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US-EU CoR Meeting, Arlington, USA, December 2013

Progress on Nano Ecotoxicology: An overview

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Aims

- Short summary of the state of knowledge across different species, mainly from the European perspective.
- Microbes and Ecosystem services
- Aquatic invertebrates, especially marine organisms.
- Terrestrial organisms- earthworms, higher plants.
- Recent research on fishes/amphibians
  - Effects on early life stages
  - Neurotoxicity, brain and behaviour

Microbes

- For microbes, lethal concentrations are known in laboratory cultures.
- Microbial functions in soil, sediment and inside host biota (e.g., the gut of fishes) is poorly understood.
- Ecosystem services from microbes.
- The data set on environmentally-relevant microbes is limited compared to model organisms like *E. coli* or *Streptococcus spp*.

Understanding the Diversity of Chemistries on the Surfaces of Organisms: Microbes

Handy et al. (2012) Ecotoxicology, 21, 933-72.

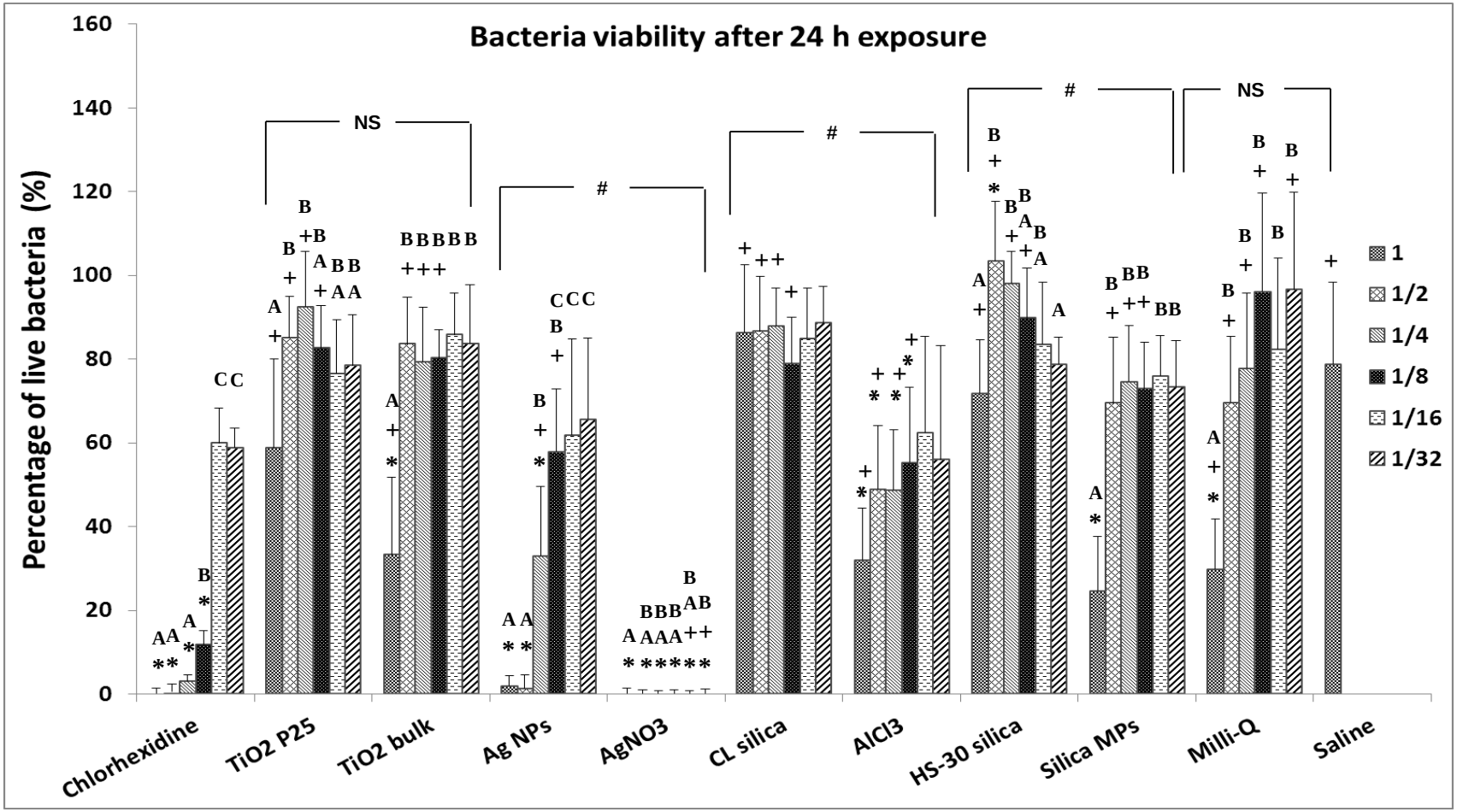
Table 3 The prokaryote envelope as a barrier to NPs

Structure	*Archaea*	Gram positive bacteria	Gram negative bacteria	Nano issue
Cytoplasmic membrane	Lipid bilayer of mainly glycerol-ether lipids. Contains membrane spanning proteins	Lipid bilayer of mainly glycerol-ester lipids. Contains membrane spanning proteins	Lipid bilayer of mainly glycerol-ester lipids. Contains membrane spanning proteins	Hydrophobic layers, pore sizes in proteins <1 nm. Only lipid dispersible, or lipid coated MNMs may associate with latter
Murein layer	Absent	Relatively thick layer, 10–50 nm wide. Peptidoglycan, teichoic acids and polysaccharides. Contains fixed polyanions and hydrophilic	Relatively thin layer, 2–3 nm wide. Mostly peptidoglycan. Contains fixed polyanions and hydrophilic	Interactions of MNMs with peptidoglycans unknown. Hydrophobic MNMs less likely to penetrate this layer
Outer membrane	Absent	Absent	A thin peptidoglycan layer, 7–8 nm thick. Contains lipopolysaccharides. Membrane spanning porins. Contains fixed polyanions and hydrophilic	Hydrophilic MNMS likely to associate with the outer membrane. Porins too small (<1 nm pore) for NPs
S-layer	Glycoprotein coat forming the outer-most cell envelope layer	Glycoprotein layer covalently linked to the murein layer. Lattice structure with a pore size 2–8 nm	Glycoprotein layer covalently linked to the outer membrane. Lattice structure with a pore size 2–8 nm	S-layer interactions with MNMs not investigated. MNMs < 8 nm may theoretically penetrate the (large pore size) lattice

Note. For clarity, the cyanobacterial cell wall is excluded, but consists in essence of a Gram positive-like murein layer with an outer layer

Sensitivity of *Streptococcus mutans* to Nanomaterials

(Besinis et al. Nanotoxicology, 2012. doi:10.3109/17435390.2012.742935)



Percentage of live *S. mutans* after a 24 h exposure to dispersions of nanomaterials compared to bulk or metal salt controls. Dilutions series of 1, 1/2, 1/4, 1/8, 1/16 and 1/32 correspond to 100, 50, 25, 12.5, 6.25 and 3.125 mg l^{-1} respectively of each material used. "*" shows significant difference from the saline negative control and "+" shows statistical difference from the corresponding dilution of the chlorhexidine positive control (one-way ANOVA, $p < 0.05$). Within a test solution, different letters indicate significant differences (one-way ANOVA, $p < 0.05$) between the dilution series whereas complete absence of letters means no statistical difference between any of the dilutions. "#" shows that the nano-solutions were significantly different (two-way ANOVA, $p < 0.05$) from their corresponding salt metal or bulk control, whereas "NS" means no significant difference. Brackets show the groups compared.

Ecosystem Services from Microbes & Plants

Grigulis et al. 2013. Journal of Ecology, 101, 47–57

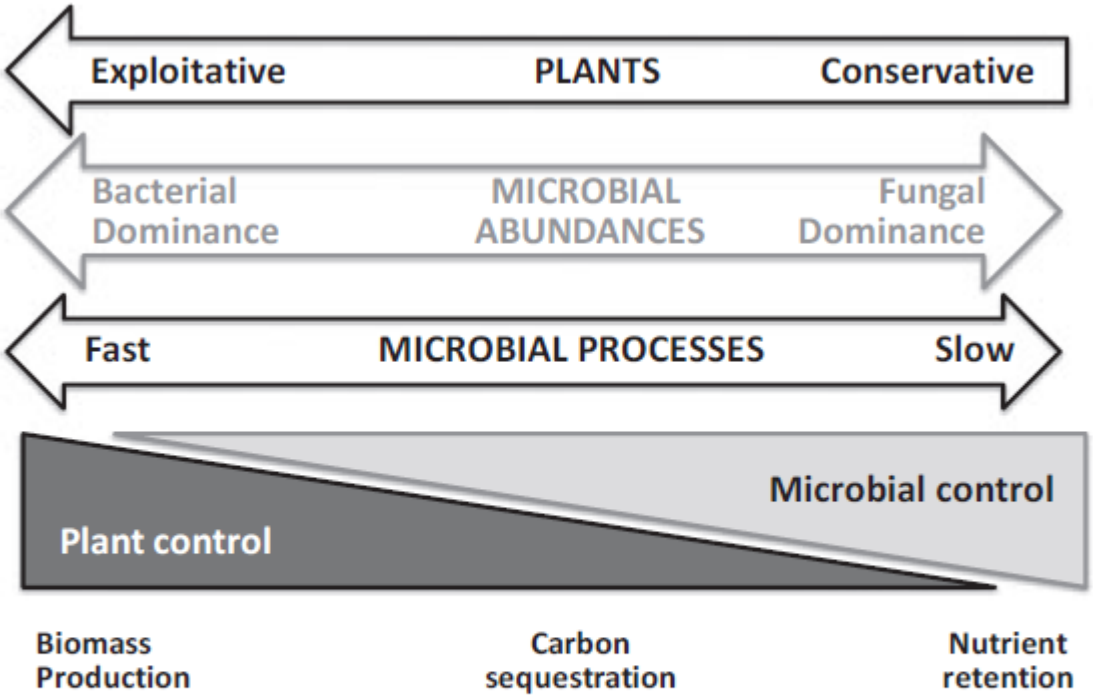


Fig. 3. Schematic overview of simultaneous variations in plant functional strategies, microbial functional composition and activities, and ecosystem processes and services.

Ag NPs from Biosolids in Terrestrial Systems

Colman et al. (2013) PlosOne, 8(2), e57189

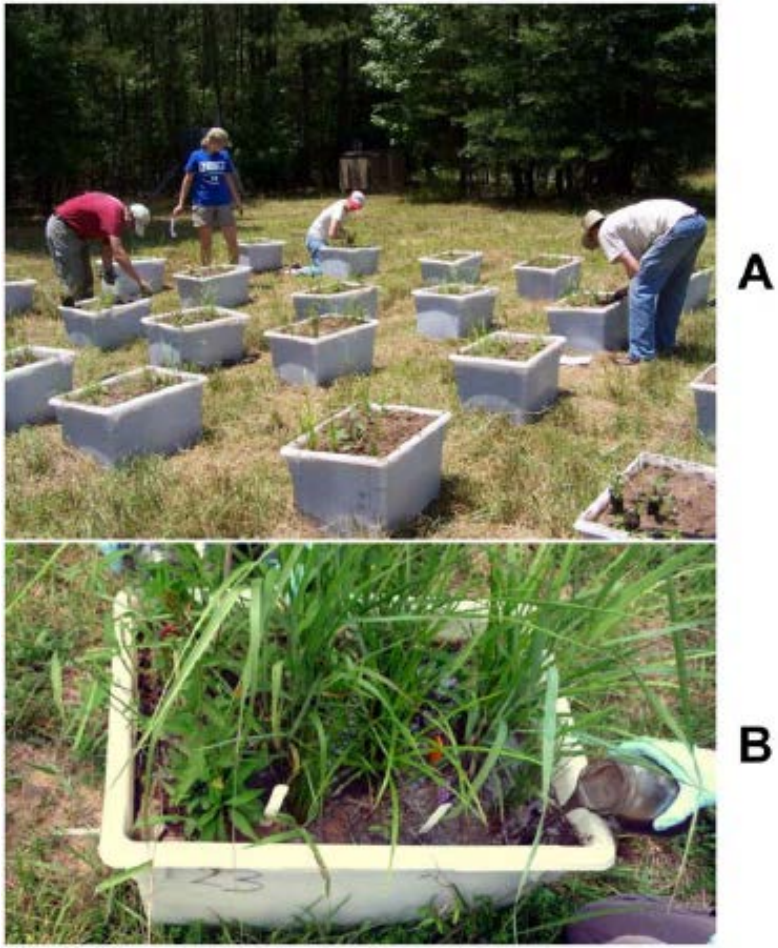
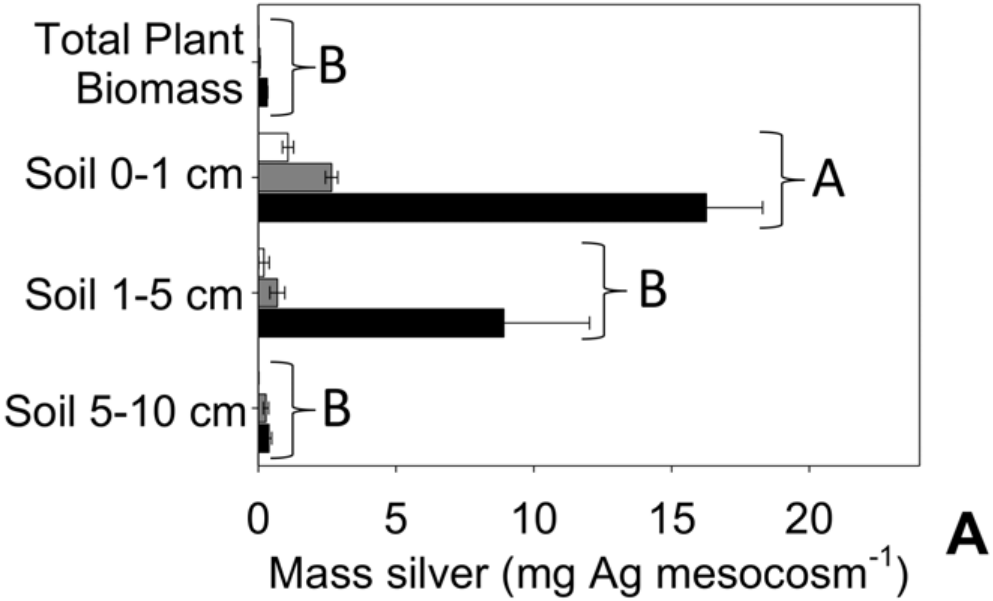


Figure 1. Terrestrial mesocosms in the Duke Forest, Durham, NC, USA. Mesocosms **A** on the day of planting, and **B** 63 days later (Day 0 of the experiment) mesocosms being amended with biosolid slurry
doi:10.1371/journal.pone.0057189.g001



Fate of the silver in the mesocosm

a single low dose of silver nanoparticles (0.14 mg Ag/kg soil)

Ag NPs from Biosolids: Plant Biomass and Soil Microbes

Colman et al. (2013) PlosOne, 8(2), e57189

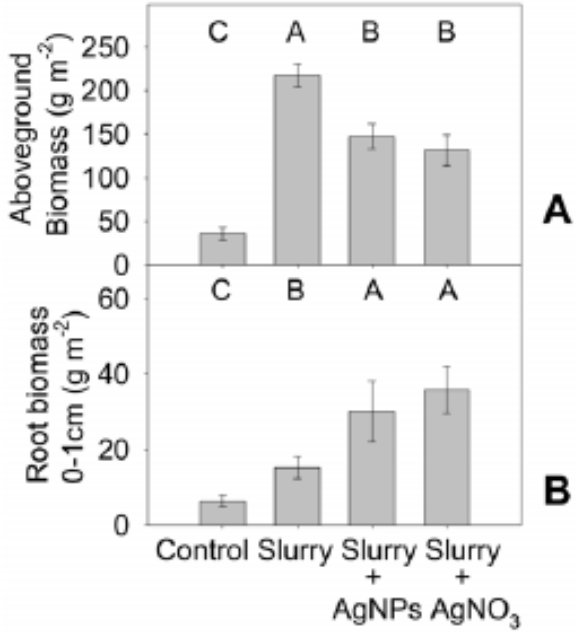


Figure 4.(A) Aboveground plant biomass of *Microstegium vimineum* (grass species), (B) root biomass in 0–1 cm soils.

