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On the uses of predictive toxicology to approve the use of engineered nanomaterials as biocidal active substances under the Biocidal Products Regulation

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Abstract. To date only two engineered nanomaterials (ENMs) have been approved to be used as biocidal active substances in the formulation of biocidal products under product type 18 (insecticides, acaricides and products to control other arthropods). Such materials are silicon dioxide (as a nanomaterial formed by aggregates and agglomerates) and synthetic amorphous silicon dioxide (nano). The use of non-animal alternative test methods has been foreseen in the Biocides Product Regulation, Regulation (EU) 258/2012 (BPR). Further, the BPR is one of the existing regulations that includes a specific definition of nanomaterials. On the present article, a review is made on the potential uses of Quantitative Structure-Activity Relationship approach (Nano-QSARs) to be used as a non-testing method for the generation of ecotoxicological data required for the approval of new active substances in the nanometric scale. Relevant challenges are to be faced in the application of computational chemistry but it could meet the needs imposed by the BPR in relation to the use of non-testing methods. However nanospecific adaptations need to be implemented further on ecotoxicological testing so that obtained results are considered a suitable input for models' building. The BPR, thus, sets the framework for innovative approaches in the regulatory approval of new chemicals that integrate special considerations derived from the chemical nature of ENMs and the application of non-testing methods but, to date, the implementation of such actions is not feasible in practical terms.

1. Introduction

The Biocidal Products Regulation (BPR, Regulation (EU) 528/2012) has specific provisions for engineered nanomaterials (ENMs). In fact, a dedicated risk assessment is needed when the nanoform of the active and non-active substances are used in a biocidal product. Furthermore, the BPR foresees the use of non-animal alternative methods.

The objective of the present assessment is twofold:



- To evaluate the possible use of computational chemistry (QSARs for ecotoxicological effects prediction) in nanomaterial forms.
- To assess uses of QSAR for the regulatory approval of ENMs within the BPR.

Within the present document technical and regulatory documentation has been reviewed and critically analysed. A reflexion is presented on the applicability of the state of the art information for the use of computational chemistry in the approval of ENMs to be used as active substances for biocidal products formulation.

2. On the regulatory framework for ENMs as active substances of biocidal products

According to the EU Biocidal Products Regulation 528/2012 (EU BPR) [1], a two steps authorization procedure is required to put biocidal products into de market. The active substances are first assessed by an evaluating Member State competent authority and the results of these evaluations are forwarded to ECHA's Biocidal Products Committee, which prepares an opinion within 270 days. The opinion serves as the basis for the decision on approval which is adopted by the European Commission. The approval of an active substance is granted for a defined number of years and is renewable. The approval is for a specific product type (PT). On the basis of approved active substances, companies will ask the authorization of biocidal products at national level. Alternatively, for Existing Biocidal Active Substances, ie, substances which were on the market on 14 May 2000 as an active substance of a biocidal product, a Review Programme was set up by the EU Biocidal Products Directive 98/8/EC (BPD) [2] and continues under the BPR and is foreseen to be completed by 2024. This means that all biocidal product manufacturers and importers must eventually seek authorization according to the procedures described in the BPR before placing a biocidal product on the European market [3, 4].

When it comes to ENMs, the BPR includes a specific definition: 'Nanomaterial' means a natural or manufactured active substance or non-active substance containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1-100 nm. Further, the BPR establishes that in order to obtain an authorization of a biocidal product that contains ENMs, a specific risk assessment has to be performed justifying that the tests used are relevant and applicable for ENMs.

To date, only silicon dioxide (as a nanomaterial formed by aggregates and agglomerates) and nanometric synthetic amorphous silicon dioxide (SAS) have been approved to be used as biocidal active substances under PT 18 (insecticides, acaricides and products to control other arthropods).

In the case of copper nanoforms, no single form has been approved for its use in the EU, to the best of author's knowledge. In fact, in the assessment reports of copper oxide II, basic copper carbonate and copper (II) hydroxide for their approval under PT 8 it is mentioned that: *The applicant is not currently placing nano forms of copper oxide on the market. Therefore, the submitted dossier and the finalised assessment report don't cover potential nanoforms of this copper compound, should such forms exist.* In the assessment report of copper flakes (coated with aliphatic acid) for substance approval under PT21 it is explicitly mentioned that *the active substance does not fulfil the criteria of article 3.1(z) of the BPR, therefore it is not a nanomaterial.* So is it in the case of granulated copper for substance approval under PT8.

As on 3 February 2017, two forms of nanosilver have been included in the Review Programme for the next substance/PT combinations: silver adsorbed on silicon dioxide (as a nanomaterial in the form of a stable aggregate with primary particles in the nanoscale) for PT9 and silver, as a nanomaterial, for PTs 2, 4 and 9. All other nanomaterial forms are explicitly excluded from the Review Program. [5].

