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# Adaptation of OECD test guidelines on ecotoxicity tests – experience gained from the EU project MARINA and further considerations.

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Kerstin Hund-Rinke

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# Introduction

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- Results regarding ecotoxicity can be influenced by test design.
  - Example:  $\text{TiO}_2$  (P25) - reproduction of *Daphnia magna*  
wide range of EC50 values (performed according to OECD 211):
    - 0.48 mg/L (Zhu et al. 2010) - ... - >> 5 mg/L (Hund-Rinke et al. 2012)
- Inappropriate for testing in the scope of regulation (e.g. REACH)
- Two approaches to guide the testing
  - Guidance documents  
General issues are addressed, recommendations to solve or work around the problems are presented.
  - Guideline  
Specific instructions are listed.



# Introduction

## ■ EU-project MARINA – WP Ecotoxicity

### ■ Specific recommendations on eight OECD test guidelines

#### ■ Algae – growth (OECD 201)

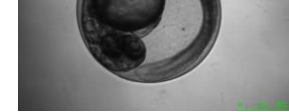
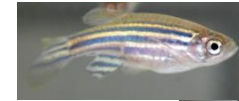
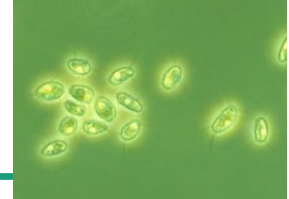
#### ■ Daphnids – immobilization (OECD 202)

#### ■ Fish embryo (OECD 210/236)

#### ■ Lumbriculus – development (OECD 225)

#### ■ Earthworm, enchytraeids – reproduction (OECD 220/222)

#### ■ Microorganisms – respiration, nitrification (OECD 216/217)



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ORIGINAL ARTICLE

Regulatory ecotoxicity testing of nanomaterials – proposed modifications of OECD test guidelines based on laboratory experience with silver and titanium dioxide nanoparticles

Kerstin Hund-Rinke<sup>1</sup>, Anders Baun<sup>2</sup>, Denisa Cupi<sup>2</sup>, Teresa F. Fernandes<sup>3</sup>, Richard Handy<sup>4</sup>, John H. Kinross<sup>5</sup>, José M. Navas<sup>6</sup>, Willie Peijnenburg<sup>6</sup>, Karsten Schlich<sup>1</sup>, Benjamin J. Shaw<sup>7</sup>, and Janeck J. Scott-Fordsmand<sup>8</sup>

<sup>1</sup>Fraunhofer Institute for Molecular Biology and Applied Ecology, Schmallenberg, Germany, <sup>2</sup>Department of Environmental Engineering, Technical University of Denmark, Kongens Lyngby, Denmark, <sup>3</sup>School of Life Sciences, Heriot-Watt University, Edinburgh, UK, <sup>4</sup>School of Biological Sciences, University of Plymouth, Plymouth, UK, <sup>5</sup>INA, Madrid, Spain, <sup>6</sup>National Institute for Public Health and the Environment, Bilthoven, Netherlands, <sup>7</sup>University Leiden, Leiden, Netherlands, and <sup>8</sup>Department of Bioscience, Aarhus University, Silkeborg, Denmark

*Hund-Rinke et al. 2016, Nanotoxicology. 2016, 1442-1447.*

# Introduction

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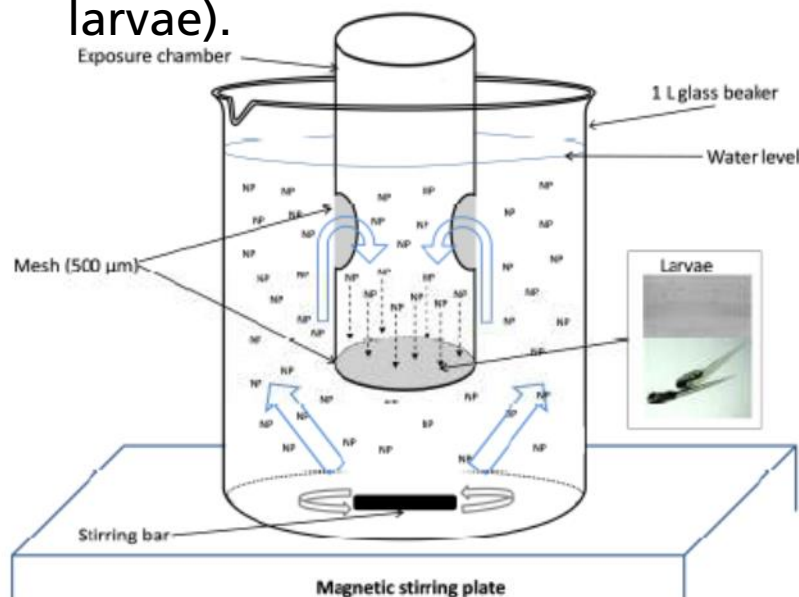
## ■ EU-project MARINA