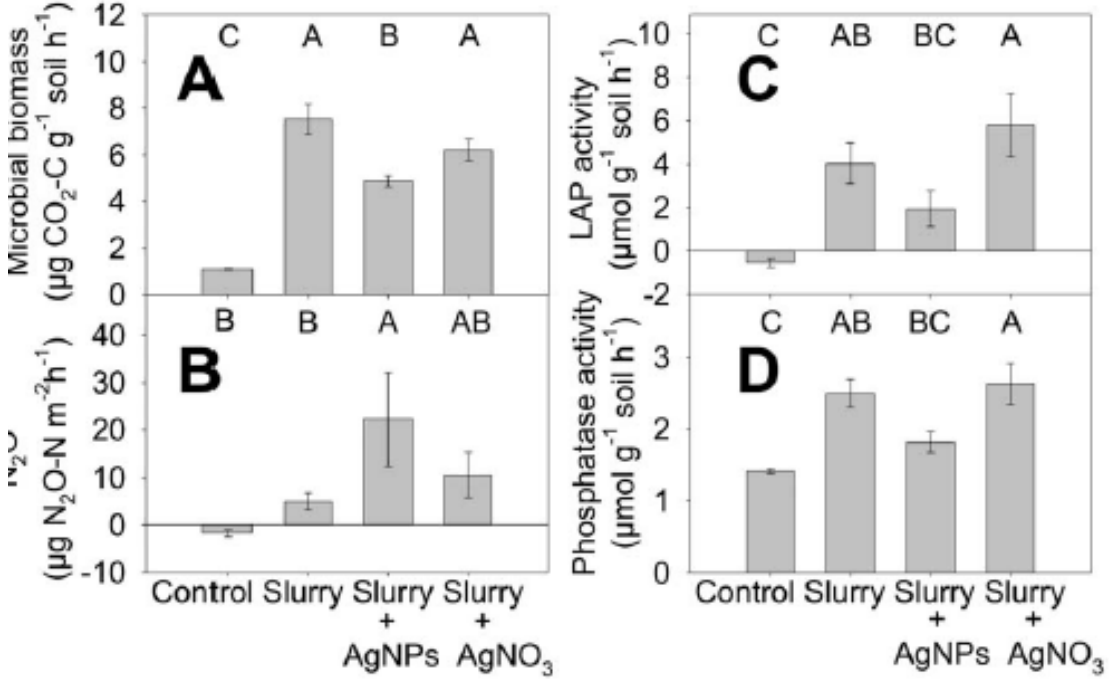
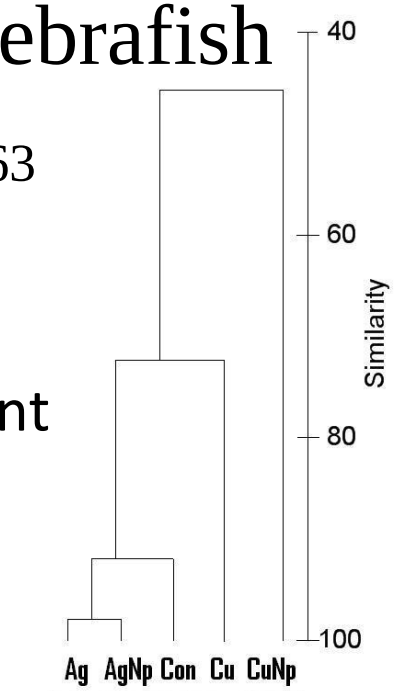


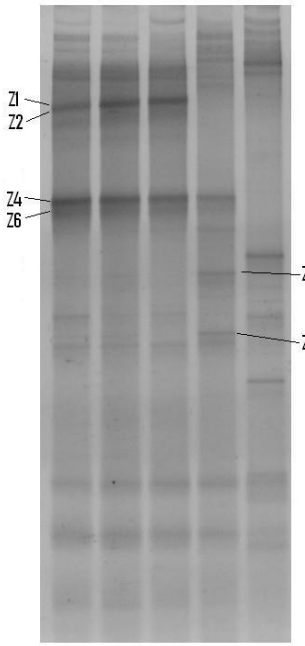
Figure 5. Microbial abundance, activity, and composition affected by Ag. A Microbial biomass; B N_2O flux from soil; C activity of the proteolytic extracellular enzyme leucine aminopeptidase (LAP); D activity of the organophosphorous degrading enzyme phosphatase

Effect of Cu-NPs on Gut Microbes in Zebrafish

Merrifield et al. (2013). Environmental Pollution 174, 157-163

Representative Dendrogram and PCR-DGGE fingerprint



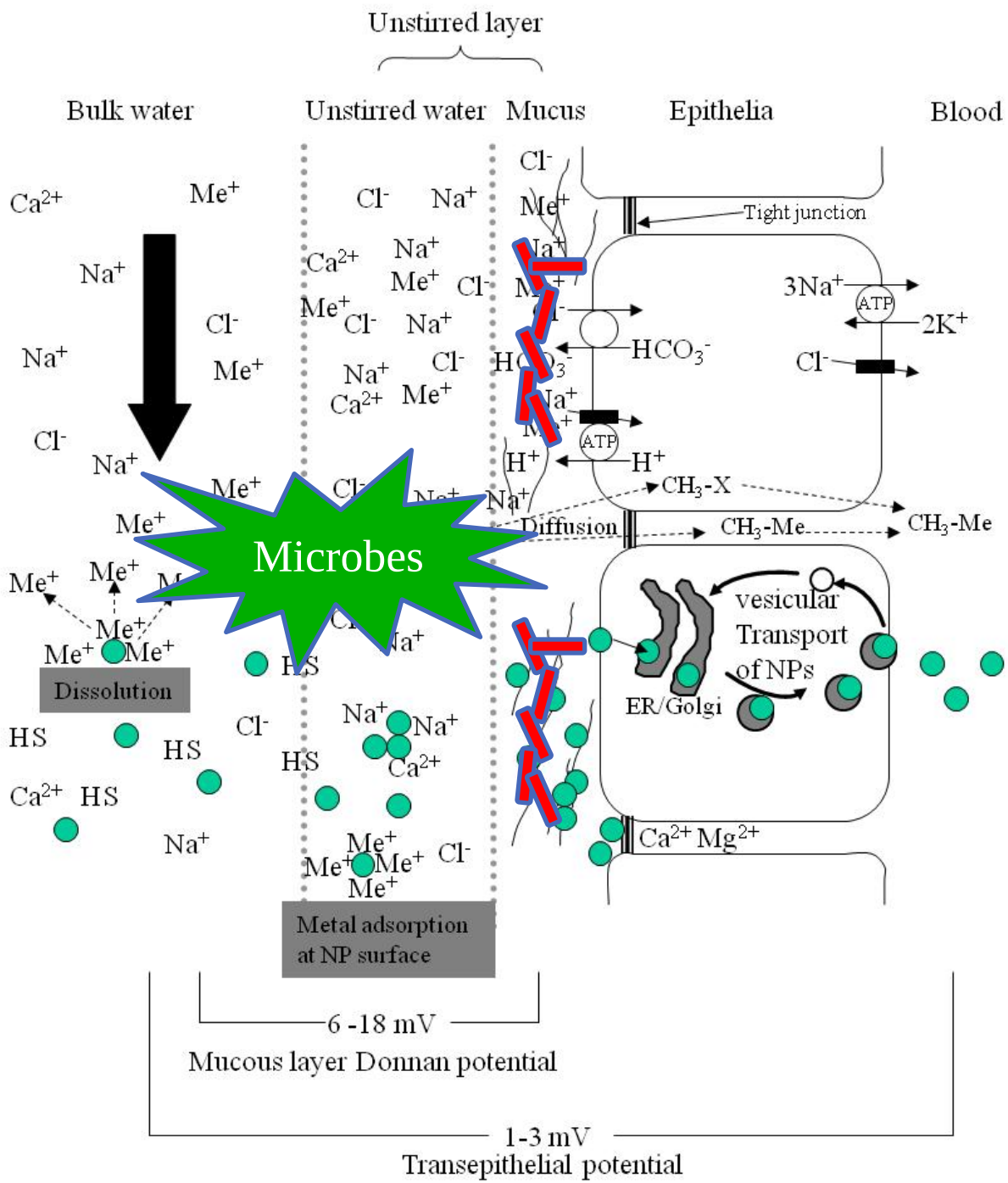


Code	Closest ID	Closest ID source	%
Z1	Uncultured bacterium clone	GIT of zebrafish	97
Z2	Uncultured bacterium clone	GIT of zebrafish	96
Z4	*Cetobacterium somerae*	GIT of fish	98
Z6	*Cetobacterium somerae*	GIT of fish	97
Z7	*Citrobacter freundii*	Estuarine sediments	95
Z9	Uncultured bacterium clone	GIT of zebrafish	97
Z10	Uncultured bacterium clone	GIT of zebrafish	94
Z13	Sphingobacteriales bacterium	Soil	100

Sensitive to Cu

Sensitive to Cu-NPs

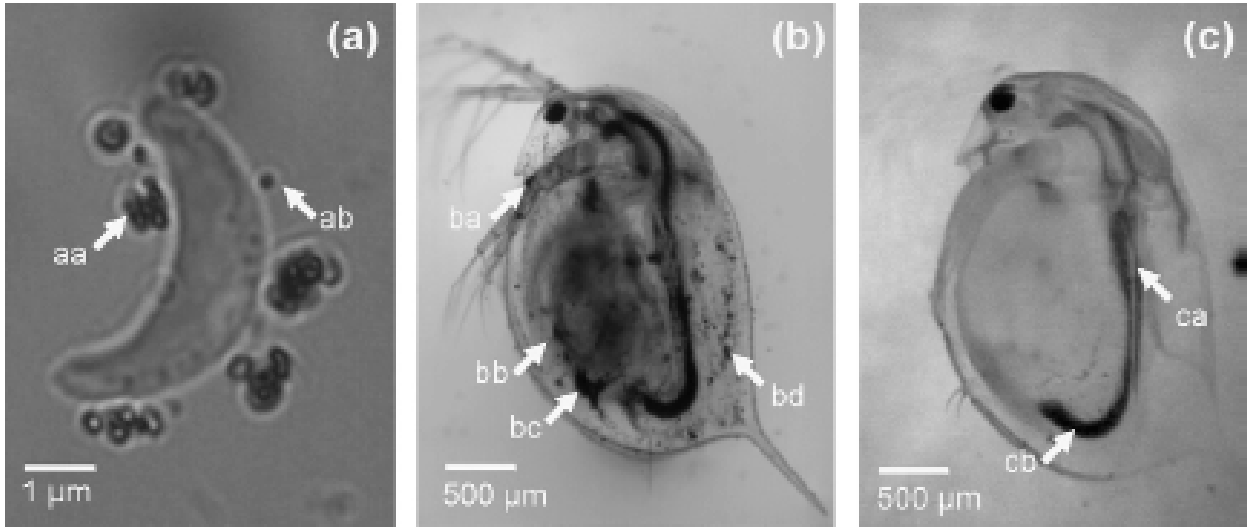
Bioavailability & Uptake Mechanisms



Shaw and Handy (2011) Environment International 37, 1083-1097.

Invertebrates –Data Gaps on Marine Species

Nano Issues with Aquatic Invertebrate Tests: mechanical suffocation?



