

Application of New Approach Methodologies (NAMs) based on *in vitro* ecotoxicity models to support the development of Safe and Sustainable by Design (SSbD) Advanced Materials

Alberto Katsumiti

GAIKER Technology Centre

Gaiker

MEMBER OF
BASQUE RESEARCH
& TECHNOLOGY ALLIANCE

REACH and CLP regulations and ecotoxicity tests

According to the requirements of **REACH Regulation (EU Reg. 1907/2006)** and **CLP (EU Reg. 1272/2008)** following Regulation (EU) No. 440/2008 of May 30, 2008 (EU TMR), and its subsequent amendments.

- **Aquatic** (freshwater, seawater) and **terrestrial**

- **Tests performed on aquatic organisms and methods used:**

- Short-term toxicity test on invertebrates (preferred species Daphnia): **OECD TG 202**
- Long-term toxicity testing on invertebrates (preferred species Daphnia): **OECD TG 211**
- Growth inhibition study on aquatic plants (algae preferred): **OECD TG 201**
- Short-term toxicity testing on fish: **OECD TG 203**
- Long-term toxicity testing on fish: **OECD TG 204**
- Fish early-life stage (FELS) toxicity test: **OECD TG 210**
- Fish, Short-term Toxicity Test on Embryo and Sac-Fry Stages: **OECD TG 212**
- Fish Embryo Acute Toxicity (FET) Test: **OECD TG 236**
- Active sludge respiration inhibition testing: **OECD TG 209**
- Biodegradability: **OECD TG 301 or 306**



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Alternative species or in vitro

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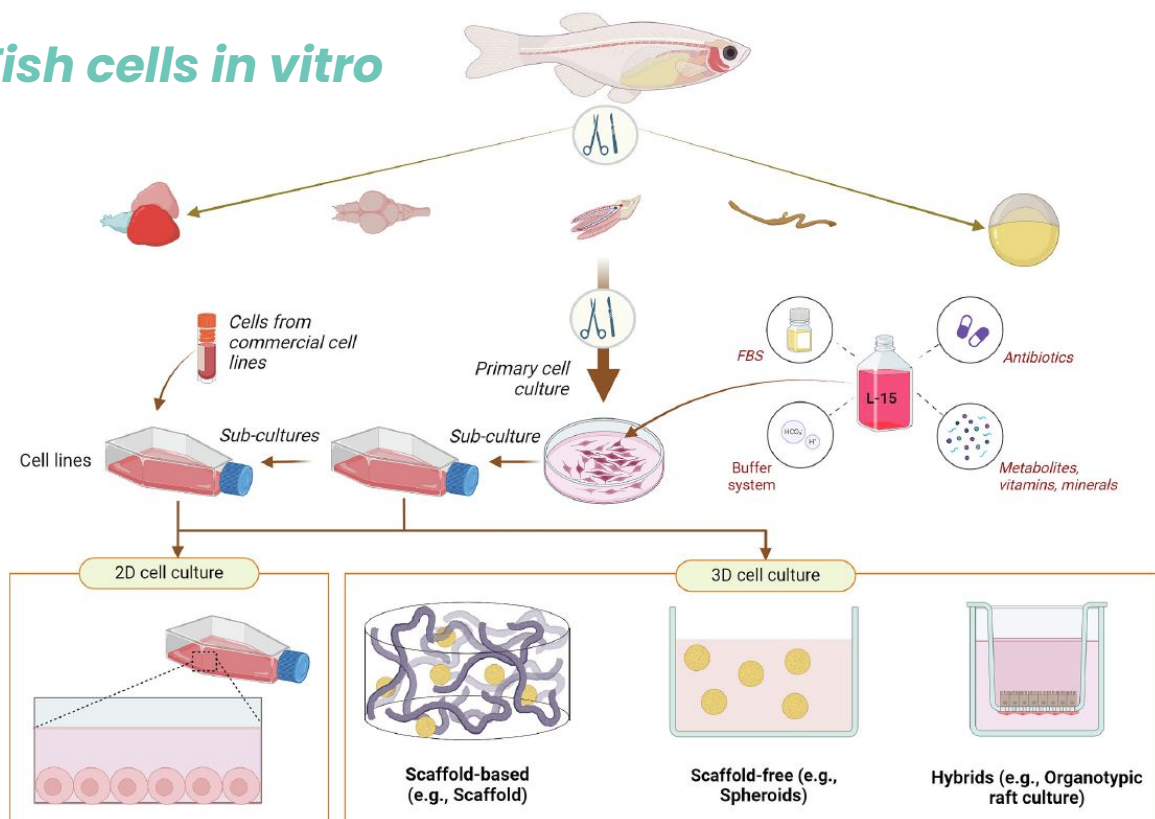
in vivo



New Approach Methodologies (NAMs) based on fish cells

New approach methodologies (NAMs) can be defined as any **in vitro, in chemico, computational (in silico)** **but also in vivo method** that when used alone, or in concert with others, enables improved chemical safety assessment through more protective and/or relevant models and as a result, contributes to the reduction or replacement of animal models.

Fish cells in vitro



Aquaculture 593 (2024) 741302



Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Review

The use of fish cell lines as *in-vitro* ecotoxicological tools: A cellular solution to aquaculture sustainability

Thao V. Nguyen^{a,b,*}, Anu Kumar^{a,1}, Phan Nguyen Trang^{c,**}

^a Environment, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Waite Campus, South Australia 5064, Australia

^b NTT Institute of High Technology, Nguyen Tat Thanh University, 300A Nguyen Tat Thanh, District 4, Ho Chi Minh City, Viet Nam