- Different subjects of interest for aquatic and terrestrial tests (general and specific topics).
  - Aquatic organisms:
    - Contact between NMs and test organisms (reduction of sedimentation; peculiarities of test organisms).
    - Algae: determination of cell number.
  - Terrestrial organisms:
    - Spiking of soil
    - Microbial nitrifiers: nitrogen source (ion-releasing NMs!)



# Aquatic tests - Fish

- Contact between organism and NMs (peculiarities of test organisms).
  - Fish (*Shaw et al. 2016, Arch Toxicol 90, 2077-2107; University of Plymouth*)
    - Mechanical turbulence tolerated → sedimentation can be reduced by stirring (protection against stirring bars).
    - Proposed modification:
      - ➔ Development of a new test chamber (effective for embryos and larvae).



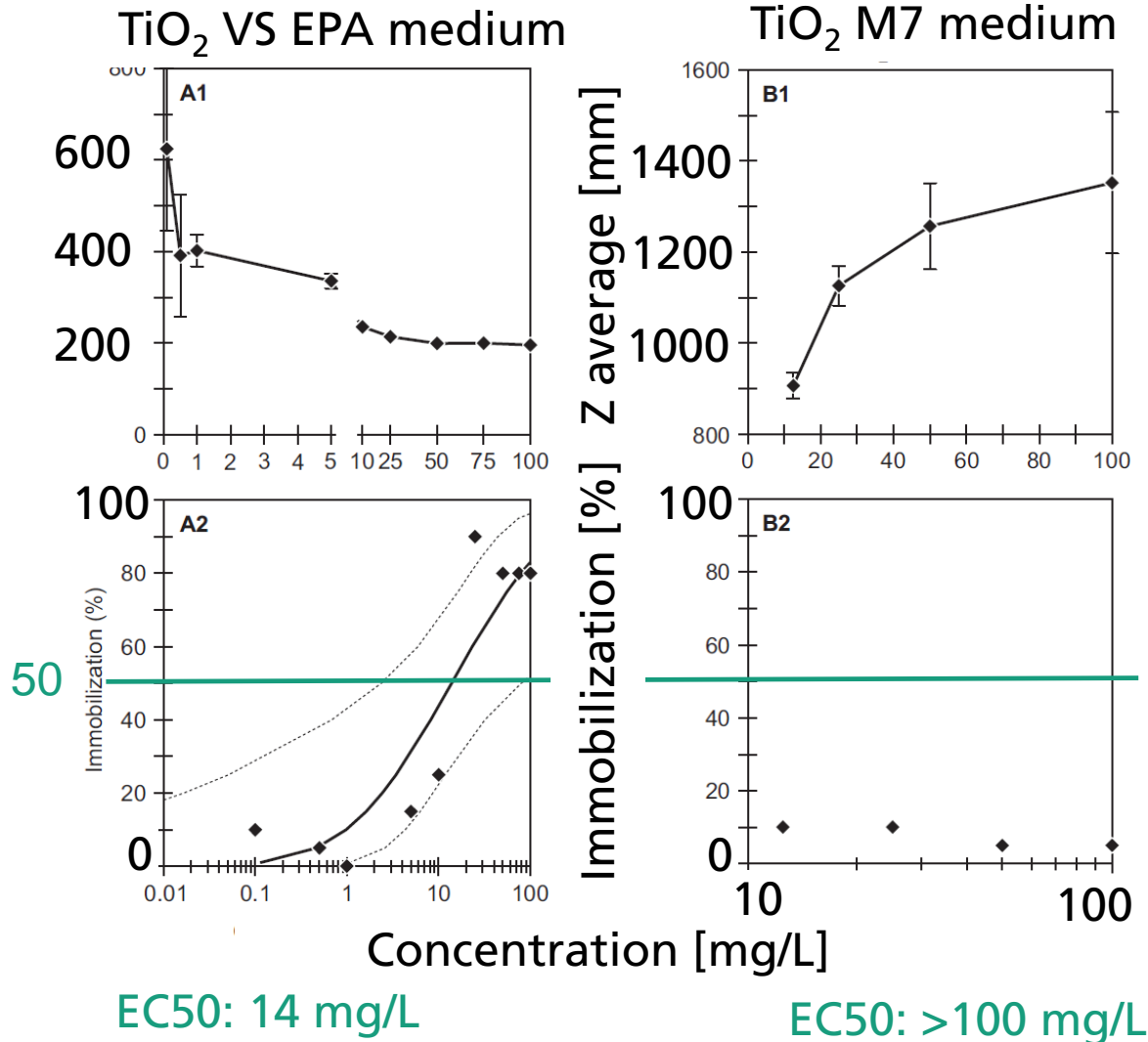
*Diagrammatic representation of the exposure chambers used to improve the dispersion of NM in the test (based upon Boyle et al. (2015))*