C60 on/in the test organism. Baun et al. Aquatic Toxicology 86 (2008) 379–387

Daphnia: Surface coating and Moult Inhibition with TiO_2

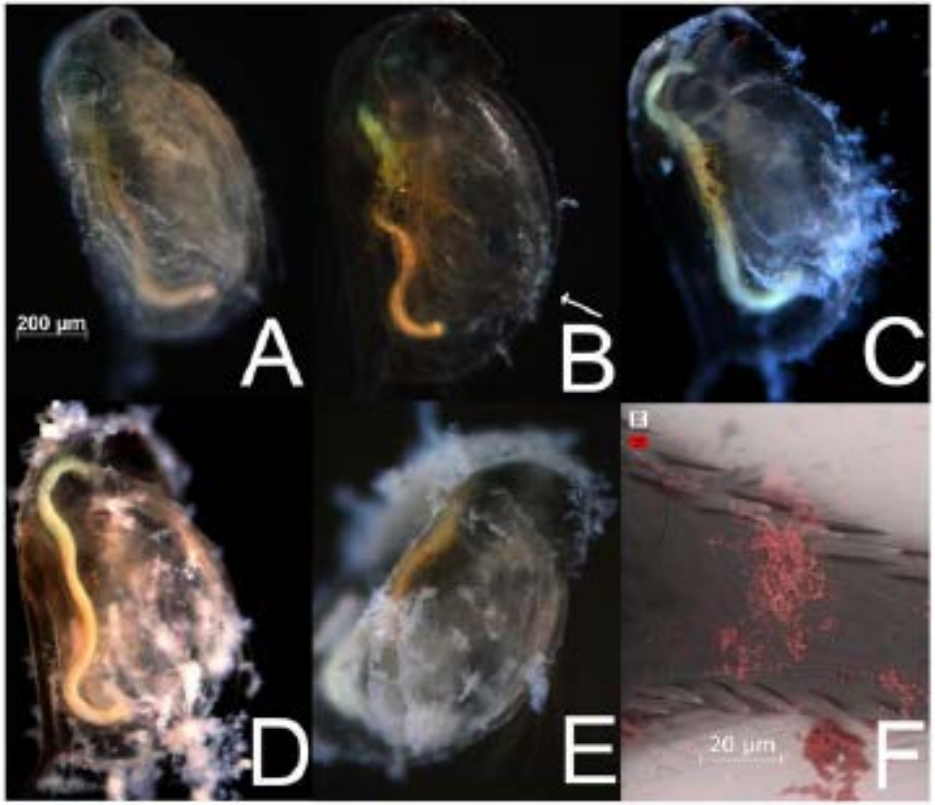
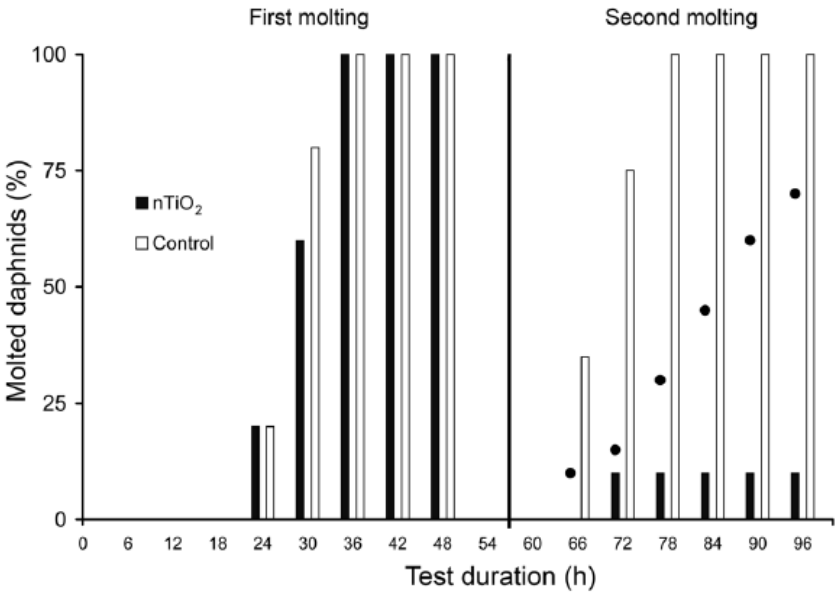


Figure 4. Development of biological surface coating in a 96-h toxicity test at 2 mg/L $nTiO_2$ in ISO-medium. (A) 24 h, before 1st molting; (B) 25 h, about 1 h after 1st molting with arrow indicating renewed particle adhesions at filtration apparatus and the lower ventral gap; (C) 48 h; (D) 72 h; (E) 96 h; (F) ESEM-EDX picture of $nTiO_2$ particle agglomerates colored in red on spine of *D. magna* following 48 h of exposure to, $nTiO_2$ particles at 2 mg/L.

Dabrunz et al PloS ONE

PLoS ONE 6(5): e20112. doi:10.1371/journal.pone.0020112



Effect of Nano Iron on Marine Organisms: Fertilisation Failure

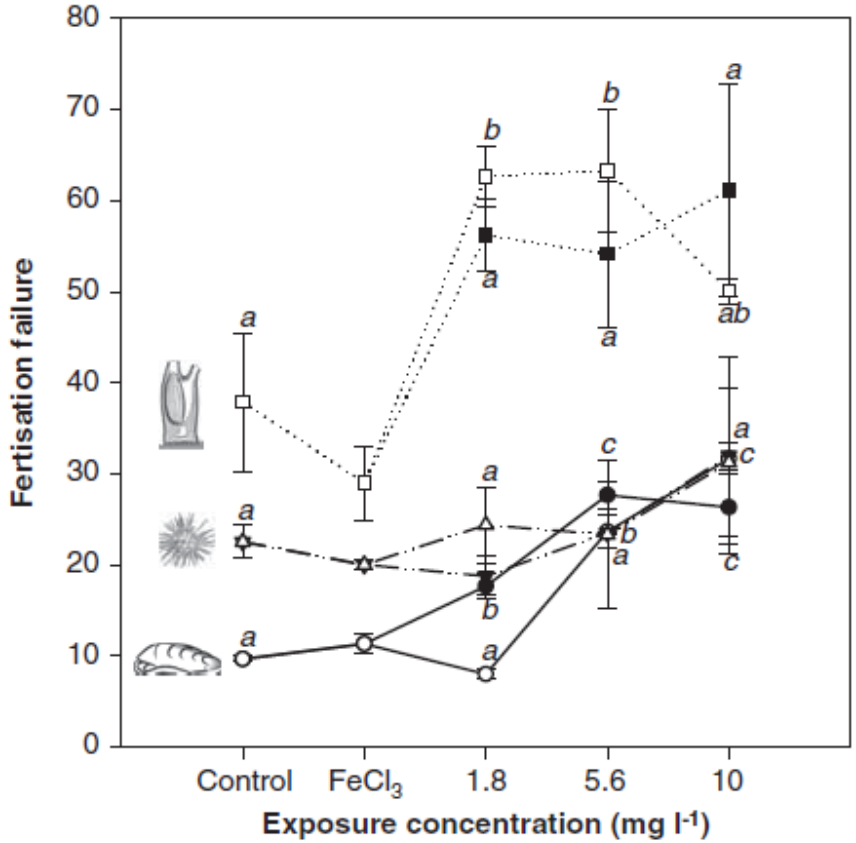


Figure 3. The effect of coated vs non-coated zero-valent iron nanoparticle (nZVI) on sperm fecundity (e.g. fertilisation failure) in three marine invertebrates: the tunicate *Ciona intestinalis* (rectangles), the sea urchin *Psammechinus milliaris* (triangles) and the common mussel *Mytilus galloprovincialis* (circles); open symbols stand for NANOFER 25S® (coated) while the full symbols denote NANOFER 25® (non-coated). Control scores were performed on both sperm exposed to the non-nano form ($FeCl_3$ equimolar to the highest exposure) concentration and unexposed sperm that was kept in the lab under same conditions as exposed ones, prior to fertilisation. The points represent mean ± SEM% of oocytes that fail to fertilise by the pre-exposed sperm; N = 3 plates (each plate contained approximately 100 oocytes). Bars with the same letters are not significantly different, according to the one way ANOVA, $p \leq 0.05$.

Kadar et al. (2012) Nanotoxicology, DOI: 10.3109/17435390.2011.647927

Effect of Nano Iron on Marine Organisms: Delayed Development

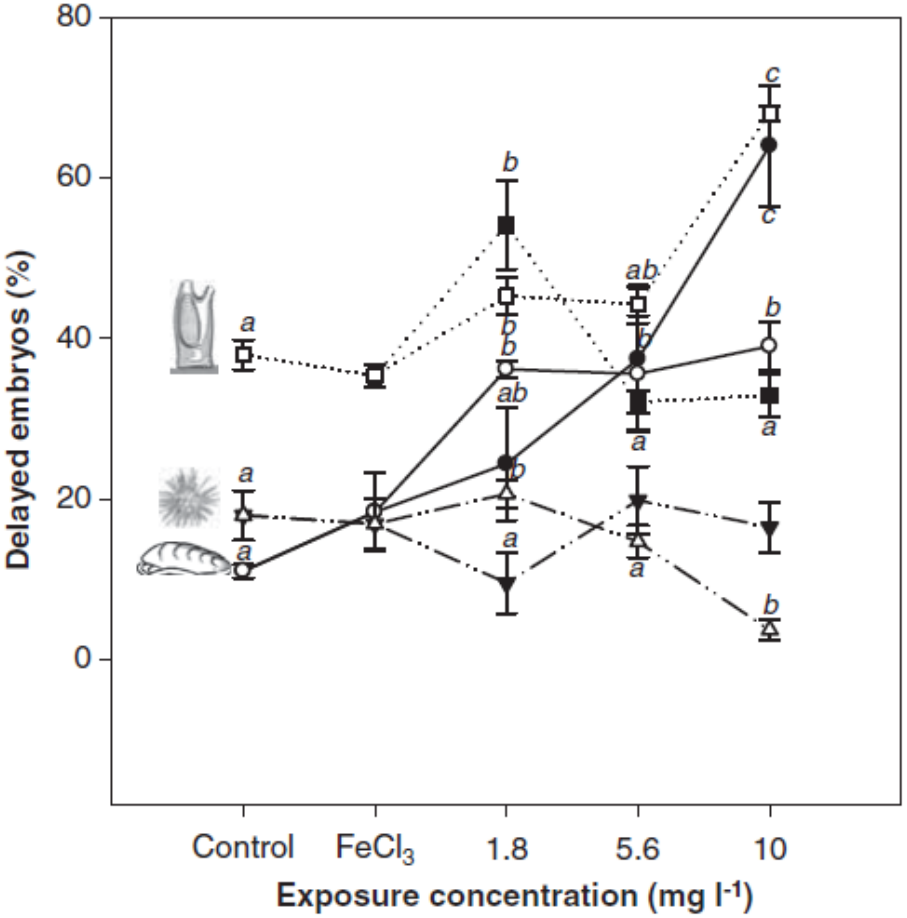
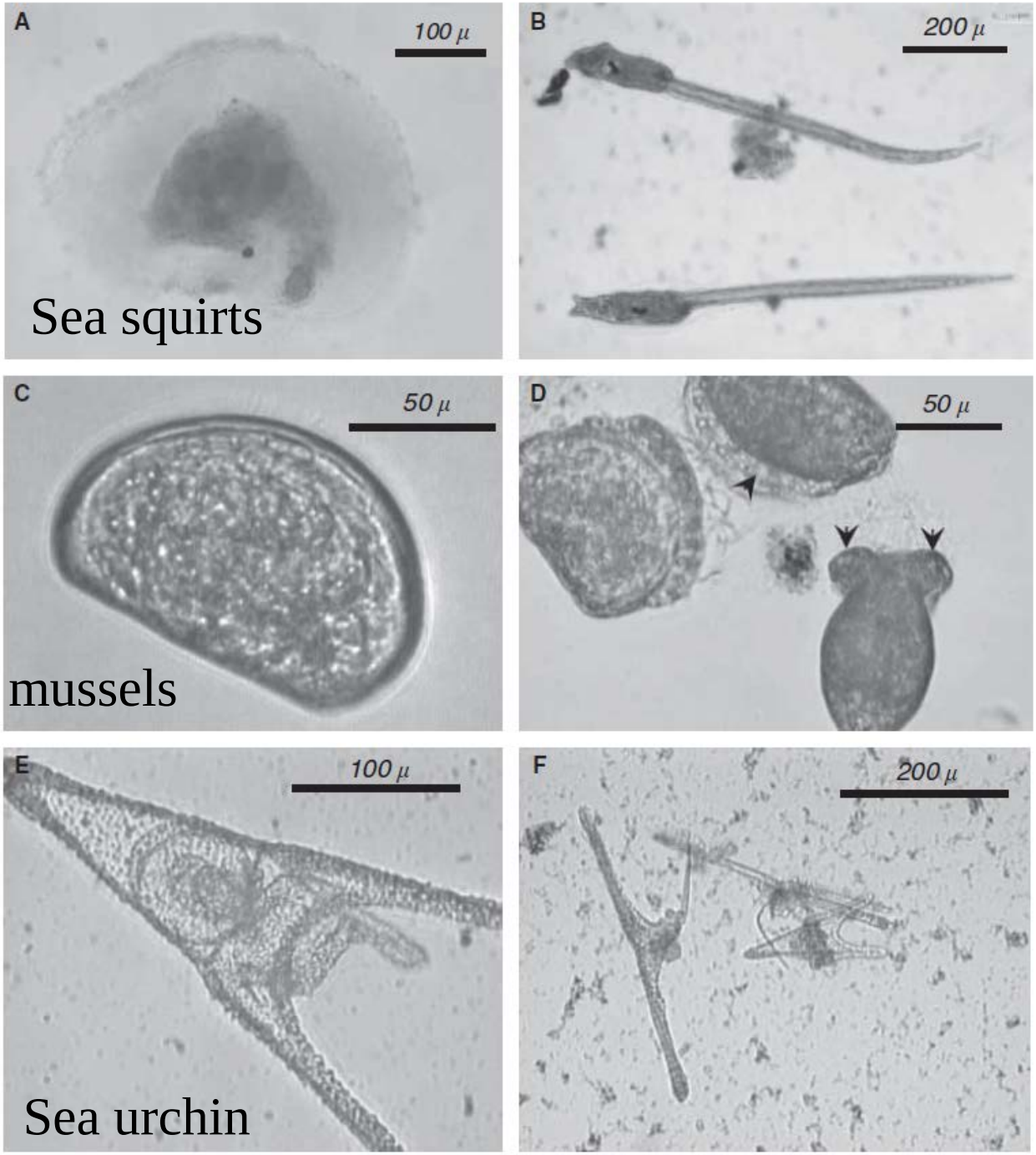


Figure 5. The effect of exposure of the male gametes to zero-valent iron nanoparticle (nZVI) on subsequent embryo development in three marine invertebrates: the tunicate *Ciona intestinalis* (rectangles), the sea urchin *Psammechinus milliaris* (triangles) and the common mussel *Mytilus galloprovincialis* (circles): open symbols stand for NANOFER 25S® (coated with an organic stabiliser) while the full symbols denote NANOFER 25® (non-coated). Control scores were performed on embryos developing from unexposed sperm and also from the sperm exposed to the non-nano form ($FeCl_3$ equimolar to the highest exposure). The points represent mean ± SEM% of delayed from total embryos, N = 3 plates (each plate contained approx. 100 embryos). Bars with the same letters are not significantly different, according to the one-way ANOVA, $p \leq 0.05$.