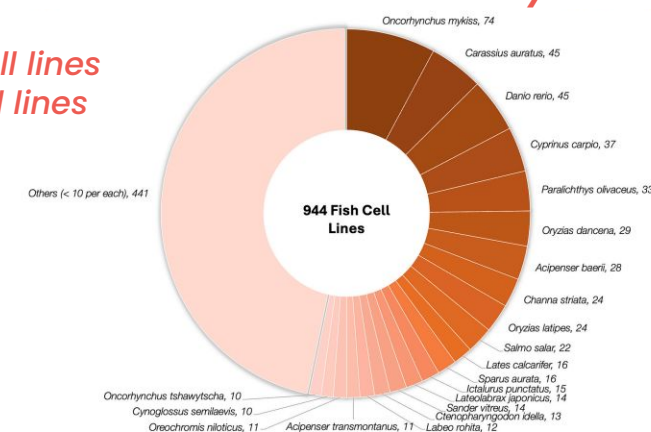
^c Department of Food Technology, Institute of Food and Biotechnology, Can Tho University, Campus II, 3/2 Street, Ninh Kieu District, Can Tho, Viet Nam



However most of them are not commercially available:

ATCC: 15 cell lines

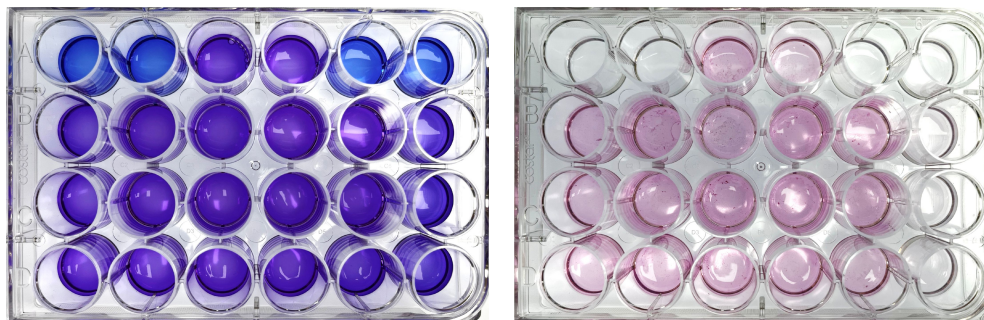
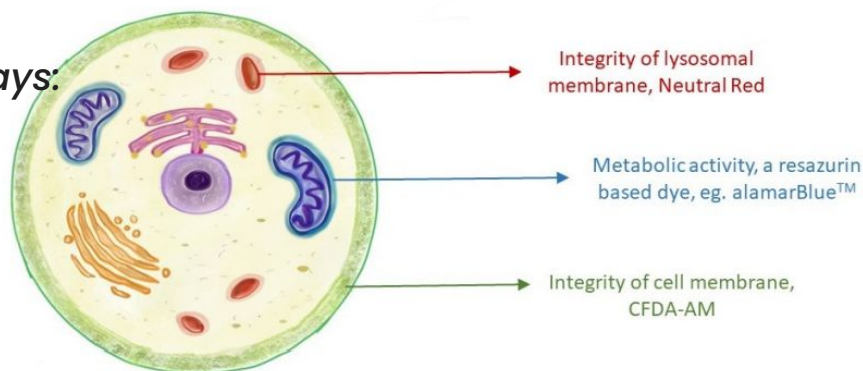
DSMZ: 5 cell lines



Funded by
the European Union

OECD TG 249 – Fish Cell Line Acute Toxicity – The RTgill-W1 cell line assay

- The only OECD guideline based on fish cells *in vitro*
- “One plate is equivalent to one fish embryo test (OECD TG 236, OECD, 2013) or one acute fish toxicity test (OECD TG 203; OECD, 2019a)” (OECD 2021, Validation report for the Test Guideline 249)
- RTgill-W1 cell line: gill cells from *Oncorhynchus mykiss* (rainbow trout)
- 24w plate format
- Three cell viability assays:
 - Neutral red
 - Alamar blue
 - CFDA-AM



Section 2
Effects on Biotic Systems

Test Guideline No. 249
Fish Cell Line Acute Toxicity:
The RTgill-W1 cell line assay

14 June 2021

OECD Guidelines for the
Testing of Chemicals



Funded by
the European Union

Application of the OECD TG 249 and other cell lines from freshwater fish

Metals

In Vitro Cytotoxicity Testing of Aquatic Pollutants (Cadmium, Copper, Zinc, Nickel) Using Established Fish Cell Lines

H. BABICH, C. SHOP SIS, AND E. BORENFREUND

Laboratory Animal Research Center, The Rockefeller University,
1230 York Avenue, New York, New York 10021

Received August 1, 1985

Pharmaceuticals



Cytotoxicity of binary mixtures of human pharmaceuticals in a fish cell line: Approaches for non-monotonic concentration-response relationships

Peter A. Bain*, Anupama Kumar
CSIRO Land and Water, Private Mail Bag 2, Glen Osmond, South Australia 5064, Australia

Nanomaterials and AdMa



Short and long-term Applicability of R

D. Hernández-Moreno
National Institute of Agricultural and Food Research and Technology (INIA), Spanish National Research Council (CSIC), Department of Environment and Agronomy, Carretera de La Coruña Km 7, Madrid, Spain



Fish cell lines as a tool for the ecotoxicity assessment and ranking of engineered nanomaterials

A. Bermejo-Nogales, M.L. Fernández-Cruz, J.M. Navas*
Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Departamento de Medio Ambiente, Carretera de la Coruña, Km 7.5, 28040 Madrid, Spain

Long-term effects (14 days)



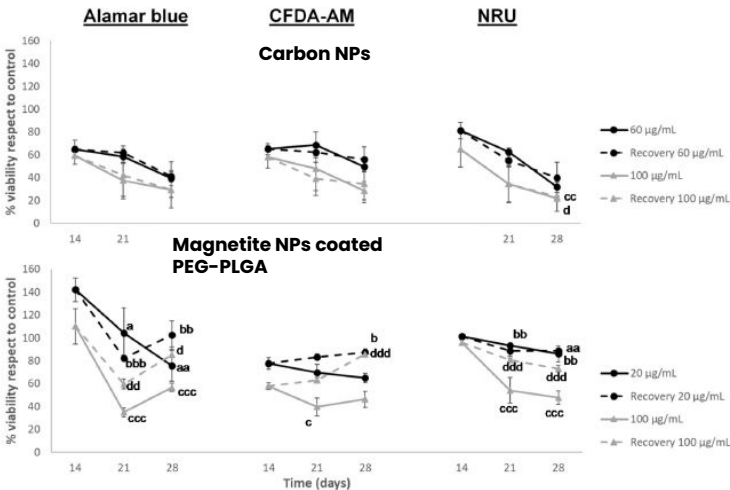
Development of a new tool for the long term in vitro ecotoxicity testing of nanomaterials