# Aquatic tests - Daphnids

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- Contact between organism and NMs (peculiarities of test organisms).
  - Daphnids (*Cupi et al. 2016, EES 127, 144-152; DTU*)
    - Mechanical turbulence is not tolerated.
    - Proposed modification:
      - ➔ Use of a test medium resulting in small agglomerates and higher stability of dispersion
        - Using a very soft medium (e.g. very soft EPA medium)
        - Testing at pH-values where more stable dispersions are obtained (zeta-potential, point of zero charge!)  
*Limited applicability; appropriate living conditions for daphnids!*

# Aquatic tests - Daphnids

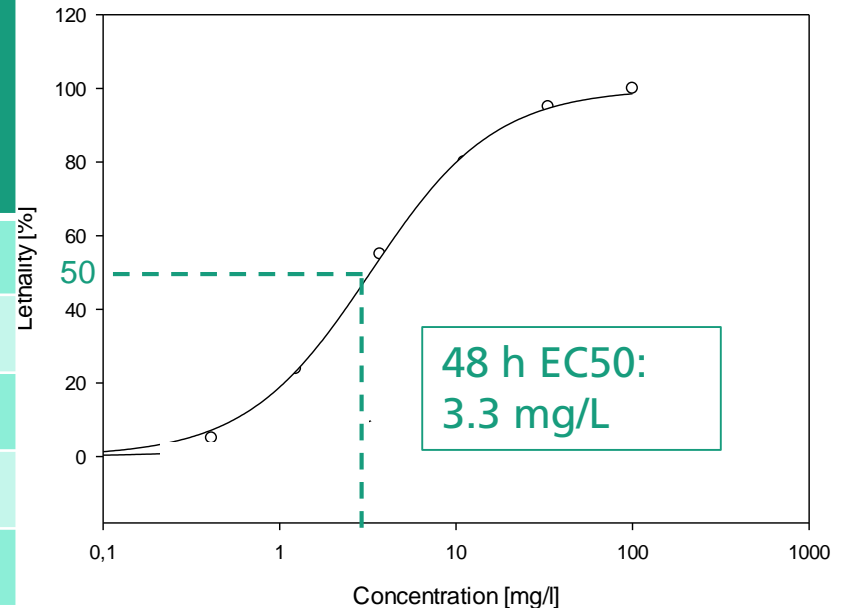


*Cupi et al.,  
2016, EES 127,  
144-152*

# Aquatic tests - Daphnids

- Exposure concentration of daphnids
  - Dispersed or sedimented NMs?
  - Example ZnO: *Daphnia magna*, immobilization (OECD 202) (Dana Kühnel; UFZ – project sponsored by German Environment Agency)

Nominal concentration [mg/L]	Analytical concentration in water phase [mg/L]
1.2	1.3
3.7	2.1
11	3.1
33	2.8
100	3.4



→ Uptake of sedimented NMs (agglomerates) which have a suitable size!