In fact, in a recent review, Mackevica et al. (2016) [3] identified on the Nanodatabase [6] 88 biocidal products containing ENMs available on the EU market including disinfecting sprays, air purifiers, cleaning products, biocidal paints and varnishes, amongst other. The ENMs that are the active substances in those biocidal products are primarily silver (46 products), with a few products containing silicon dioxide (3 products) and a large fraction of them claiming the presence of nanomaterials but without any identification of their chemical composition (39 products).

3. On the suitability of the existing information on the environmental hazard of ENMs from a regulatory perspective

Besides being the first piece of legislation to adopt the definition of ENMs recommended by the European Commission, the BPR is also the first to specify that an approval of an active substance does not cover a corresponding ENM form, except when this is explicitly mentioned. The reasoning for this provision is that ENMs will be used as a biocide because of their different properties compared to the bulk form of the substance. These different properties may also result in different toxicities, and therefore ENMs require a separate assessment [7].

Table 1: Ecotoxicological studies to evaluate the toxicity of biocides to aquatic species (Regulation 528/2012) Core data set (CDS) // Additional data set (ADS).

| INFORMATION REQUIRED | | SPECIFIC RULES |
|---|-----|--|
| 9.1 Toxicity to aquatic organisms | CDS | |
| 9.1.1. Short-term toxicity testing on fish | CDS | Not needed if a valid long-term aquatic toxicity study on fish is available |
| 9.1.2. Short-term toxicity testing on aquatic invertebrates | CDS | |
| 9.1.2.1. Daphnia magna | CDS | |
| 9.1.2.2 Other species | ADS | |
| 9.1.3. Growth inhibition study on algae | CDS | |
| 9.1.3.1. Effects on growth rate of green algae | CDS | |
| 9.1.3.2. Effects on growth rate of cyanobacteria or diatoms | CDS | |
| 9.1.4. Bioconcentration | CDS | |
| 9.1.5. Inhibition of microbial activity | CDS | The study may be replaced by a nitrification inhibition test if available data show that the substance is likely to be an inhibitor of microbial growth or function, in particular nitrifying bacteria |
| 9.1.6. Further Toxicity studies on aquatic organisms | ADS | If the results of ecotoxicological studies, studies on fate and behaviour and/or the intended use(s) of the active substance indicate a risk for the aquatic environment, or if long-term exposure is expected, then one or more of the tests described in this Section shall be conducted |
| 9.1.6.1. Long term toxicity testing on fish | ADS | |
| 9.1.6.2. Long term toxicity testing on invertebrates | ADS | |
| 9.1.7. Bioaccumulation in an appropriate aquatic species | ADS | |
| 9.1.8. Effects on any other specific, non-target organisms (flora and fauna) believed to be at risk | ADS | |
| 9.1.9. Studies on sediment-dwelling organisms | ADS | |
| 9.1.10. Effects on aquatic macrophytes | ADS | |

For active substance approval, the dossier submitted to the competent authority must fulfil specific information requirements which are specified in Annex II and III. According to Annex II of the BPR the data submitted to support the approval of an active substance must be obtained according to the methods specified in the Test Methods Regulation [8]. These methods normally refer to test guidelines validated by the Organisation for Economic Co-operation and Development (OECD). If a test method is considered inadequate or not included in this regulation, it is possible to use other scientifically suitable methods; however, justification for the appropriateness of these alternative methods is required. On

Table 1 the ecotoxicological information required by the BPR is included specifying rules to obtain such information when available. A differentiation is made when the data are required for the Core Data Set (CDS) or for the Additional Data Set (ADS). To provide this information, studies should be conducted following the OECD Test Guidelines detailed in Table 2 to indicate the possible acute toxicity of the substance or in

Table 3 for chronic toxicity.

Table 2: OECD Guidelines for acute assays

| Acute assays | | |
|----------------|----------------|---|
| Species | OECD Guideline | Assay |
| Fish | 203 | Fish Acute Toxicity Test |
| | 210 | Fish, Early-life Stage Toxicity Test |
| | 212 | Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages |
| | 236 | Fish Embryo Acute Toxicity (FET) Test |
| Invertebrates | 202 | Daphnia sp. Acute Immobilisation Test |
| | 218 | Sediment-Water Chironomid Toxicity Using Spiked Sediment |
| | 219 | Sediment-water Chironomid toxicity Test using Spiked water |
| | 225 | Sediment-Water Lumbriculus Toxicity Test Using Spiked Sediment |
| | 235 | Chironomus sp., Acute Immobilisation Test |
| | 239 | Water-Sediment Myriophyllum Spicatum Toxicity Test |
| Algae | 201 | Freshwater Alga and Cyanobacteria, Growth Inhibition Test |
| Microorganisms | 209 | Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation) |
| | 224 | Determination of the Inhibition of the Activity of Anaerobic Bacteria |
| | 244 | Protozoan Activated Sludge Inhibition Test |