Kadar et al. (2012) Nanotoxicology, DOI: 10.3109/17435390.2011.647927

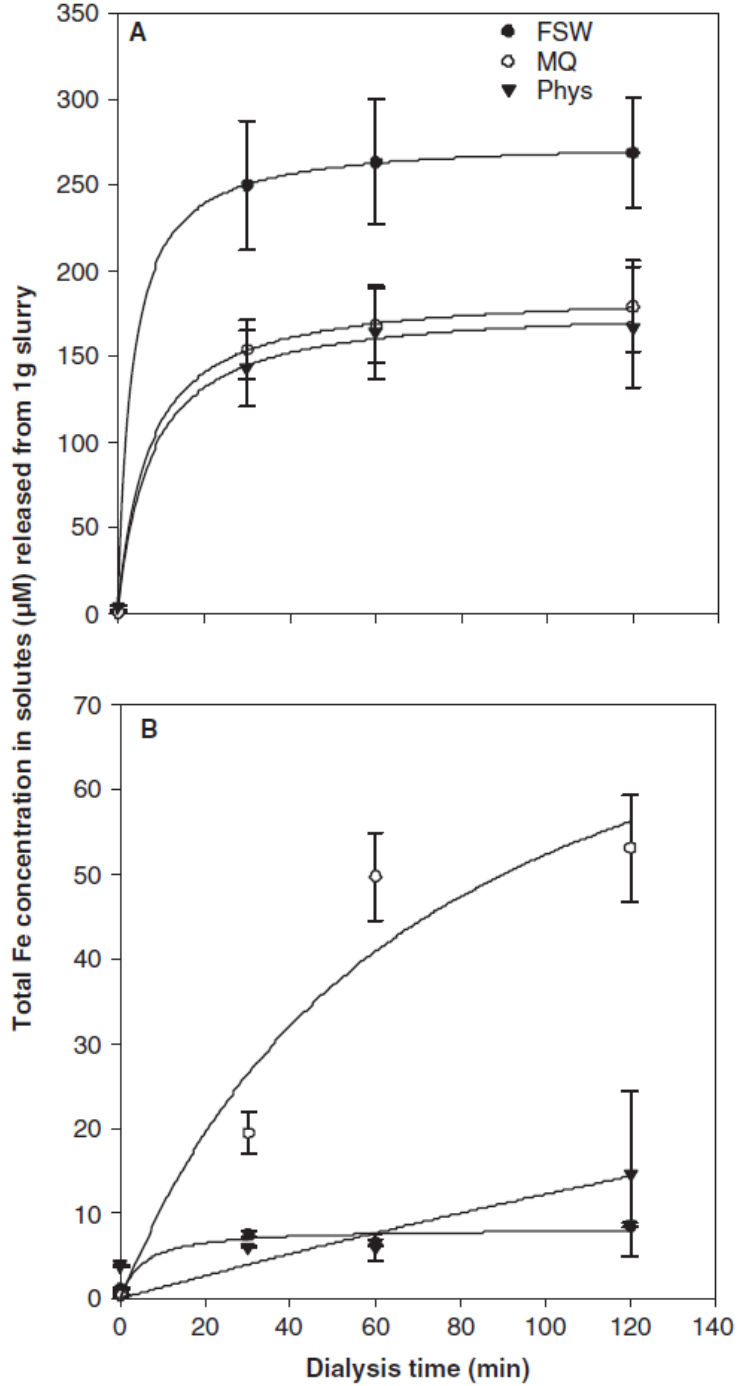


Effect of Nano Iron on Marine Organisms: Abnormal Larvae

Kadar et al. (2012) Nanotoxicology, DOI: 10.3109/17435390.2011.647927

Behaviour of Iron Nanoparticles in Seawater

Kadar et al. (2012) Nanotoxicology, DOI: 10.3109/17435390.2011.647927



■ S-nZVI ▒ nZVI □ FeCl3

Mean: 131 nm Mode: 37 nm SD: 89 nm D10: 29 nm D50: 101 nm D90: 246 nm Conc. 0.45 x 10^8 particles/ml	Mean: 171 nm Mode: 87 nm SD: 101 nm D10: 31 nm D50: 231 nm D90: 277 nm Conc. 0.30 x 10^8 particles/ml	Mean: 586 nm Mode: 84 nm SD: 614 nm D10: 73 nm D50: 285 nm D90: 1624 nm Conc. 1.08 x 10^8 particles/ml

Figure 2. Concentration of total Fe in solutes during 120-min dialysis of A) SnZVIs (zero-valent iron nanoparticles coated with a Na-acrylic copolymer) and B) nZVI (uncoated) for three different dialysis media: filtered seawater (FSW), ultra-pure water (MQ) and physiological saline (Phys), and are averages ± SEM, N = 3.

Behaviour of Silver Nanoparticles in Seawater

Khan et al. (2012) Environ. Sci. Technol. 46, 7621−7628

Table 1. Ag NP Behavior Measured as Hydrodynamic Diameter (nm) and Suspension Stability (Zeta Potential in mV) in Deionized, Estuarine (17 ‰), and Marine (33 ‰) Waters over 96 h (n = 3 Replicates Per Measure)[a]

	hydrodynamic diameter (nm)			Zeta potential (mV)		
water	0 h	1 day	7 days	0 h	1 day	7 days
d-H_2O	32 ± 1	32 ± 2	31 ± 1	−41 ± 1	−32 ± 2	−28 ± 2
estuarine	79 ± 13	145 ± 6	164 ± 6	−12 ± 2	−16 ± 1	−16 ± 1
marine	162 ± 21	339 ± 6	266 ± 3	−13 ± 1	−15 ± 2	−14 ± 2

[a]Results demonstrate an increase in aggregation at higher salinities. The consistency of the zeta potentials over time would indicate that colloidal instability continues over time and hydrodynamic diameters are subject to fluctuations.

Silver Uptake from Ag NP Exposures in Marine Snails

Khan et al. (2012) Environ. Sci. Technol. 46, 7621−7628

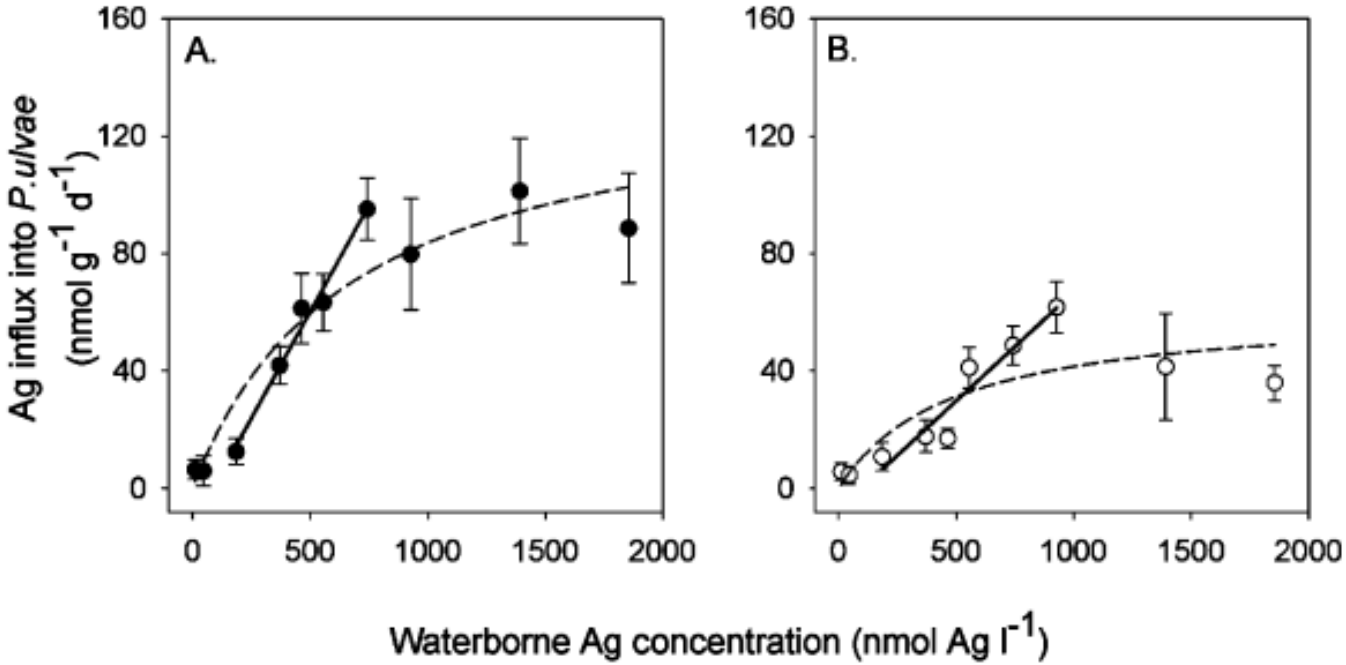
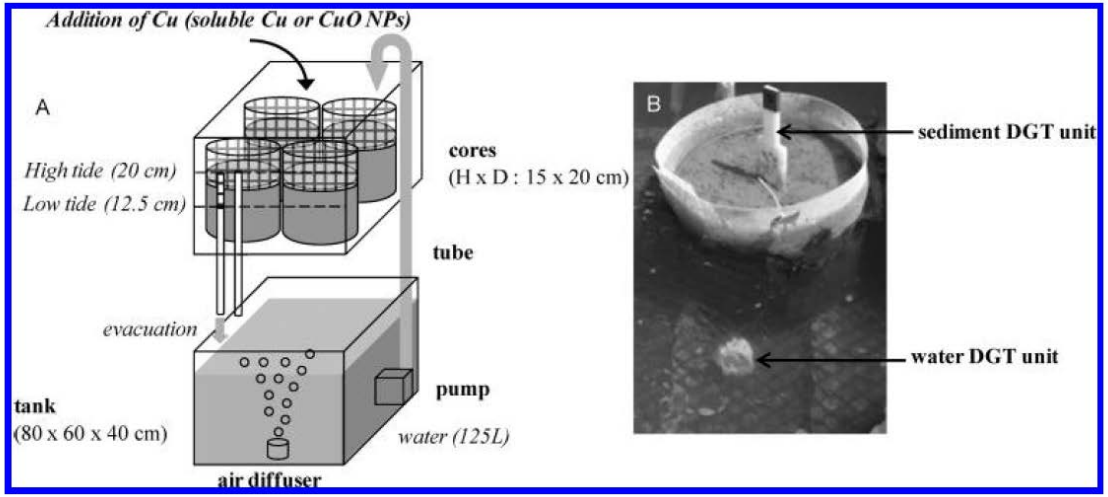
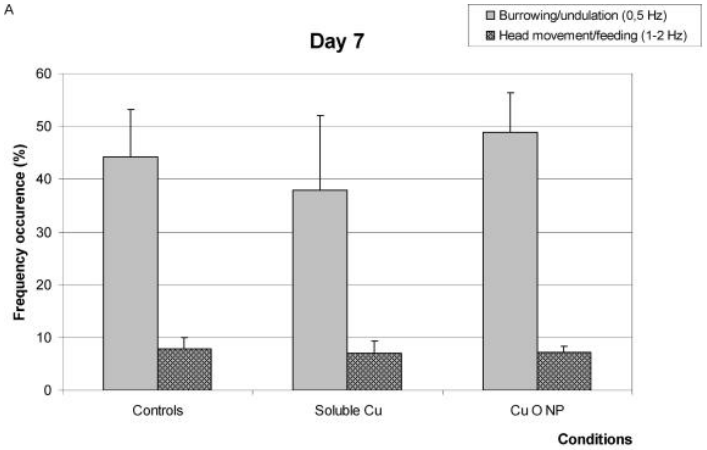


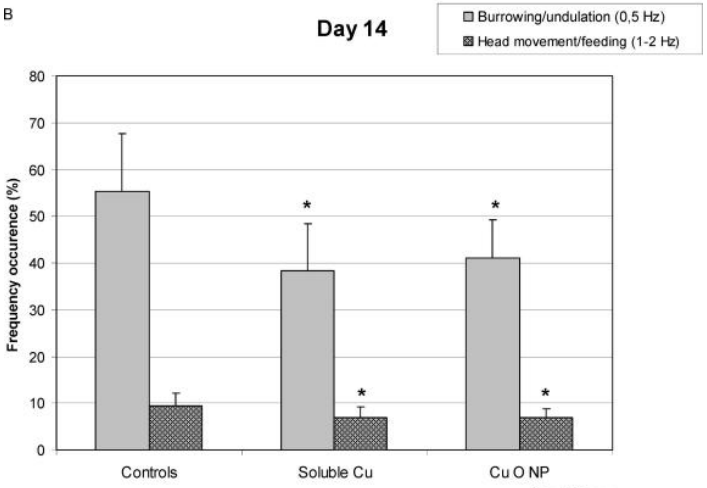
Figure 1. Ag influx into Peringia ulvae soft tissue following 1 day waterborne exposures to dissolved Ag (A, black circles) and Ag NPs (B, open circles) for (mean values (nmol g^{-1} (dw) d^{-1}) ± S.D., n = 6 pooled replicates per data-point from 48 individuals). Linear regression (solid line) was used to determine the uptake rate constants .

Marine Mesocoms Study –Nano CuO

Buffet et al. (2013) Environ. Sci. Technol. 47, 1620−1628







Changes in burrowing behaviours of benthic invertebrates?

