It has been observed that some particle agglomeration, such as that resulting from NMs interaction with NOM or the biomolecules secreted by daphnids into the medium during their water filtering, leads to enhanced recognition of the particles as food, especially if the agglomerates are close in size to the algae that daphnids normally consume, i.e., around 1 μm (Nasser and Lynch, 2016, 2019). *Daphnia* usually consume particles from around 1 μm up to 50 μm, although particles of up to 70 μm in diameter may be found in the gut content of large individuals (Ebert, 2005). Since our study utilised neonates (<24 h) and monitored the impact of NMs on them over 48 h, it is expected that agglomerates larger than 40–50 μm in diameter would be excluded. Thus, it is not surprising that both TEM (core size) and DLS (combining core and coating size plus the hydration layer, hence also called the hydrodynamic diameter), which give valuable information about the dispersion stability of NMs in a specific medium (Bae et al., 2010; Giusti et al., 2019), are found to be important parameters in driving the acute toxicity of the NMs to daphnids.

The surface charge of NMs affects their interactions with other NMs or organic matter of the medium and is influenced by these interactions. Depending on whether the particles are electrostatically stabilized only or are also sterically stabilized due to the presence of a polymeric coating, medium composition will play an important role in the NMs stabilization. High salt content media may have a destabilizing effect on NMs by neutralizing their surface charges leading to agglomeration. On the other hand, the presence of NOM in the medium potentially acts as a dispersant, coating the particles and providing them with a negative charge (since NOM is primarily composed of humic and fulvic acids) as well as introducing steric stabilization (Markiewicz et al., 2018). Zeta-potential encodes the NMs surface charge in relation to the "local" conditions (medium pH, concentration, suspension conductivity etc.). Thus, the electrostatic stability, the steric stabilization and thereby the extend of agglomerates formation can be correlated to zeta-potential (Lowry et al., 2016; Mikolajczyk et al., 2015; Vogel et al., 2017). Generally, higher zeta-potential values either positive or negative, lead to stable NMs suspensions, while on the contrary, lower zeta-potential values produce suspensions of agglomerated NMs (Lin et al., 2010), although this neglects the role of steric stabilization of NMs. Interestingly, in all cases, the zeta potential of all of the Ag NMs was negative and remained negative during the ageing in all media, although become slightly less negative in most cases, while the TiO_2 NMs, irrespective of coating charge, were positive initially and became negatively charged during aging; in the case of the Class I and Class V waters, this was likely the result of NOM binding resulting in formation of an eco-corona, while in the case of the salt-only HH Combo medium, this was likely a result of some loss of the coatings over time, and interaction of the oxide groups with the salts in the medium. Inorganic anions including phosphate, carbonate, and to a lesser extent, sulfate were found to decrease the isoelectric point (IEP) of TiO_2 NMs and stabilized the NMs suspensions owing to specific surface interactions, which was not observed for nitrate and chloride ions, and a decrease in hydroxyl radical generation was observed for all inorganic anions (Farner Budarz et al., 2017). Thus, the reactivity of TiO_2 NMs is strongly influenced by the makeup of the waters they are released into. The HH Combo medium contains phosphate, carbonate, and sulfate (Table S1), which likely contributes to the passivation of the TiO_2 NMs observed as a result of ageing, and interaction of these anions also explains the acquisition of a negative zeta potential even in the absence of NOM (Table S3) as well as the significant agglomeration observed upon ageing (Table S2).

As it can be clearly denoted, agglomeration process (depending on the combination of surface charge, NM size, medium conductivity, salt composition and/or presence of NOM) plays a key role in the final toxicity expression of the various NMs towards *D. magna*, as also demonstrated by the experiments described in this study. The agglomeration of the NMs during ageing, whether driven by interactions with anions or NOM contained in the medium, reduces their reactivity due to the limitation of their specific surface area (Lin et al., 2010) and in addition leads to possible sedimentation of the agglomerates, thus removing them from the water column and reducing their interaction with, and bioavailability to, living organisms.

5.4. Discussion on grouping and read-across

In order to reinforce our choice of grouping and read-across methodology, we have used the entire dataset of pristine and aged NMs (without formulating a grouping hypothesis) and different modelling techniques (J48, *k*NN and random forests) and we compared the results to the previously presented models. The models developed using the entire dataset had lower predictive accuracy in external validation, than the ones developed using a grouping hypothesis (based on NM age) and the *k*NN methodology. In addition, PCA analysis was run on the entire dataset (see Figure S2) using 3 principal components which explain up to 60% of the variation of the data. The grouping of the NMs in two separate groups of pristine and aged particles is obvious from the PCA analysis, which further reinforces our grouping hypothesis.

6. Data availability

The full dataset is available through the NanoPharos (2021) database https://db.nanopharos.eu/Queries/Datasets.zul) developed under the H2020 NanoSolveIT (Afantitis et al., 2020a) and NanoCommons (Nanocommons, 2021) projects, in compliance with the FAIR data principles (i.e., Findable, Accessible, Interoperable and Re-useable) and is ready for further computational analysis.

7. Conclusions

In this work we have developed ecotoxicological read-across models for predicting NM acute toxicity, following ECHA's recommended strategy for grouping of NMs as a means to explore *in silico* the effects of a panel of freshly dispersed versus environmentally aged Ag and TiO_2 NMs on the freshwater zooplankton *D. magna*. The computational modelling procedure was performed by combining the KNIME Platform and Enalos Chem/Nano informatics tools, which facilitate the manipulation of big data, the modelling, the validation, and the virtual screening processes. The selected grouping hypotheses were that NMs ageing reduces their toxicity and that the degree of agglomeration, which is dependent on the combination of surface charge, NM size and surface conductivity, plays a key role in the final toxicity expression of the NMs to *Daphnia*. The agglomeration of NMs reduces their reactivity, reduces dissolution potential for soluble NMs such as Ag NMs, and above a certain size reduced bioavailability to the daphnids, both by agglomerates larger than the size of their mandible being too large to be taken up by the daphnids, and as a result of agglomerates settling out of the water column in which the daphnids exist. The predictive power of the proposed models has been evaluated according to OECD criteria and a QMRF report has been produced and made available in the supplementary information. The dataset is included in the supplementary files and via NanoPhasros database and has been curated in manner to be suitable for aadditional nanoinformatics studies.

Author contributions

Dimitra-Danai Varsou performed the model development and validation, drafted the manuscript & prepared the graphics; **Laura-Jayne Ellis** performed the biological experimental exposures, the NMs characterization and drafted the manuscript; **Antreas Afantitis** conceptualized the research goals, supervised DDV, performed the model development & validation and drafted and edited the manuscript; **Georgia Melagraki** conceptualized the research goals, supervised DDV, performed the model development & validation and drafted and edited the manuscript & prepared the graphics; **Iseult Lynch** conceptualized the research goals, supervised LJE, and drafted and edited 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.

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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.2021.131452.

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