Table 3: OECD Guidelines for chronic assays

| Chronic assays | | |
|----------------|----------------|---|
| Species | OECD Guideline | Assay |
| Fish | 215 | Fish, Juvenile Growth Test |
| | 229 | Fish Short Term Reproduction Assay |
| | 230 | 21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition |
| | 234 | Fish Sexual Development Test |
| | 305 | Bioaccumulation in Fish: Aqueous and Dietary Exposure |
| Invertebrates | 211 | Daphnia magna Reproduction Test |
| | 233 | Sediment-Water Chironomid Life-Cycle Toxicity Test Using Spiked Water or Spiked Sediment |
| | 315 | Bioaccumulation in Sediment-dwelling Benthic Oligochaetes |

For testing of ENMs it is stated that “when test methods are applied to nanomaterials, an explanation shall be provided of their scientific appropriateness for NMs, and where applicable, of the technical adaptations/adjustments that have been made in order to respond to the specific characteristics of these

materials” (see paragraph 5 of Annex II and Annex III of the BPR). Effectively, the OECD test guidelines (TG) for ecotoxicity testing can be used for ENMs but through methodological adaptations. In fact, the European Chemical Agency (ECHA) is in the process of revising its guidance documents on how industry is to complete chemical safety assessments to address the challenges that nanoparticles pose for ecotoxicological testing. A revision of the state of the art in relation with the appropriateness of these ECHA guidelines is available in Hansen et al. 2017. [9].

In the case of synthetic amorphous silicon dioxide (SAS), the applicant states that exposition under conditions of normal handling and use will be to aggregates but not to the nanoforms as SAS aggregates quickly with the surrounding medium forming stable aggregates. For this reason they only provide a risk assessment for the SAS under its aggregated form. No assessment of the individual particles of silicon dioxide with nanometric size have been included in the dossier for this reason but just for *synthetic amorphous silicon dioxide as a nanomaterial in the form of stable aggregated particles of particle size > 1 μ m, with primary particles of nanosize*. The applicant notes that the present position might be updated in the light of newly emerging knowledge. An additional justification of the applicant for the present circumstance is that SAS was evaluated according to the BPD prior to the existence of nanospecific requirements in the BPR (1 September 2013).

On its side, for silicon dioxide (as a nanomaterial formed by aggregates and agglomerates) no specific assessment report exists which is not the case for the rest of approved biocidal active substances as included on the ECHA’s list of biocidal active substances [10]. It might be the case that the present substance is assimilated to SAS in terms of substance approval; however, it is not explicitly stated.

As it can be derived, only one assessment report publicly exists for biocidal active substances in the nanoform although the specificities of the hazard associated to nanoforms have been excluded.

Brinch et al. 2016 [7] have claimed that for subsequent evaluations under the BPR the procedure followed in the case of SAS might be precedent, that is, applicants could include the same statement in other reports when nanoparticles form stable aggregates avoiding the inclusion of hazard data on the nanoforms. However, this claim doesn’t have much basis since in the BPR definition of nanomaterial and also in the EU recommendation of definition of nanomaterial [11], the aggregate state is also envisaged. This is because the nanoparticles although aggregated/agglomerated can behave as if they were not or can be separated easily once in contact with the surrounding medium. In addition, in the SAS assessment report it is indicated that *the position will be updated with the evolution of knowledge and specific regulations about nanomaterials or with complementary data showing that use of the active substance leads to exposure to individual particles of silicon dioxide with nanometric size*.

4. On the uses of QSAR in nanomaterial forms

Computational toxicology is a subdiscipline of toxicology that aims to use advanced chemometric methods combining statistics, mathematics and computational chemistry to predict the toxic effects of chemicals on human health and/or environment. *In vivo* experiments require much time for preparation and implementation, and are expensive and ethically questionable. In opposite, *in silico* models have the ability to predict the physical, chemical or biological properties of compounds without necessarily carrying out chemical synthesis in the laboratory. The use of *in silico* approaches allows for the decreasing cost, time and the number of necessary testing on laboratory animals, following the 3Rs principle (Replace, Reduce, Refine of laboratory animals) [12]. What’s equally important the computational studies can offer a better understanding of the possible mechanisms by which a given chemical induces harm as well as that these methods could also be crucial in establishing safe-by-design principles at early stages of new nanomaterial development.