Terrestrial Organisms

Earthworms

Higher Plants

Earthworm Pathologies from Exposure to C60

Van der Ploeg et al. (2012) Nanotoxicology; Early Online
DOI: 10.3109/17435390.2012.668569

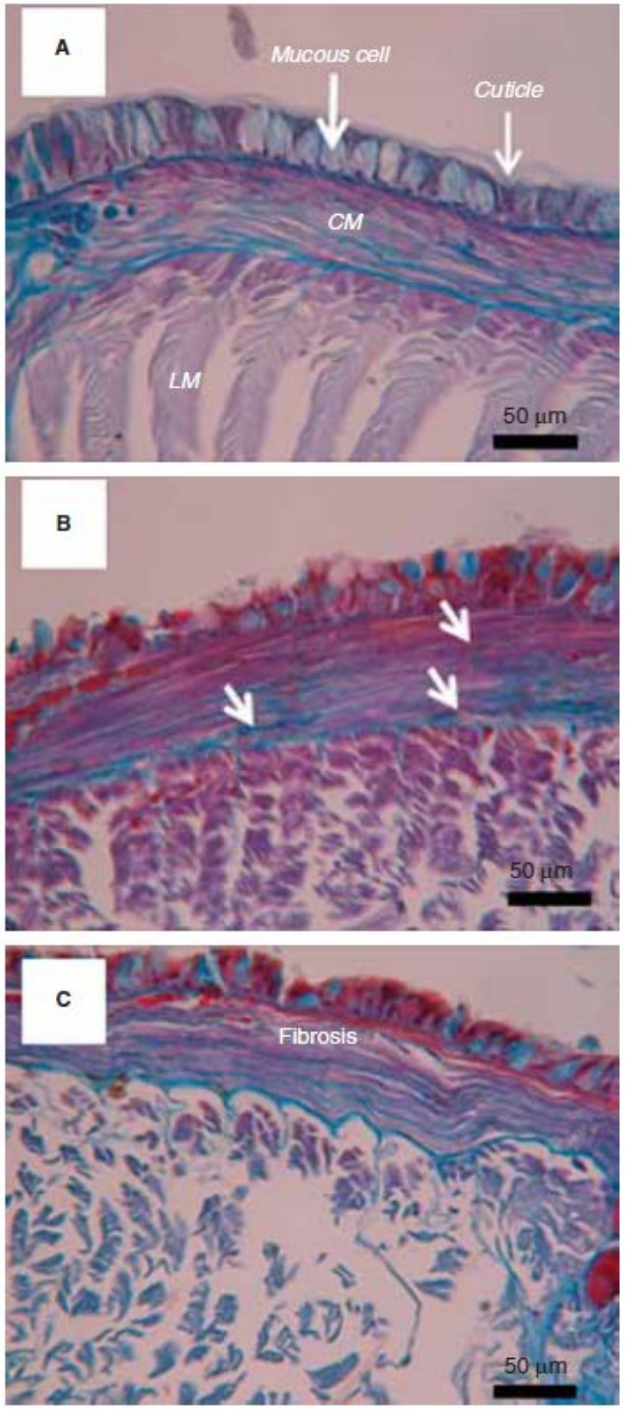


Figure. Transverse sections of segments from the anterior region of earthworms exposed to (A) control, (B) 15 and (C) 154 mg/kg of C60 nanoparticles.

Earthworm Exposure to C60: Only Minor Life Long Effects on Gene Expression

Van der Ploeg et al. (2012) Nanotoxicology; DOI: 10.3109/17435390.2012.668569

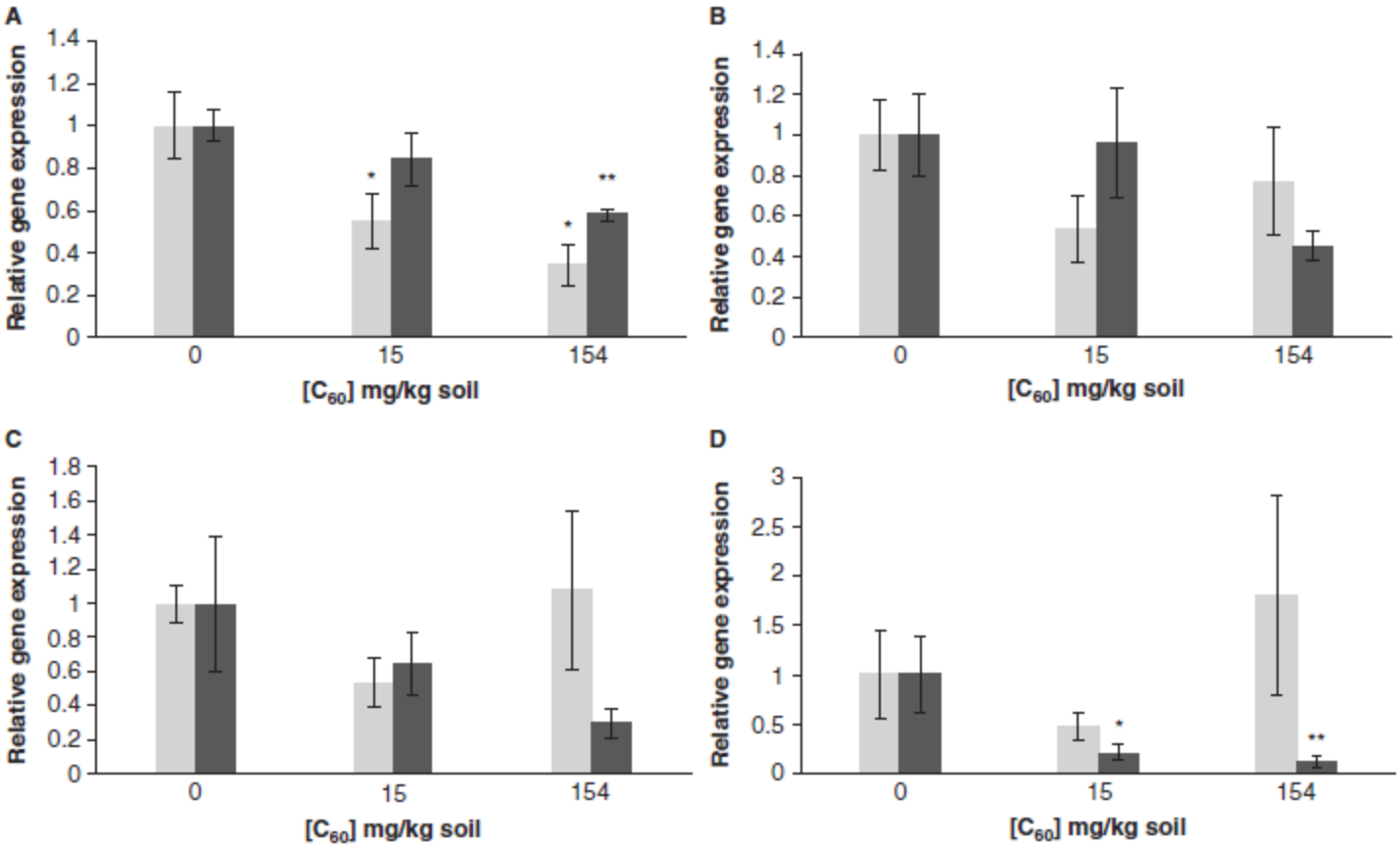


Figure 1. Relative gene expression levels of (A) heat shock protein 70 (HSP70), (B) catalase, (C) glutathione S-transferase (GST) and (D) coelomic cytolytic factor 1 (CCF-1), for the four-week- (light grey) and for the lifelong (dark grey)-exposed earthworms.

Earthworm Exposure to C60: No Effects on Enzymes Related to Oxidative Stress

Van der Ploeg et al. (2012) Nanotoxicology; DOI: 10.3109/17435390.2012.668569

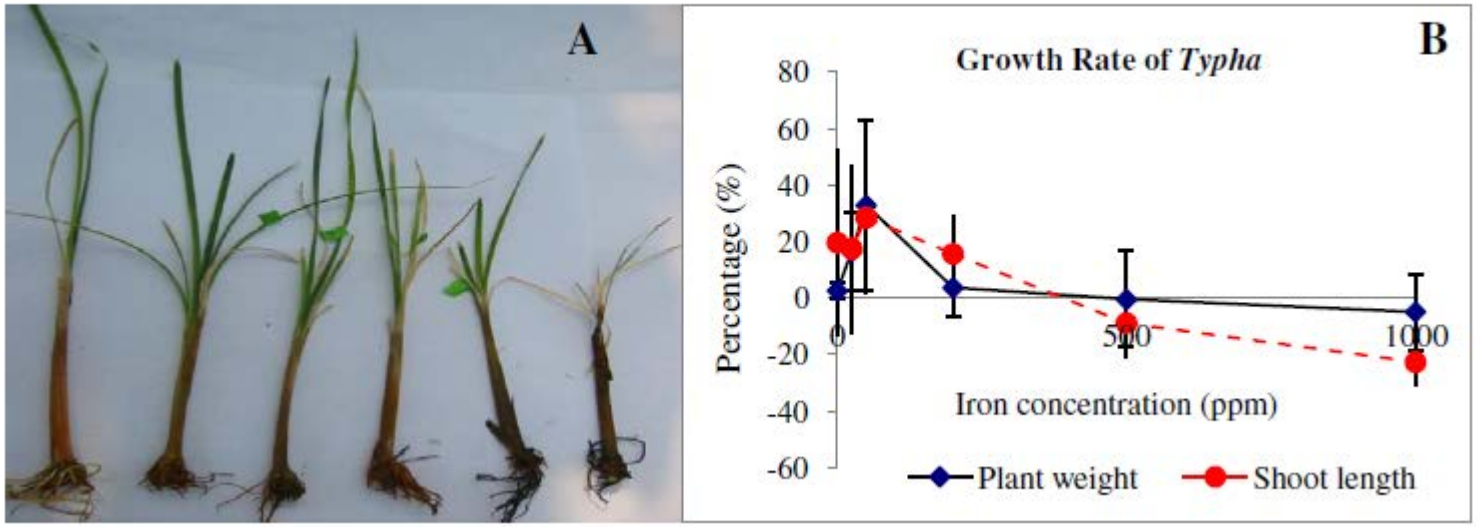
Table III. Measured levels of catalase and glutathione-*S*-transferase for the different treatment groups.

Exposure period	[C60] (mg/kg soil)	Catalase (nmol/min/mg protein)	GST (nmol/min/mg protein)
Four weeks	0	295 ± 32	184 ± 53
	15	318 ± 48	154 ± 62
	154	327 ± 67	158 ± 49
Lifelong	0	394 ± 38	212 ± 89
	15	464 ± 51	133 ± 56
	154	298 ± 48	148 ± 40

Mean values ± S.E. No significant differences were demonstrated between treatments, within the assays (alpha = 0.05).

Iron Nanoparticle Effects on Higher Plants

X. Ma et al. / Science of the Total Environment 443 (2013) 844–849



Plants grown hydroponically in green houses with different concentrations of nZVI (0–1000 mg/L) for four weeks.

Fig. 1. A. Image of Typha seedlings (the Cattail plant) four weeks after their exposure to different concentrations of nZVI; B. Percentage of plant weight and shoot height change after four weeks of exposure. Error bars represent standard deviation, n=4.

Iron Nanoparticle On The Roots of the Cattail Plant

Ma et al (2013) Science of the Total Environment 443, 844–849

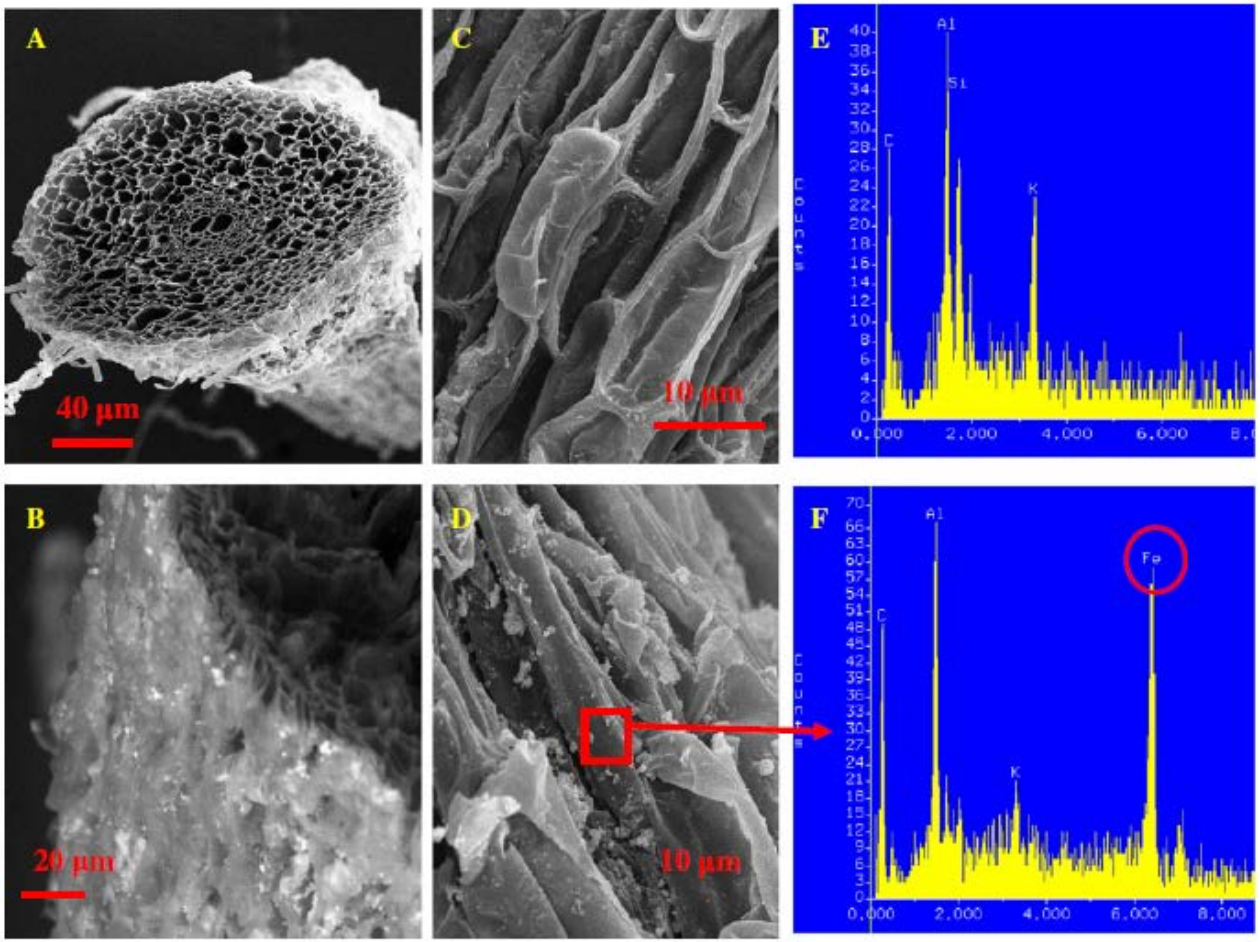
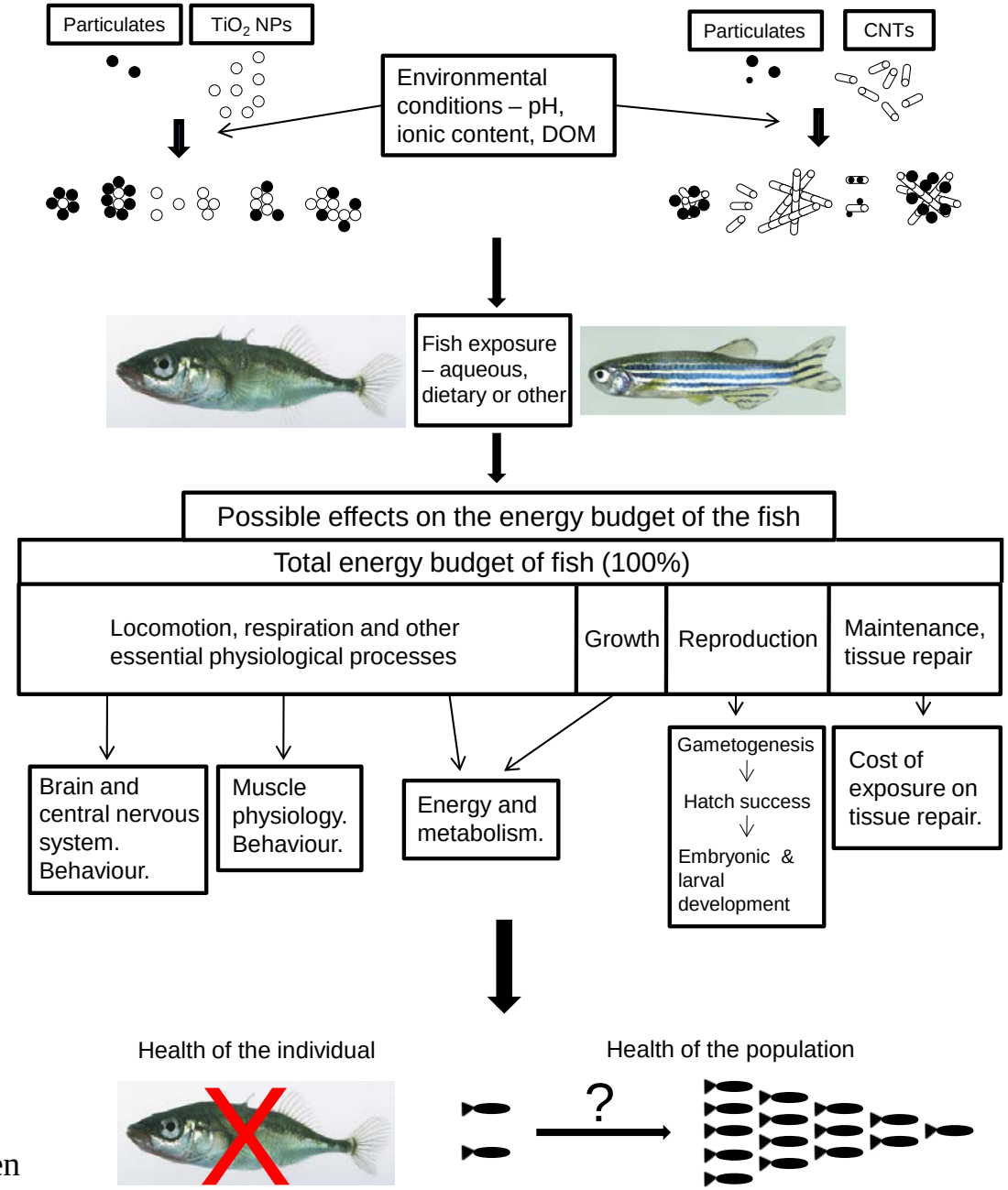