L. Galbis-Martín
* INIA, Dpt. de Medio Ambiente
* CIEMAT, Chemical Division



Short and long-term effects of nanobiomaterials in fish cell lines. Applicability of RTgill-W1

D. Hernández-Moreno, J.M. Navas, M.L. Fernández-Cruz*
National Institute of Agricultural and Food Research and Technology (INIA), Spanish National Research Council (CSIC), Department of Environment and Agronomy, Carretera de La Coruña Km 7, Madrid, Spain



Hernández-Moreno et al. 2022

Predictivity of in vivo effects



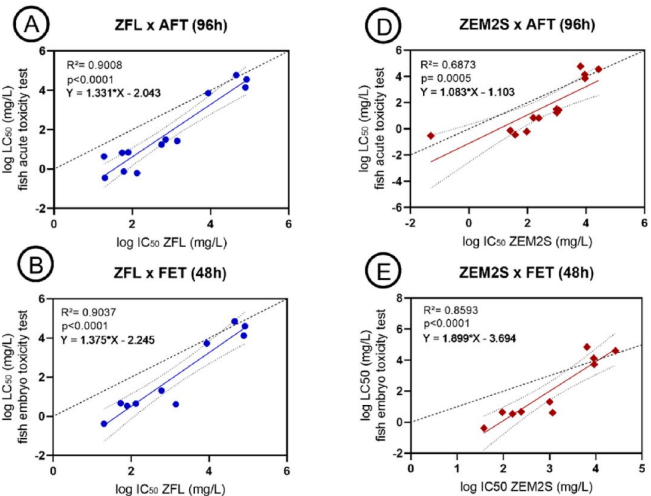
Short and long-term Applicability of R

D. Hernández-Moreno
National Institute of Agricultural and Food Research and Technology (INIA), Spanish National Research Council (CSIC), Department of Environment and Agronomy, Carretera de La Coruña Km 7, Madrid, Spain



Prediction of acute fish toxicity (AFT) and fish embryo toxicity (FET) tests by cytotoxicity assays using liver and embryo zebrafish cell lines (ZFL and ZEM2S)

Isidoros Rodrigues de Souza^a, Júlia Beatriz Vaz de Oliveira^a, Tainá Wilke Sivek^a, Natália de Albuquerque Vita^b, Andrezza Di Pietro Micali Canavez^b, Desirée Cigaran Schuck^b, Marta Margarete Cestari^a, Márcio Lorencini^b, Daniela Moraes Leme^{a,*}
^a Graduate Program in Genetics, Department of Genetics, Federal University of Paraná (UFPR), Curitiba, Paraná, Brazil
^b Grupo Biotérios, Safety of Product Department, São José dos Pinhais, Paraná, Brazil



Rodrigues de Souza et al. 2024

Application of cell models from marine species

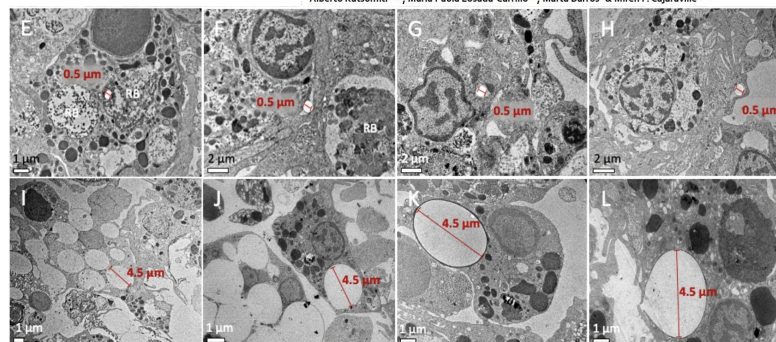
MNPs

scientific reports

www.nature.com/scientificreports

Polystyrene nanoplastics and microplastics can act as Trojan horse carriers of benzo(a)pyrene to mussel hemocytes in vitro

Alberto Katsumiti^{1,2,3}, Maria Paula Losada-Carrillo^{1,2}, Marta Barros¹ & Miren P. Cajaravilla^{1,2,3}



PAHs

Science of the Total Environment 670 (2019) 1084–1094

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

In vitro toxicity testing in hemocytes of the marine mussel *Mytilus galloprovincialis* (L.) to uncover mechanisms of action of the water accommodated fraction (WAF) of a naphthenic North Sea crude oil without and with dispersant

Alberto Katsumiti^a, Greta Nicolussi^a, Dennis Bilbao^b, Ailette Prieto^b, Nestor Etxebarria^b, Miren P. Cajaravilla^{a,*}

Nanomaterials and AdMa

Nanotoxicology

<http://informahealthcare.com/nan>
ISSN: 1743-5390 (print), 1743-5404 (electronic)
Nanotoxicology, Early Online: 1–9
© 2015 Informa UK Ltd. DOI: 10.3109/17435390.2015.1039092

informa
healthcare

ORIGINAL ARTICLE

Cytotoxicity of Au, ZnO and SiO₂ NPs using *in vitro* assays with mussel hemocytes and gill cells: Relevance of size, shape and additives

Alberto Katsumiti¹, Inmaculada Arostegui², Miriam Oron³, Douglas Gilliland⁴, Eugenia Valsami-Jones⁵, and Miren P. Cajaravilla¹



Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox



Research Paper

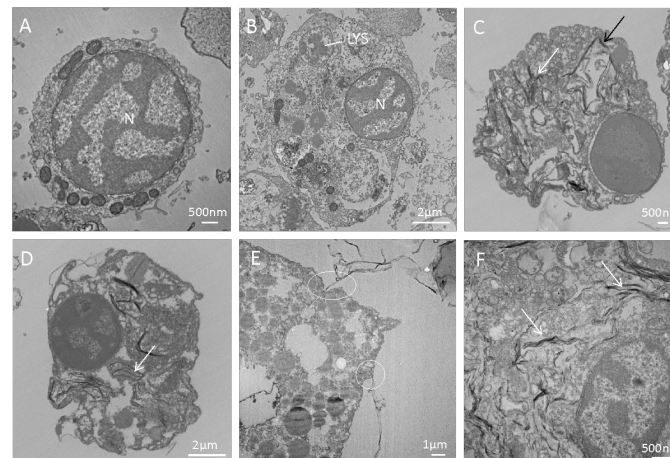
Intracellular localization and toxicity of graphene oxide and reduced graphene oxide nanoplatelets to mussel hemocytes *in vitro*

Alberto Katsumiti^a, Radmila Tomovska^{b,c}, Miren P. Cajaravilla^{a,*}

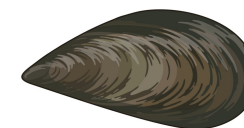
^a CBET Research Group, Dept. Zoology and Animal Cell Biology, Faculty of Science and Technology and Research Centre for Experimental Marine Biology and Biotechnology PIE, University of the Basque Country UPV/EHU, Basque Country, Spain

^b POLYMAT and Dept. Applied Chemistry, Faculty of Chemistry, University of the Basque Country UPV/EHU, Basque Country, Spain

^c IKERBASQUE, Basque Foundation for Science, Basque Country, Spain

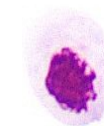


Mussels (*M. galloprovincialis*)

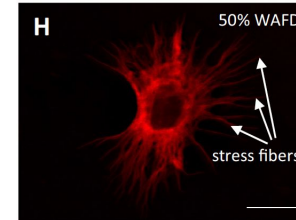
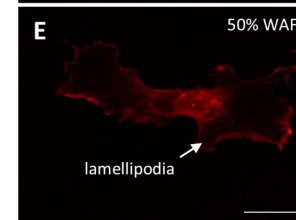
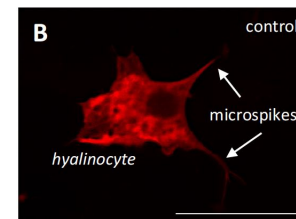
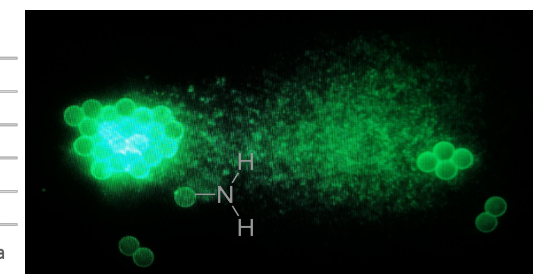
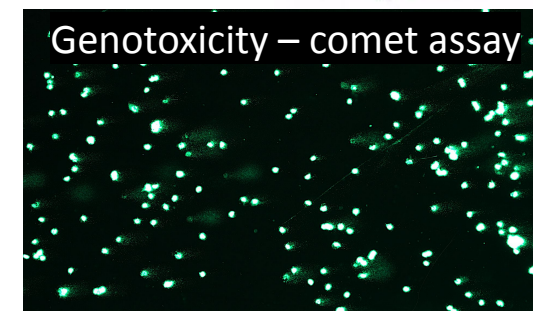


Granulocytes

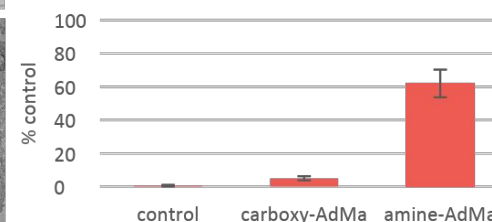
Hyalinocytes



Genotoxicity – comet assay



Comet assay (haemocytes)



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Application of cell models from terrestrial species

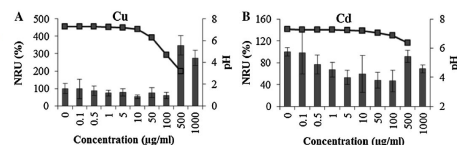
Metals

Ecotoxicology (2015) 24:1004–1013
DOI 10.1007/s10646-015-1441-9



Establishment of toxicity thresholds in subpopulations of coelomocytes (amoebocytes vs. eleocytes) of *Eisenia fetida* exposed in vitro to a variety of metals: implications for biomarker measurements

Amaia Irizar · Carlos Rivas · Nerea García-Velasco · Felipe Goñi de Cerio · Javier Etxebarria · Ionan Marigómez · Manu Soto



Ecotoxicology
DOI 10.1007/s10646-016-1710-2



Uptake route and resulting toxicity of silver nanoparticles in *Eisenia fetida* earthworm exposed through Standard OECD Tests

Nerea García-Velasco¹ · Maite Gandariasbeitia¹ · Amaia Irizar¹ · Manuel Soto¹

Comparative Biochemistry and Physiology, Part C 231 (2020) 108735



Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Effects of elevated temperatures and cadmium exposure on stress biomarkers at different biological complexity levels in *Eisenia fetida* earthworms