In light of this, the computational nanotoxicology, in particular Quantitative Structure–Activity Relationship models, have recently received much attention. QSARs are, in essence, statistical/probabilistic approaches which rely on defining mathematical dependencies between the variance in molecular structures, encoded by so-called molecular descriptors, and the variance in biological activity in a set of compounds. Once the correlation between chemical structure and (eco)toxicological parameter is established, it can be used to predict this toxicological feature in new

molecules whose chemical structures are known. Several articles have pointed out the relevance of this method in computational toxicology [13], as well as the regulatory level [14].

In its annex IV, the BPR refers that results obtained from valid qualitative or quantitative structure-activity relationship models ((Q)SARs) may indicate the presence, but not the absence of a given dangerous property. Results of (Q)SARs may be used instead of testing when the following conditions are met:

- the results are derived from a (Q)SAR model whose scientific validity has been established,
- the substance falls within the applicability domain of the (Q)SAR model,
- the results are adequate for the purpose of classification and labelling and risk assessment, and
- adequate and reliable documentation of the applied method is provided.

An in depth review of literature shows these computational methods still suffer from many limitations. These are mainly related to the paucity of the systematic data available for specific groups of ENMs necessary for the development and validation of such models. Moreover, in the previous contributions the application of Nano-QSAR models was limited rather to simple cases, where usually *in vitro* toxicity endpoint was strongly related to one or two simple structural properties of materials that do not depend on the external conditions (i.e. intrinsic properties). In the further perspective, QSAR methodology should be further developed to consider extrinsic (system-dependent) properties and behaviour of ENMs dependently on the external conditions (dispersion media, presence of other chemical species and ENMs etc.).

Because the ENMs suitable to be used as biocidal active substances are relatively simple nanoforms (mainly Ag, SiO₂, Cu), though relevant challenges are to be faced in the application of Nano-QSAR models to predict ENMs ecotoxicity, computational science could be a viable option.

5. On the suitability of the existing information on the ecological hazard of ENMs from the perspective of computational chemistry for the purposes of the BPR

The use of experimental data for the development of computational models has a series of inherent constrictions, starting from the fact that any model represents a simplification of the reality.

- Experimental data are obtained from animal models. Model implies, for instance, putting a certain number of fish in a tank, which is much simpler than any ecosystem, where many organisms are present and in much more complex conditions. For human toxicity the situation is even more complex, since data are extrapolated from animal species to humans.
- Any experimental value is associated with an uncertainty value. If results of tests addressing one particular endpoint are compared (acute toxicity for *Daphnia magna*, for instance), the value can vary within different ranges even if the entities performing such tests have used the same protocol and have implemented good laboratory practices. Since experimental values have an uncertainty, the model built upon this data will also have an uncertainty value. It is therefore important to have access to multiple values for the same chemical, since that is a way to compare and integrate data.
- Since the models need to be applicable from a regulatory perspective, strong efforts are needed to check the inputs required to build up the model (regulatory endpoint). The different chemicals fall under different regulatory frameworks depending on their particular application (an example are biocides and Plant Protection Products –PPP– since some active substances are affected by both regulations but the specific requirements of each of them differ). The question is: do models need to be created having the BPR in mind and then additional models are needed to comply with the PPP Regulation? Or should models be generated independently of the regulatory classification of the different chemicals?
- An additional example deals with the input on which the model is based on; the data used for models building up can consider an average value or a worst case value and the output of the model will vary depending on this criterion.

This is: models' building up for "traditional" chemicals implies a series of difficulties that are inherent to the use of computational chemistry. In the case of ENMs such difficulties increase due to the

uncertainties associated to the results obtained in the ecotoxicological tests for this group of chemicals. An international consensus on how to assess the ecotoxicological hazard of ENMs is urgently needed since sound data obtained in agreement with recognized protocols are the basis for the development of non-testing methods.

6. Conclusions and future perspectives

Conclusions of the present assessment are summarized as follows:

- The BPR has specific provisions for the authorization of ENMs to be used as active substances in biocidal products and to place on the market biocidal products containing those.
- Despite in force regulation, several studies have pointed out the widespread use of certain ENMs as biocidal active substances which, to the best of the author's knowledge are not currently included in the EU list of biocidal active substances, be it approved or under review.
- On the basis of the existing information it cannot be claimed that all the biocidal products containing ENMs have undergone the approval process required by the BPR.
- To date, only an assessment report with specific data on ENMs is publicly available.
- The BPR anticipates the use of non-testing methods for the approval of biocidal active substances.
- The application of Nano-QSAR/ models is -in principle- a viable option for the ecotoxicological assessment of ENMs.
- Despite the fact that the BPR requires nanospecific testing, and although ECHA has made available some guidance documents on testing on nanomaterials, these documents are still under revision and further considerations need to be taken into account for the testing of ENMs. As a consequence, each of the different entities undertaking such tests might implement different adaptations to meet the specific requirements of this type of chemicals. This implies that significant differences can be obtained by different laboratories which hinders the development of robust Nano-QSAR models.

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