Fig. 2. SEM showing the deposition of nZVI on root surface. A. control plants; B Typha root exposed to 200 mg/L of nZVI. C and D are control and Typha roots exposed to 200 mg/L of nZVI. E and F are EDX analyses.

Fishes and other vertebrate animals

NP toxicity – the big picture



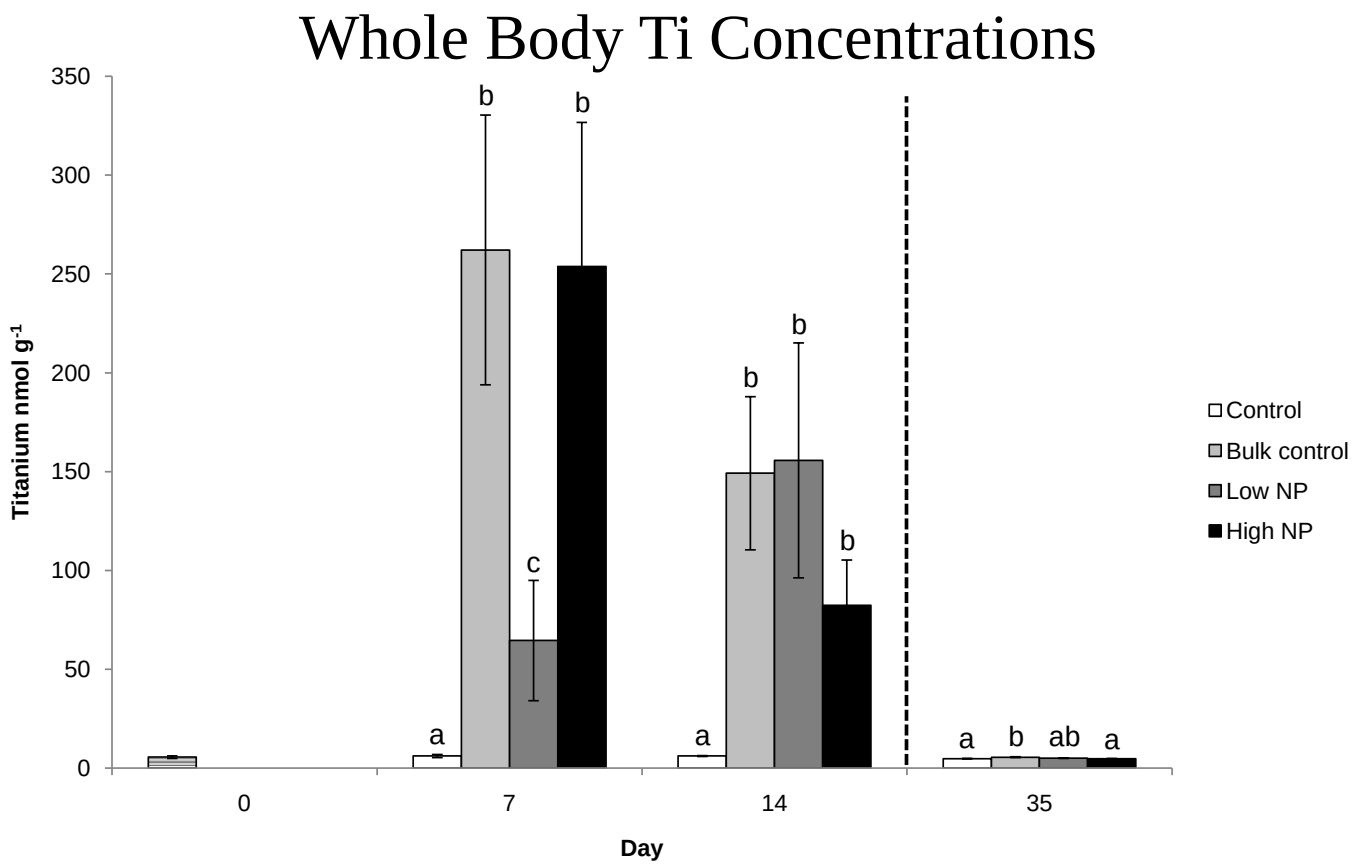
Slide: Dr Chris Ramsden

Early Life Stages of Fishes and Amphibians

The Effects of TiO_2 NPs on Adult Zebrafish

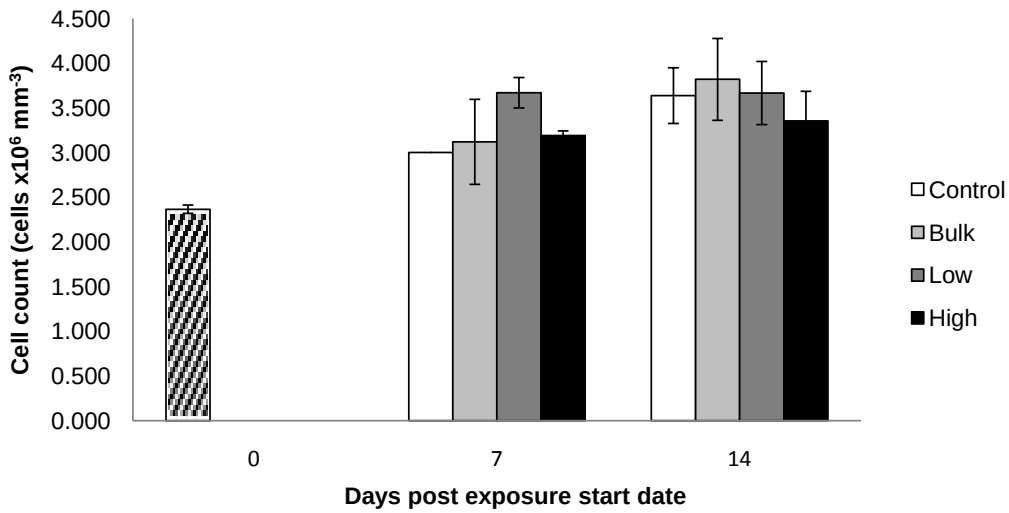
Ramsden et al. (2013) Aquatic Toxicology 126, 404– 413.

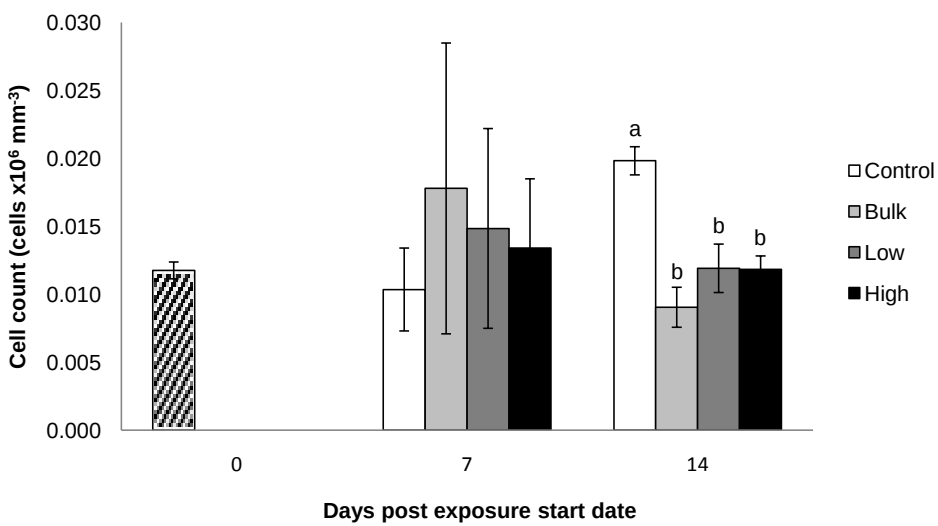
Control, 1 mg l^{-1} bulk TiO_2, 0.1 mg l^{-1} TiO_2 NPs, 1 mg l^{-1} TiO_2 NPs. Fish exposed for 14 days, followed by a recovery period.



Haematology of Adult Zebrafish

Ramsden et al. (2013) Aquatic Toxicology 126, 404– 413.





- No significant effect on red blood cells
- White blood cell counts significantly lower for all Ti exposed fish at day (ANOVA, $P < 0.05$).

Embryo Quality: Reproduction TiO_2

Ramsden et al. (2013) Aquatic Toxicology 126, 404– 413.

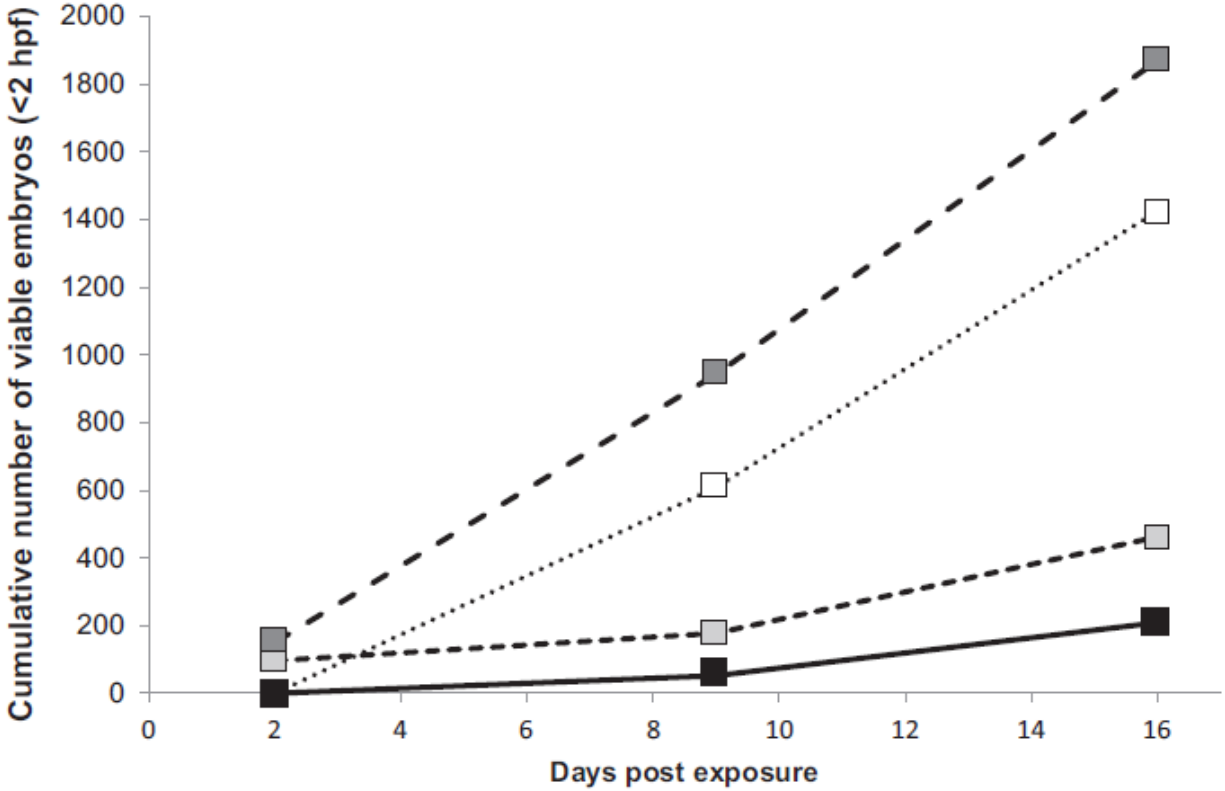
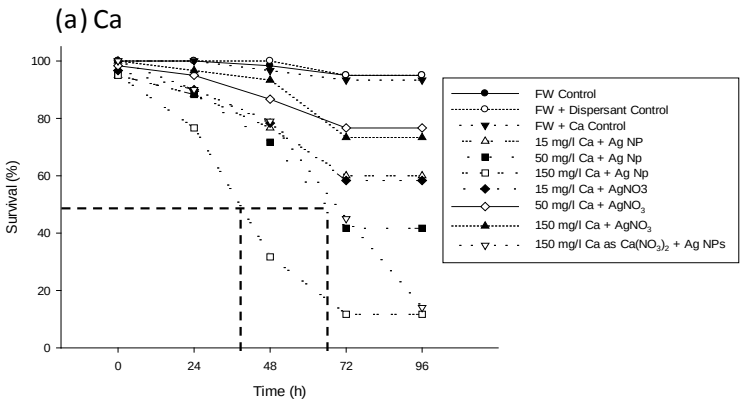
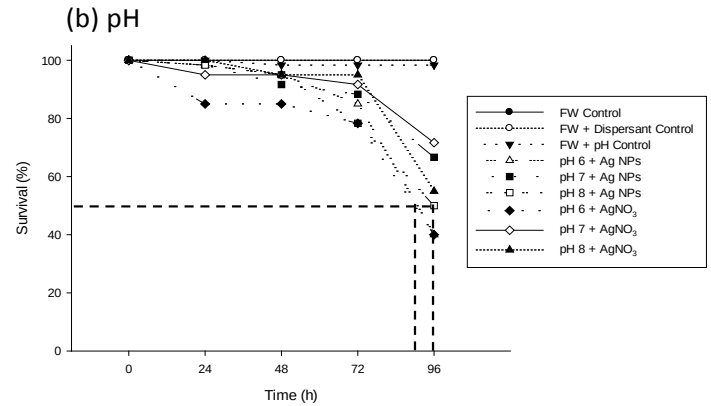