# Aquatic tests - Algae

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- Determination of biomass / growth rate
  - TG 201: determination of cell number → interference with NMs
    - Algae (*Teresa Fernandes; Heriot-Watt-University*)
      - MARINA: determination of fluorescence (in-vivo; in-vitro)  
in-vitro preferred; any potential interference with NMs excluded.
    - Further publications
      - TiO<sub>2</sub>: no problem with in-vivo (less work and less time intensive; results obtained the same day) (*Hund-Rinke et al., 2006*).
      - CNT: in-vivo / in-vitro fluorescence is unsuitable (sorption of CNTs to algae) → microscopic determination (*Farkas & Booth, 2017, Nanotoxicology 569-577*)
  
- ➔ Best method for the determination of biomass / growth rate is specific for the NMs; has to be figured out in pre-tests.

# Terrestrial tests

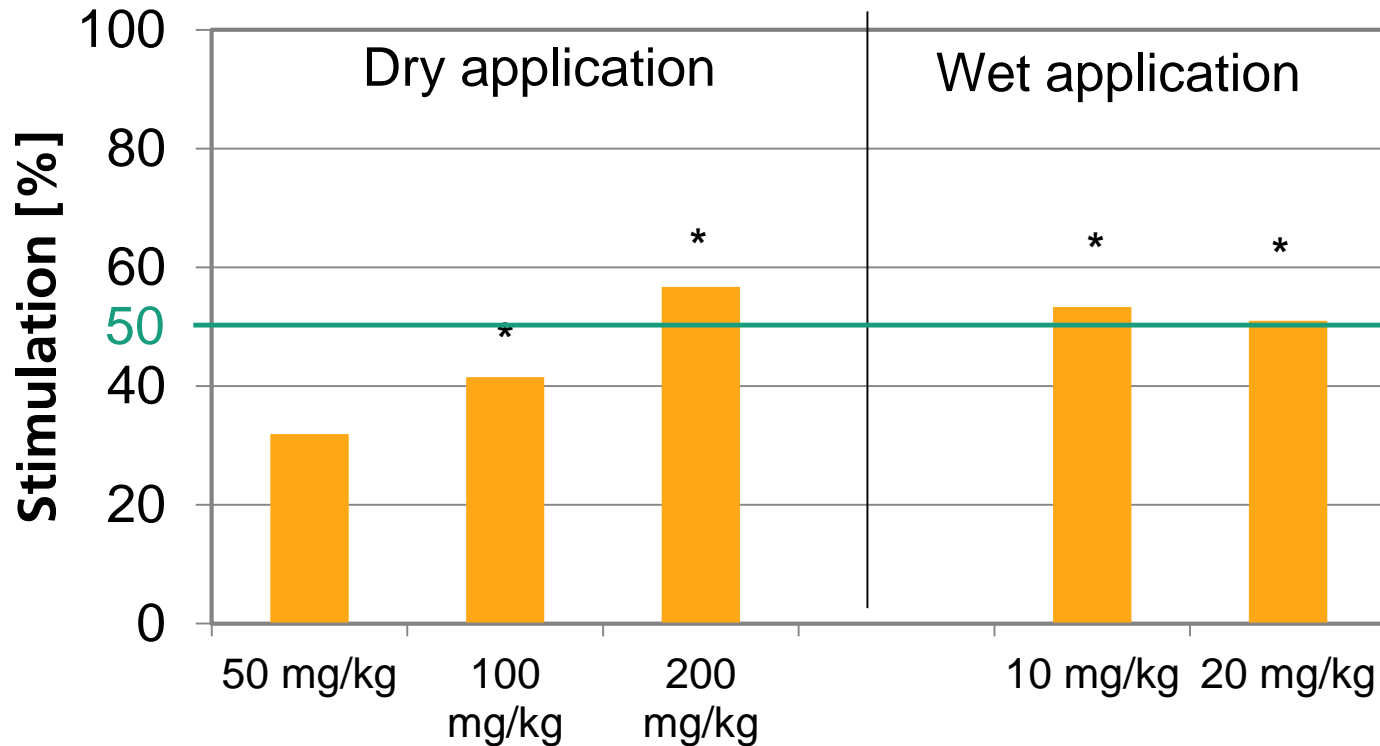
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- EU-project MARINA
  - Invertebrates (Janeck Scott-Fordsmand – Aarhus University)
  - Soil microflora (Kerstin Hund-Rinke – Fraunhofer IME)
  - Spiking!  
Dry spiking / wet spiking

# Terrestrial tests



- $\text{TiO}_2$  - reproduction test with earthworms (OECD 222)