Erik Urionabarrenetxea, Nerea García-Velasco, Ionan Marigómez, Manu Soto^{*}



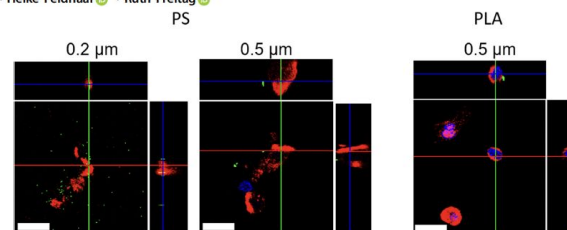
Nanomaterials and MNPs

Ecotoxicology (2022) 31:221–233
<https://doi.org/10.1007/s10646-021-02495-2>



In vitro cultivation of primary intestinal cells from *Eisenia fetida* as basis for ecotoxicological studies

Simon A. B. Riedl¹ · Matthias Völkl¹ · Anja Holzinger² · Julia Jasinski³ · Valérie Jérôme¹ · Thomas Scheibel³ · Heike Feldhaar² · Ruth Freitag¹



Ecotoxicology and Environmental Safety 183 (2019) 109545



Contents lists available at ScienceDirect

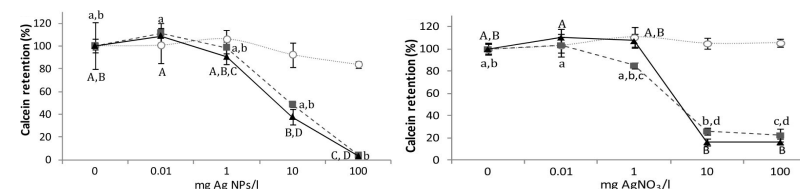
Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

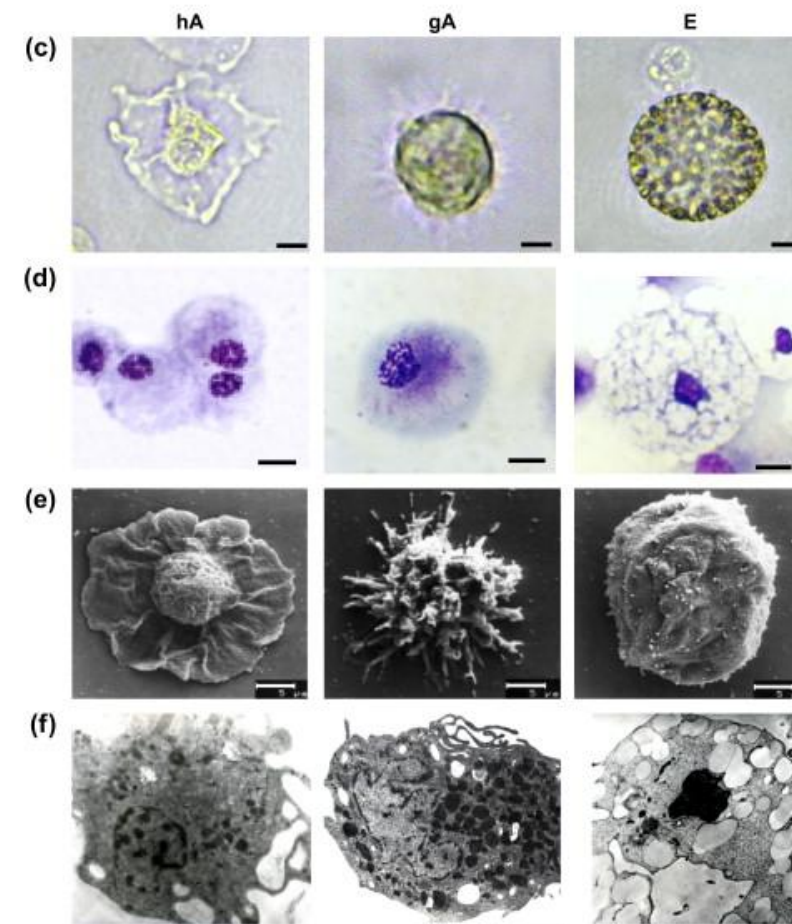


Selection of an optimal culture medium and the most responsive viability assay to assess AgNPs toxicity with primary cultures of *Eisenia fetida* coelomocytes

N. García-Velasco^{a,*}, A. Irizar^b, E. Urionabarrenetxea^a, J.J. Scott-Fordsmand^b, M. Soto^a



Earthworms (*Eisenia fetida*)



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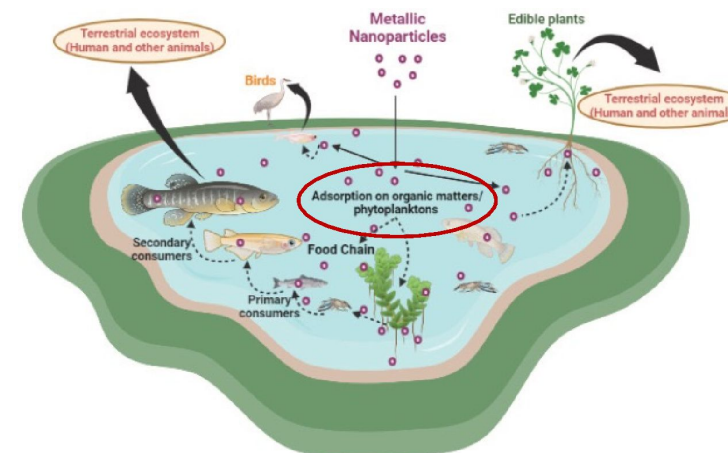
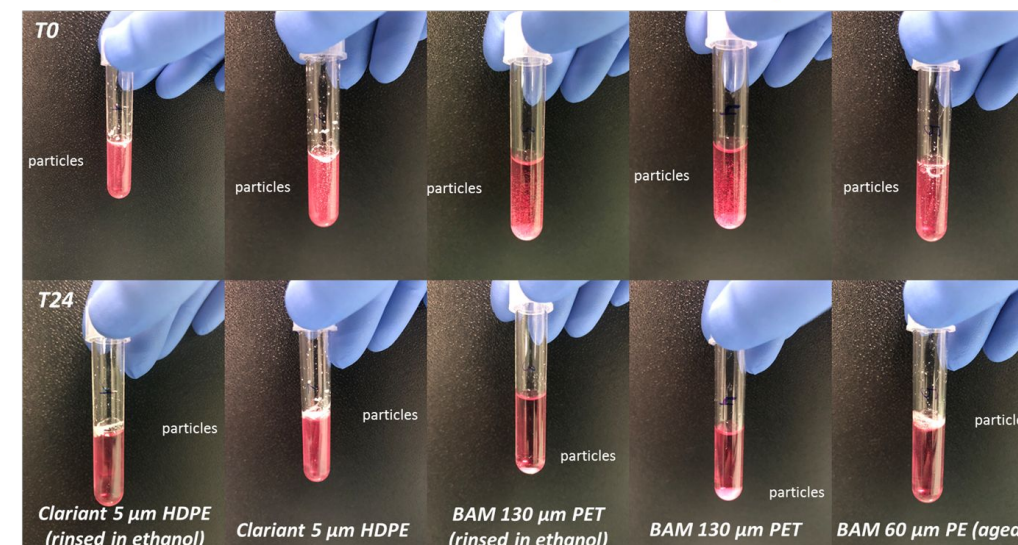
Application of OECD TG 249 for testing challenging materials (AdMa)