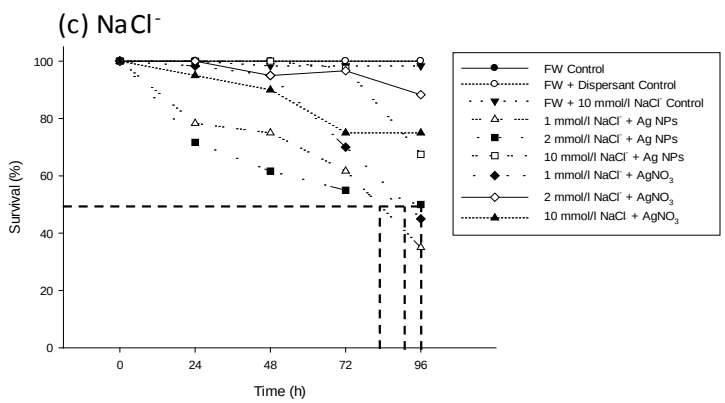
Fig. 5. Cumulative number of viable embryos (<2 hpf) produced by control (clear squares with dotted line), 1.0 mg l^{-1} bulk TiO_2 (light grey squares with close-dashed line), 0.1 mg l^{-1} TiO_2 NPs (dark grey squares with dashed line) and 1.0 mg l^{-1} TiO_2 NPs (black squares with solid line) treatment groups of zebrafish after the exposure period.

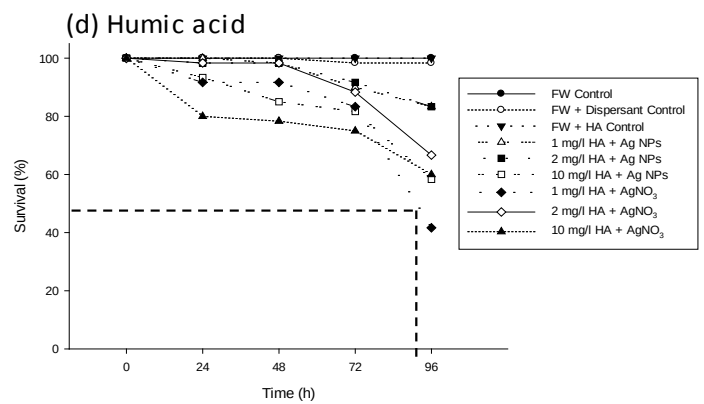
FP7 MARINA: Progress on water quality factors affecting NP toxicity to Zebrafish Larvae

Shaw and Handy, unpublished data





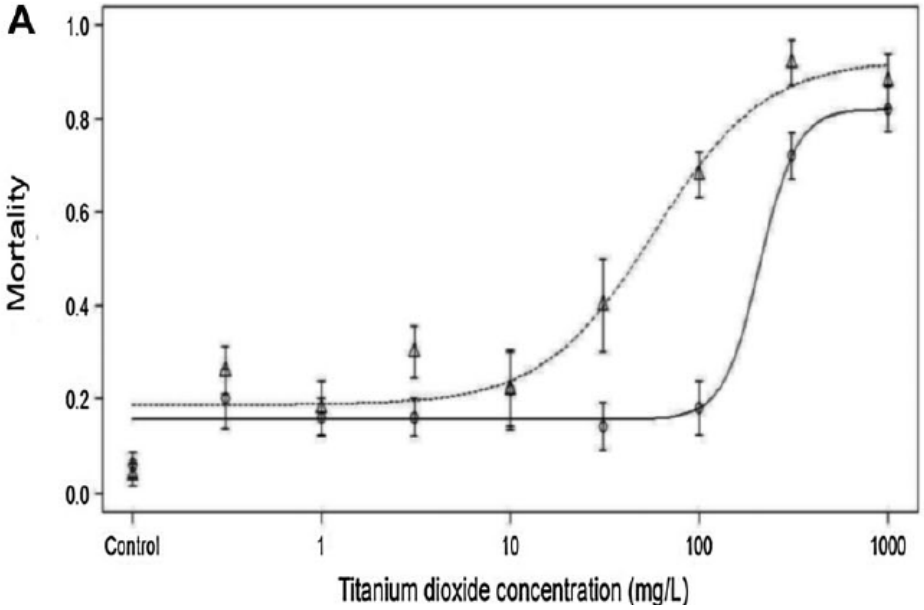


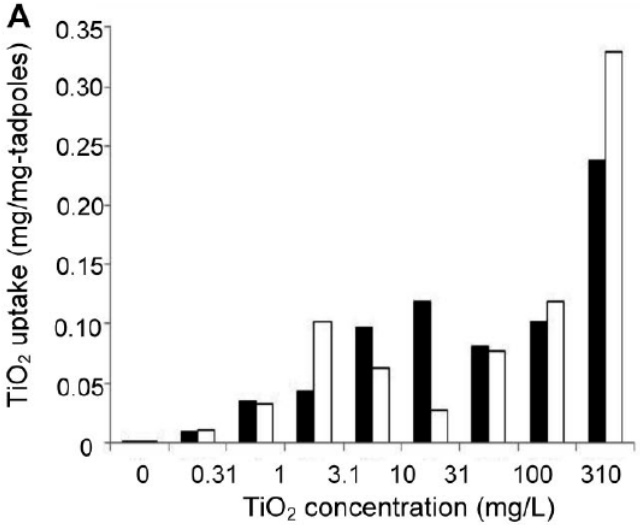


TiO_2 and UV Effects on *Xenopus* larvae

Zhang et al. (2012). ET&C 31,176–183

Dose–response curves of *Xenopus laevis* tadpole mortality to titanium dioxide nanomaterials for day 14: 5 nm material. white light (circles), ultraviolet light (triangles).





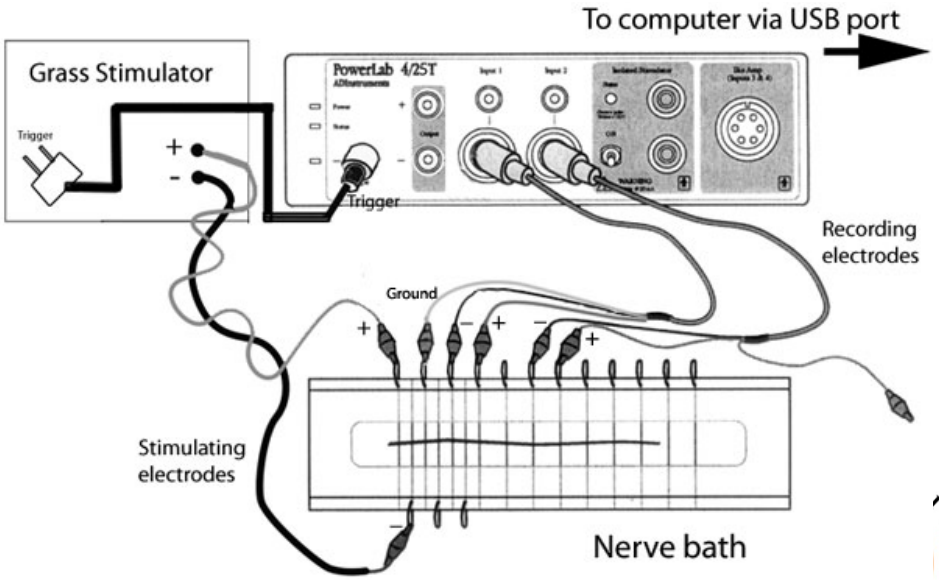
white light (solid bars), ultraviolet light (open bars).

Neurotoxicity, Brain and Behaviour

Do Nanomaterials Alter Action Potentials in Nerves?

Windeatt and Handy (2012) Nanotoxicology

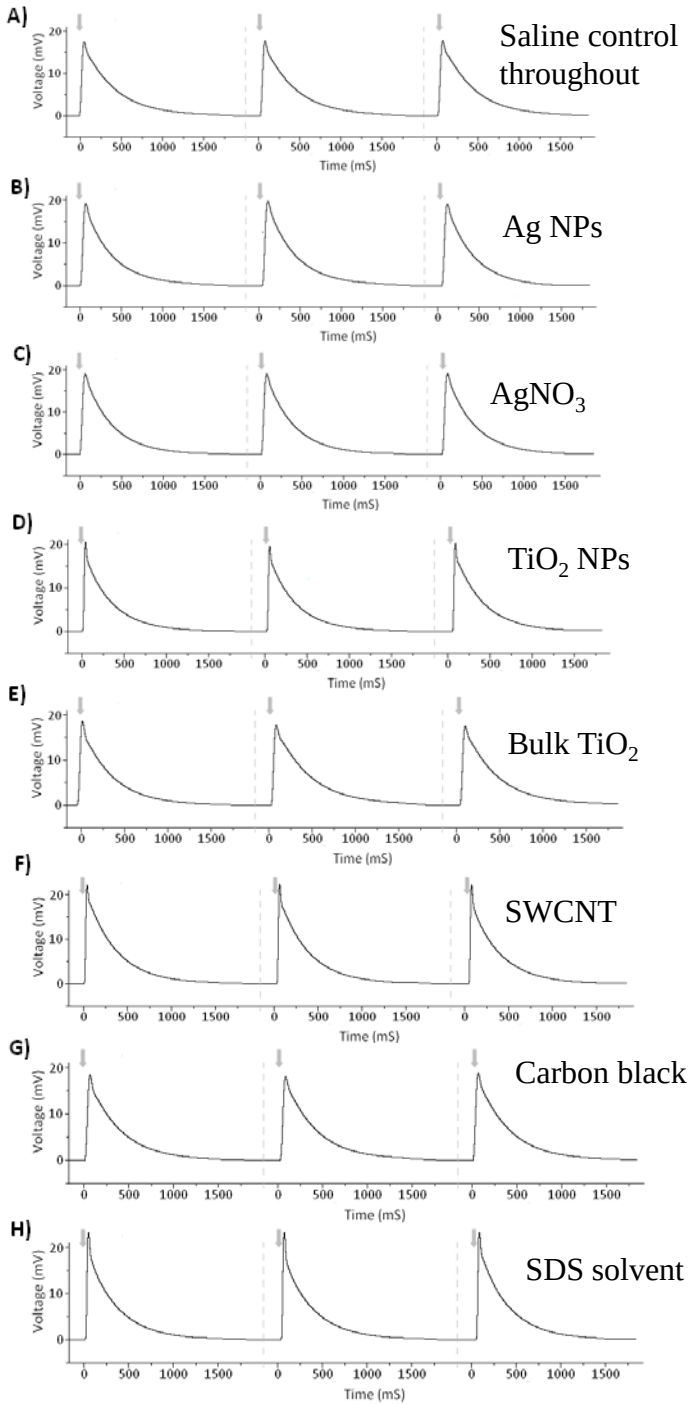
- Compound action potentials from shore crabs.
- Classic action potential experiment.
- Control saline-NP exposure-control saline.



http://www.medicine.mcgill.ca/physio/vlab/cap/recording.htm

Single Action Potential Recordings

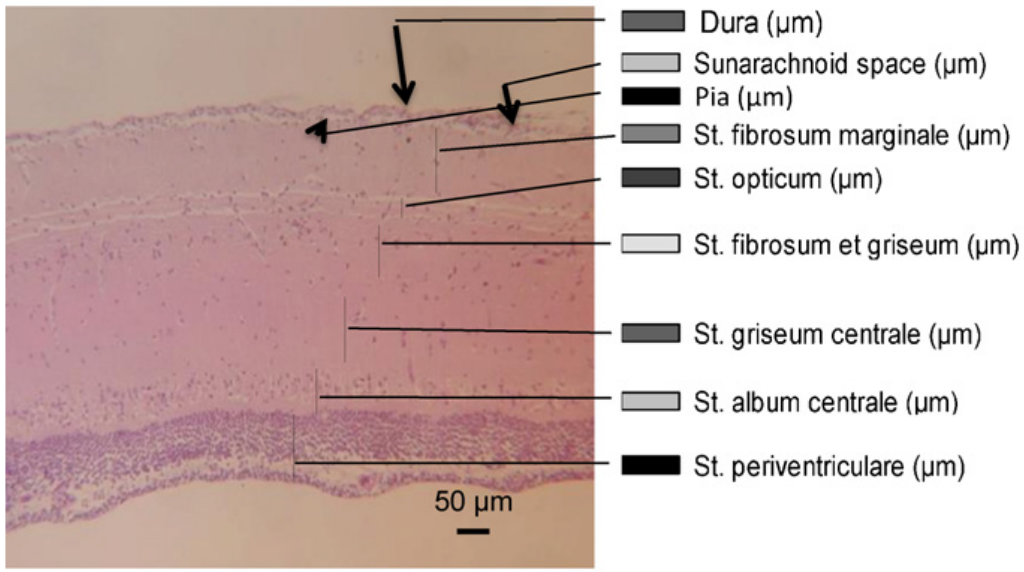
Windeatt and Handy (2012) Nanotoxicology