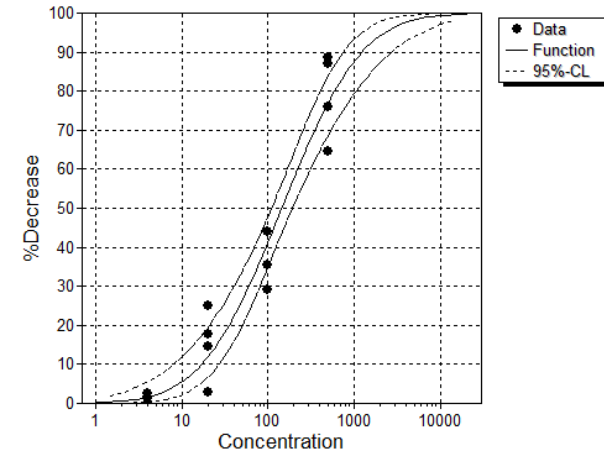


- $\text{TiO}_2$  - wet application: higher bioavailability → higher effect  
but: no difference between the two concentrations.

# Terrestrial tests

- Soil microflora: potential ammonium oxidation (project nanoGRAVUR)

ZnO	Dry spiking EC50 [mg/kg]		Wet spiking EC50 [mg/kg]	
NM110	183	[138 - 244]	145	[85 - 259]
NM111	167	[131-211]	128	[100-165]

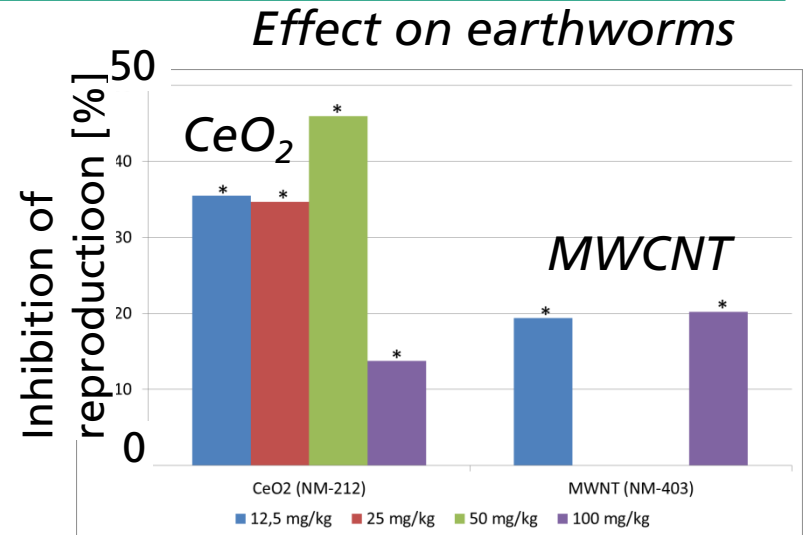
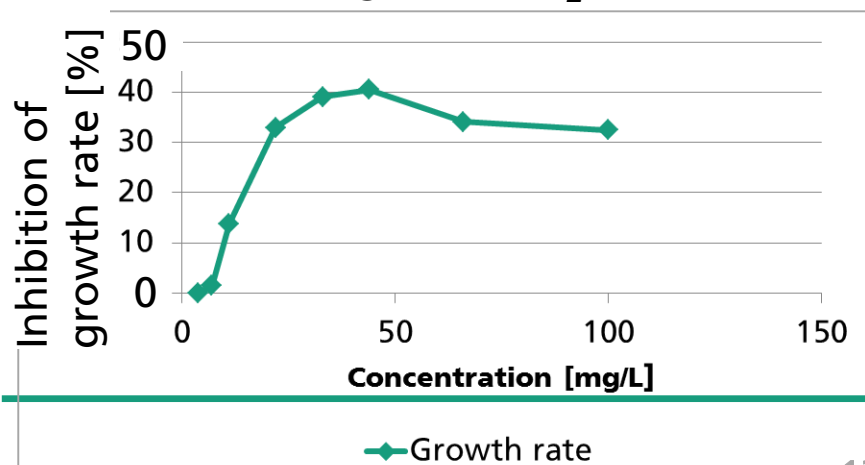
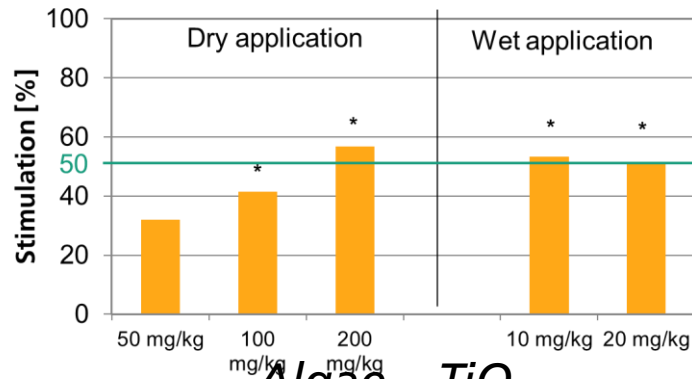