Optimum conditions

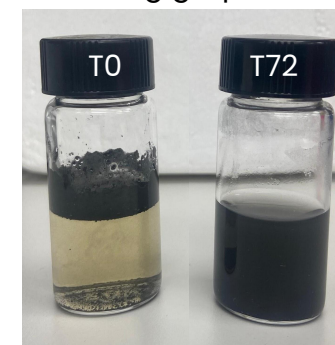
- **Accurate exposure:** concentration and form (dispersed or aggregated) directly influence toxicity
- **Reliable testing:** standard ecotoxicity tests require a homogeneous and stable dispersion
- **Environmental Relevance:** reproduce environmental conditions

Dispersion stability

- **Aggregation, agglomeration, sedimentation, floatation**
- **Dispersion methods**
 - **2% BSA:** relevant to human not to the environment
 - **Sonication:** may generate ROS
- **Optimised protocol for dispersion of hydrophobic materials**
 - Adapted from Lizonova et al. 2024
 - Stock (10mg/mL) in AFW + NOM (20mg/L)
 - Stir at 500 rpm for 72h
 - Mix with cell culture medium
 - Vortex and expose the cells



Stirring graphene

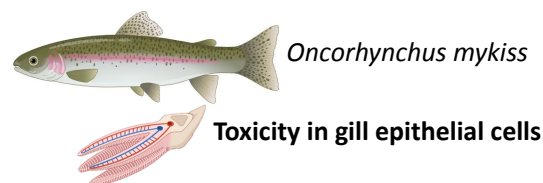
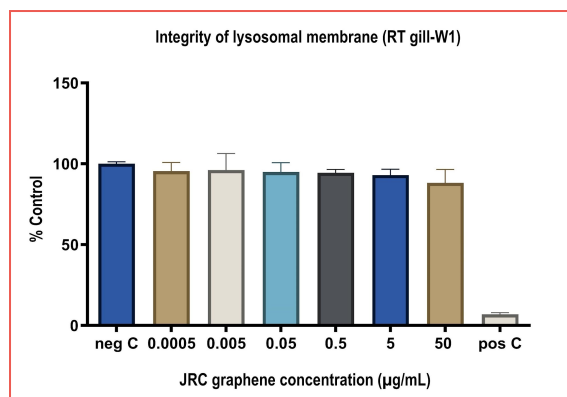
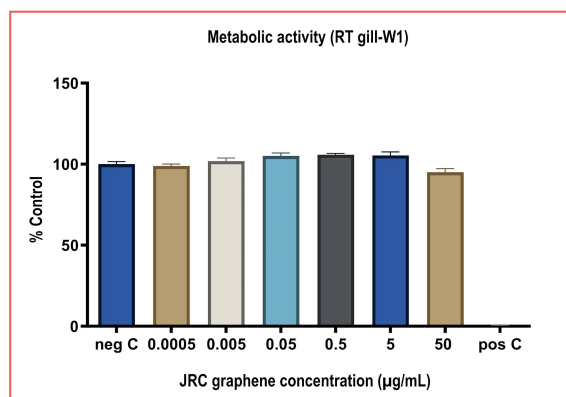
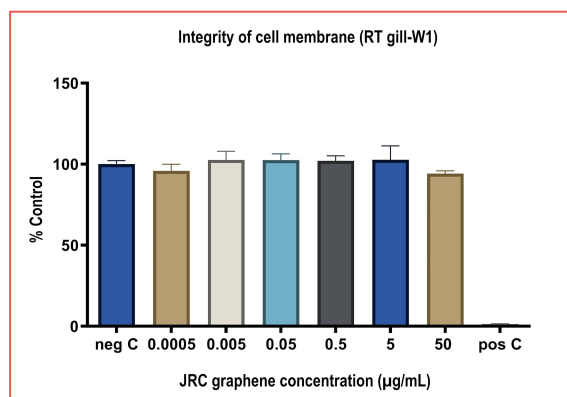


Application of OECD TG 249 for testing challenging materials (AdMa)