No measurable effects

Brain Injury: Waterborne Copper Nanoparticles

Al-Bairuty et al. (2013) Aquatic Toxicology 126 (2013) 104– 115



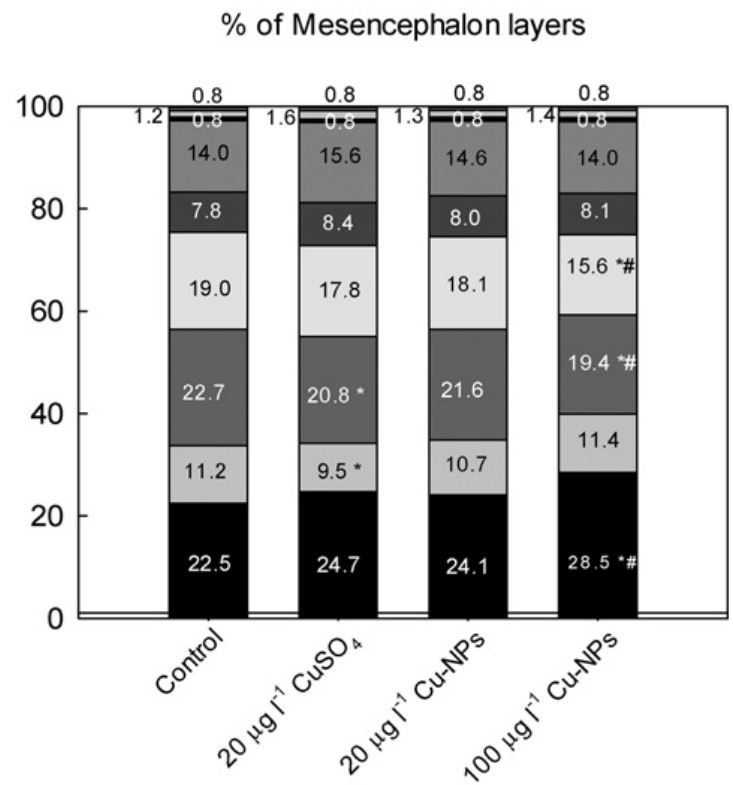


Fig. 5. The proportion of alteration in the thickness of mesencephalon tissue layers of the brain in rainbow trout following waterborne exposure to control, 20 ug l^{-1} of Cu as $CuSO_4$, 20 or 100 ug l^{-1} of Cu as Cu-NPs for 10 days. Data are proportion means as a % ± S.E.M., n = 6 fish/treatment. (*) Significant difference from control within treatment (ANOVA, $P < 0.05$). (#) Significant difference between low and high concentration of Cu-NPs (t-test, $P < 0.05$).

Immune Activity in the Kidney: Waterborne Copper Nanoparticles

Al-Bairuty et al. (2013) Aquatic Toxicology 126 (2013) 104– 115

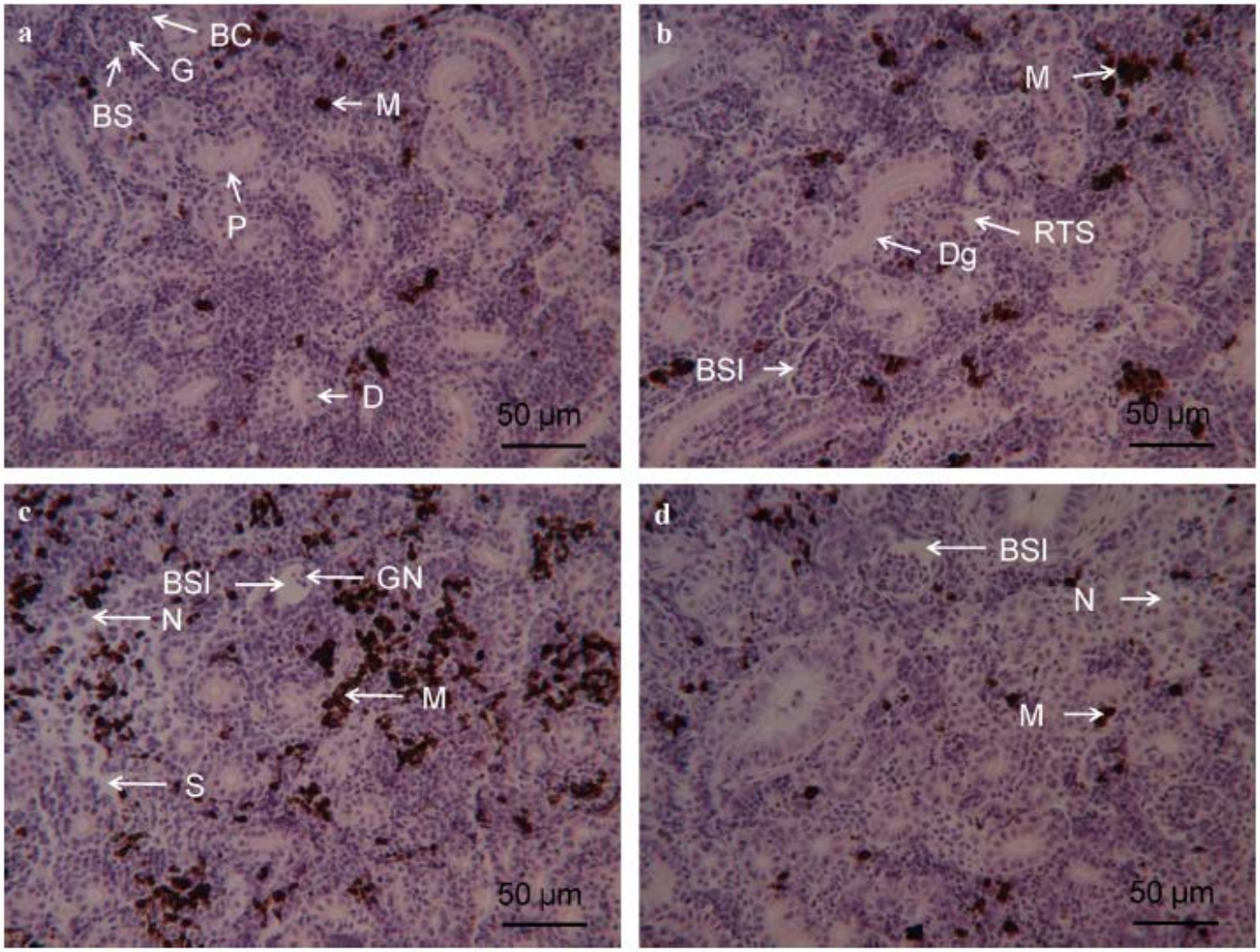
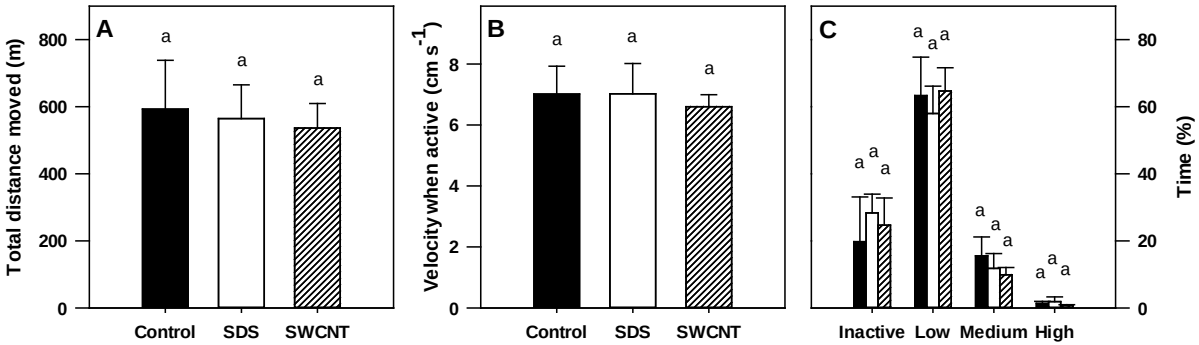


Fig. 4. Kidney morphology in rainbow trout following waterborne exposure to (a) control (b) 20 ug l^{-1} of Cu as $CuSO_4$, (c) 20 ug l^{-1} of Cu as Cu-NPs, and (d) 100 ug l^{-1} of Cu as Cu-NPs for 10 days.

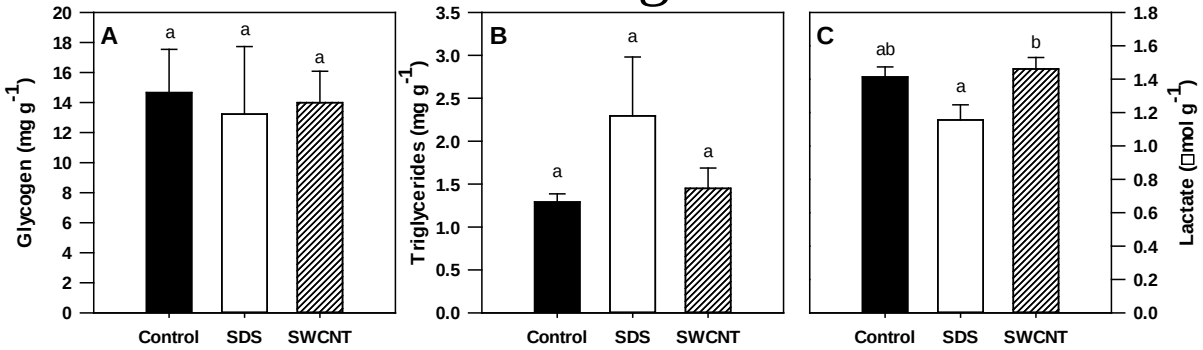
Minimal effects of waterborne exposure to SWCNTs on behaviour of juvenile rainbow trout.

Boyle et al. 2013. Aquatic Toxicology, in press

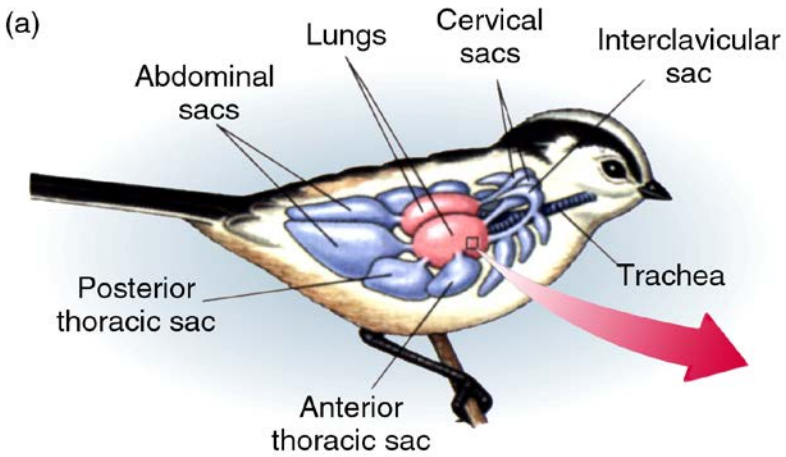
Locomotor Behaviours



Bioenergetics



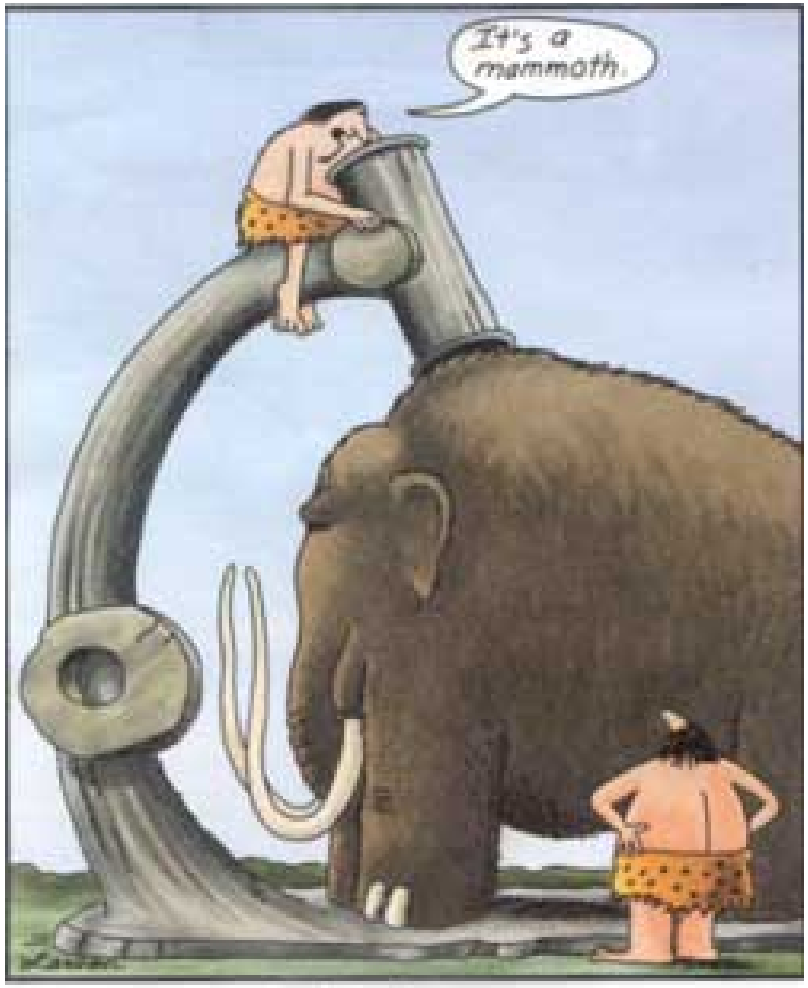
Knowledge Gaps on Species



Conclusions- What's New?

- Soil organisms and ecosystem services
  - Need more work on ecologically-relevant soil microbes.
  - Earthworms: life long sub-lethal pathologies, but with not much genomic or biochemical change.
  - Mesocosm studies: plant biomass and soil functions are altered.
- Aquatic invertebrates
  - Altered development and toxicity to marine larvae/planktonic stages.
  - More understanding of particle behaviour in marine systems.
  - More marine/freshwater mesocosm studies needed.
- Fishes and amphibians
  - Effects on unexposed off spring (Zebrafish)
  - Amphibian data set remains small. UV effects?
  - Animal behaviour: neurotoxicity may or may not translate into an effect locomotor behaviours (consider bioenergetics).
- Lots of data gaps on vertebrate species.

Any Questions?



Early microscopes