Adapting current *in vitro* ecotoxicity standard methods to allow more realistic and environmentally relevant exposure conditions

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1. Introduction

When assessing the ecotoxicity of Advanced Materials (AdMa), current assays are hampered by a number of issues, including challenges to disperse these materials, as many AdMa are highly hydrophobic, or challenges to expose the test organisms or cells mimicking exposure routes. This work highlights some major issues encountered when setting up *in vitro* ecotoxicity assays for testing poorly water-soluble AdMa. Here, we propose potential solutions that could be explored and developed in order to overcome the existing challenges.

2. Materials and Methods

In this work we tested different dispersion protocols (e.g. low and high energy dispersion methods), dispersion media (e.g. cell culture medium, DMSO, BSA, PVP, conditioned medium, artificial freshwater) and exposure protocols (e.g. submerged, quasi-ALI) to improve the stability of carbon-based AdMa (graphene) and allow more realistic exposure conditions. The OECD TG 249 (Fish Cell Line Acute Toxicity) was then used to evaluate the cytotoxicity of graphene suspensions on RTgill-W1 cells.

3. Results

Results showed that graphene materials disperse poorly in DMSO, artificial freshwater and in cell culture medium, floating away from cells over time. Resulting graphene suspension showed a very low toxicity possibly because they were not accessible to the cells. Graphene dispersed with PVP showed good stability over time, however PVP was cytotoxic to the cells at relatively low concentrations. BSA and conditioned medium slightly improved the stability of graphene in culture medium. Cytotoxicity of graphene dispersed in conditioning medium was higher than that of graphene dispersed in cell culture medium, possibly due to a higher bioavailability to the cells. Cells exposed at quasi-ALI condition showed higher sensitivity to graphene compared to cells exposed at submerged conditions, certainly because quasi-ALI favour a closer contact of cells with the materials. High energy dispersion methods (bath or tip sonication) did not improve

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the dispersion of the materials. Low energy dispersion (long-term stirring) showed better results than sonication.

4. Conclusions and future steps

Results indicated that low energy dispersion (long time stirring) of graphene in the presence of mucopolysaccharides and cell culture medium components (conditioning medium) seems to the best suspension protocol. In addition to that, conditioning medium provides protein corona compounds that facilitate the interaction of AdMa with the cells. More realistic exposure conditions are being explored including dispersing graphene in the standard Suwannee River NOM in conditioning medium.

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