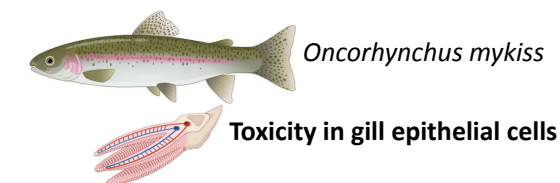
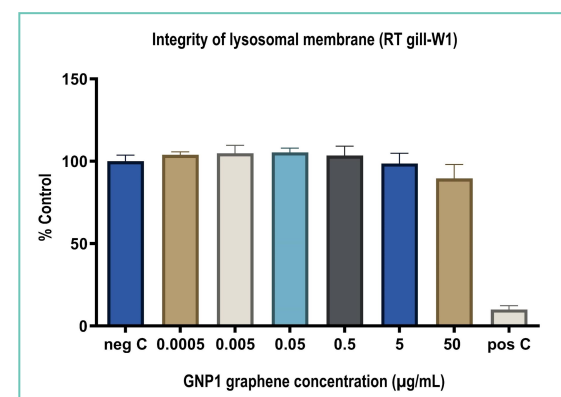
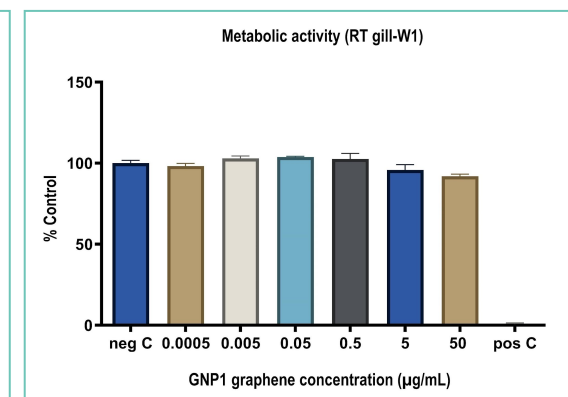
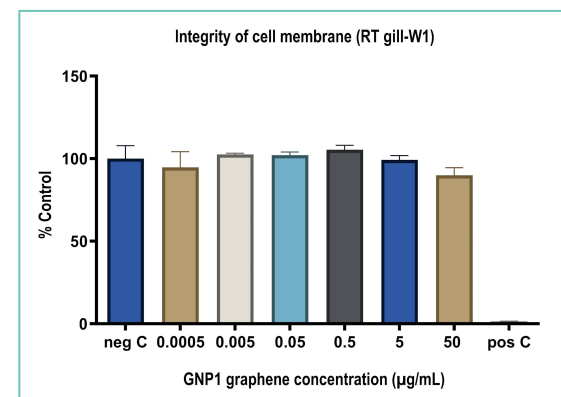
Graphene (representative, JRC)

Gill cells, 24h



Graphene (industrial application, construction)

Gill cells, 24h



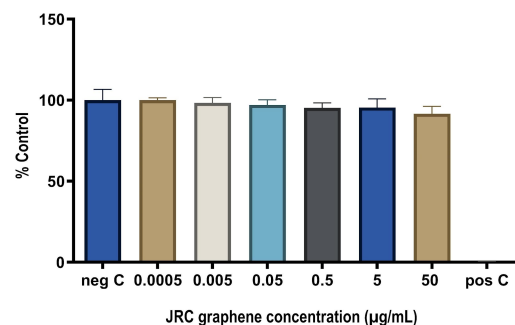
Expanding OECD TG 249 scope: different fish species



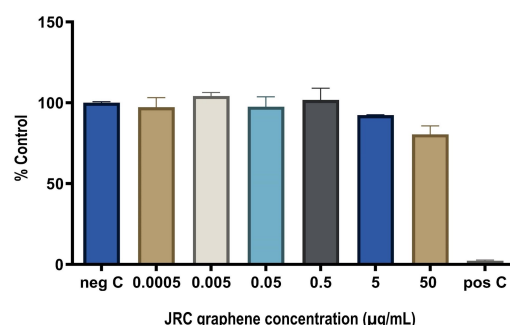
Graphene (representative, JRC)

Plasma membrane integrity, 24h

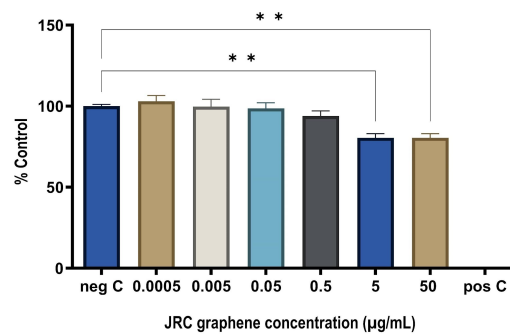
Gonad cells



Liver cells



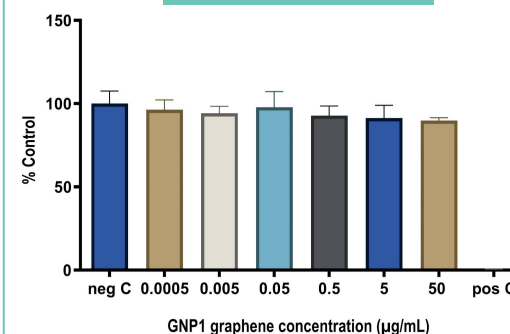
Brain cells



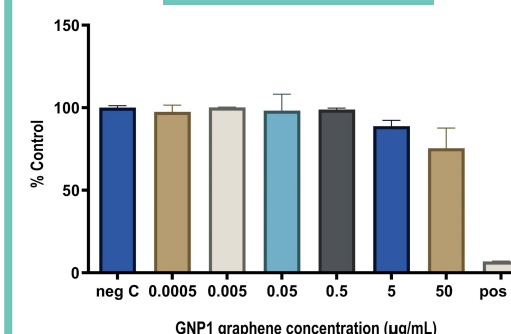
Graphene (industrial application, construction)

Plasma membrane integrity, 24h

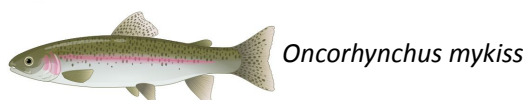
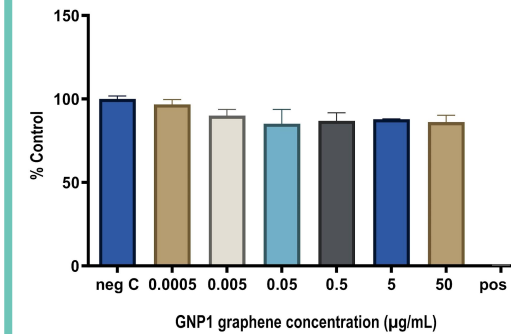
Gonad cells