- Indications, that differences between wet and dry spiking depend on NMs (ion-releasing / non-ion-releasing)  
Differences between the two spiking methods might be more obvious for non-ion releasing NMs.

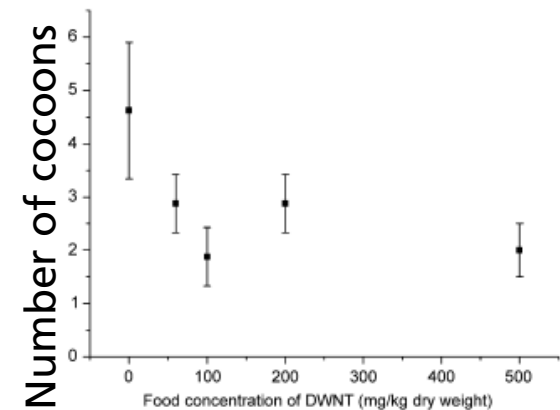
→ Recommendation: Testing for regulation: use of both spiking procedures – use of lower EC<sub>x</sub>

# Concentration-effect relationships

- Several examples: constant effect below 100 % even with increasing test concentrations. Effect can even decrease.



## Effect of DWNT on earthworms



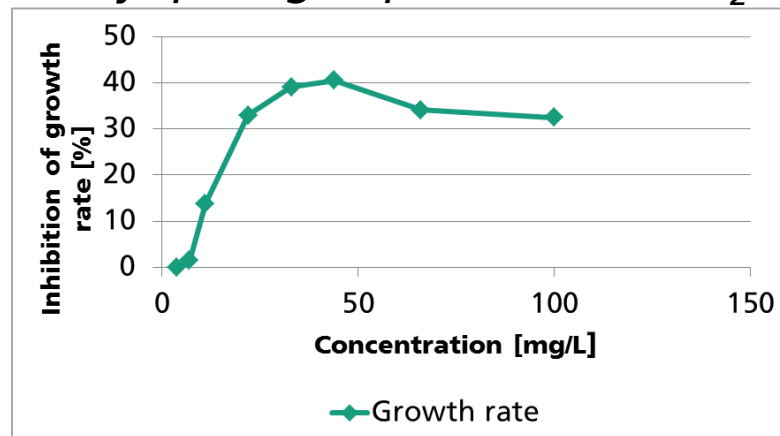
Scott-Fordsmand et al., 2008

# Limit-tests (testing of one high concentration)

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- Traditional chemicals: limit tests are accepted.
- NMs: similar effect / reduced effect at high test concentrations cannot be excluded.
- ➔ Recommendation: No limit test for NMs; testing of several test concentrations!

*Dry spiking, aquatic test – TiO<sub>2</sub>*



# Further support in testing

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- OECD Series on Testing and Assessment:

GUIDANCE DOCUMENT ON AQUATIC AND SEDIMENT TOXICOLOGICAL TESTING OF NANOMATERIALS.

- Chapter 4 ANALYTICAL AND MEASUREMENT TECHNIQUES

- Chapter 5 MEDIA PREPARATION

- Chapter 6 CONDUCT OF THE BIOASSAY

- Chapter 7 DATA ANALYSIS AND REPORTING (NANOMATERIAL-SPECIFIC)

- Testing approaches to reduce animal testing (alternative testing approaches) are discussed.

- ➔ Conclusion: alternative tests not recommended in the scope of regulation; however: suitable to elucidate fundamental knowledge.

# Summary / Conclusion

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- Much experience as well as general and specific recommendations regarding the ecotoxicological testing of nanomaterials are available.
- Regulatory testing: the regulatory bodies have to decide which test approaches they accept.
- Research projects: Test approach has to be selected according to the problem to be solved (advantages and limitations of the selected approach have to be considered).