Liver cells



Brain cells



Oncorhynchus mykiss



Gonadal toxicity



Hepatotoxicity



Oreochromis mossambicus

Neurotoxicity



Oncorhynchus mykiss



Gonadal toxicity



Hepatotoxicity



Oreochromis mossambicus

Neurotoxicity

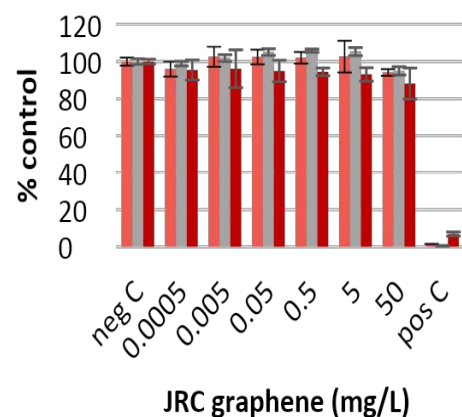
Expanding OECD TG 249 scope: short vs. long-term



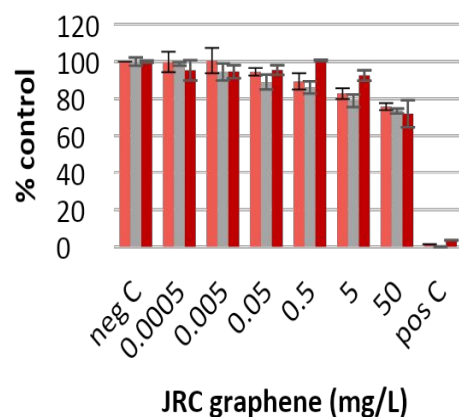
24 hours

28 days

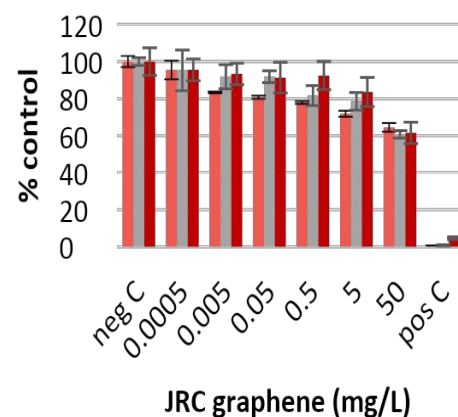
RTgill-W1 (24 h)



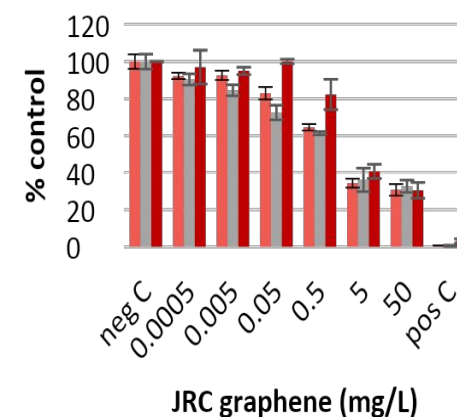
RTgill-W1 (7 d)



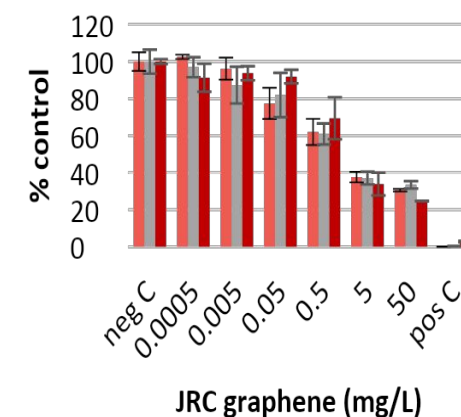
RTgill-W1 (14 d)



RTgill-W1 (21 d)



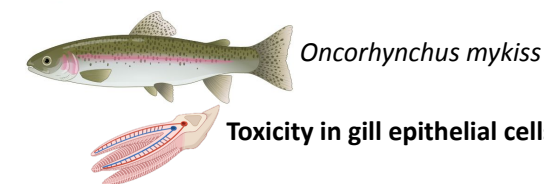
RTgill-W1 (28 d)



■ Cell membrane

■ Metabolic activity

■ Lysosomal membrane



Toxicity in gill epithelial cells

Take-home messages

- **Fish cell lines represent good candidates for the development of NAMs**
 - **Cell models from different organs:** gonads, brain, liver
 - **Different fish species:** freshwater and seawater
 - **More complex in vitro systems:** co-cultures, 3D, microfluidic
- **Model sensitivity**
 - **Brain** cells > **Liver** cells > **Gill** cells > **Gonad** cells (address tissue-specific toxicity)
- **Short and long-term exposures**
 - **Short-term:** environmentally relevant and high concentrations
 - **Long-term:** environmentally relevant, repeated exposures
- **Testing materials**
 - **Soluble chemicals:** directly applicable
 - **AdMa:** dispersion protocol + dosimetry

Cost analysis

“One plate is equivalent to one fish embryo test (OECD TG 236, OECD, 2013) or one acute fish toxicity test (OECD TG 203; OECD, 2019a)” (OECD 2021, Validation report for the Test Guideline 249)”

	OECD 249 Fish Cell Line Acute Toxicity – The RTgill-W1 cell line assay	OECD 236 Fish Embryo Acute Toxicity (FET) Test	OECD TG 203 Short-term toxicity testing on fish
Exposure type	Acute (24 hours)	Acute (96 hours)	Acute (96 hours)
Duration	3-4 days	1 week	> 1 week
Endpoint	Viability	Mortality	Mortality
Cost/6 samples	1800 €	7550 € (9300 € under GLP)	14000 € (17500 € under GLP)
<i>In vitro/in vivo</i>	<i>In vitro</i>	<i>In vitro</i>	<i>In vivo</i>

- **Cost-effective alternative for screening formulations (Tier 2 method)**

- **Animal-free**



Thanks | Muchas gracias | Eskerrik asko

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