Standardized Analysis of Morphology & Toxicology Effects in Freshwater Organisms to Nano-plastic Exposure

SAMTEFONE

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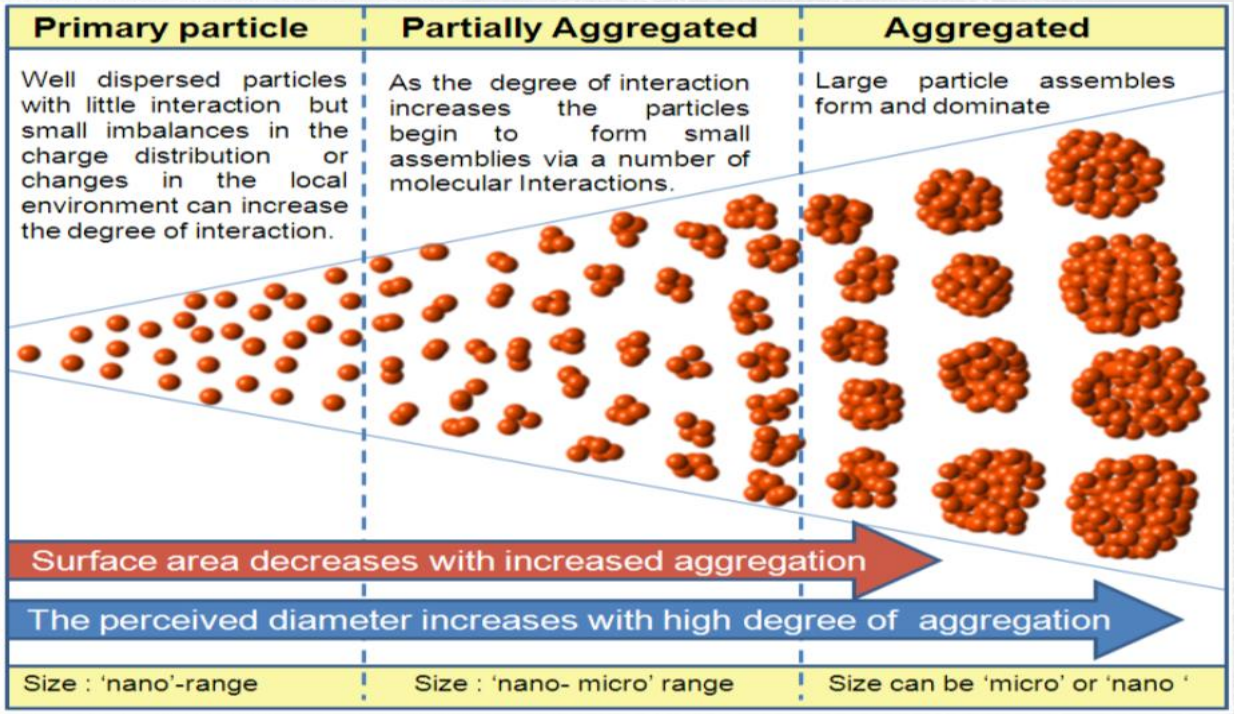
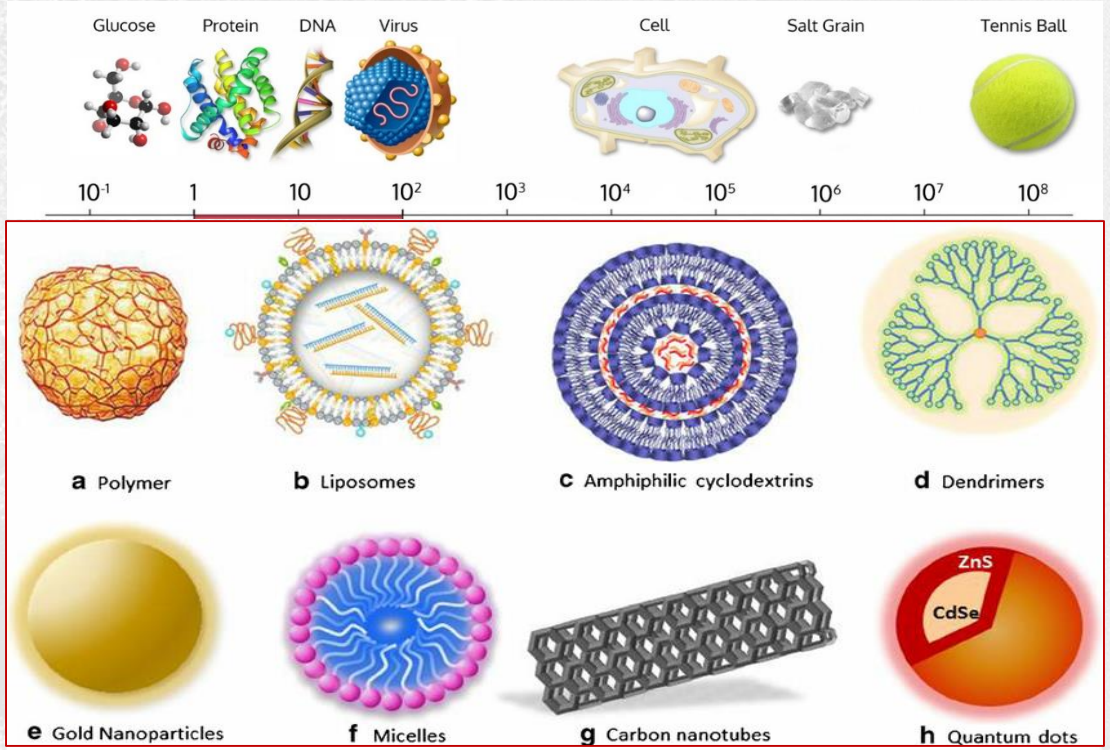
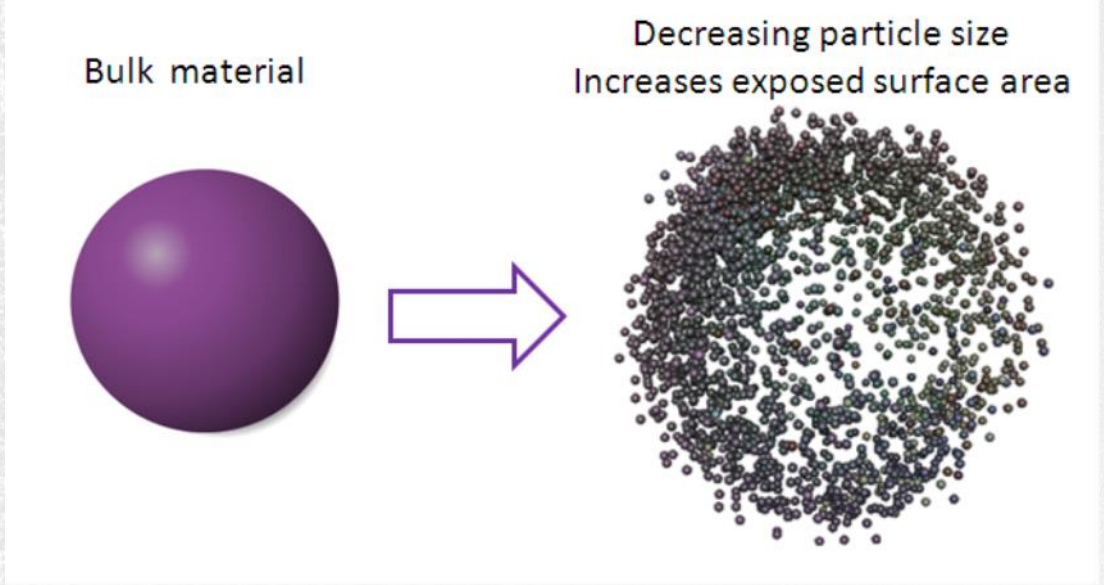
Nano-Ecotoxicology

- Nanoparticle Definition: Any particle created in either isolated or coagulated formats between 1 - 100nm in at least one dimension
- Examines alternate routes of toxicity of nanoparticle compared to bulk chemical in the environment
- Toxicity dependant on structural properties and integrity of the nanoparticles in the environment

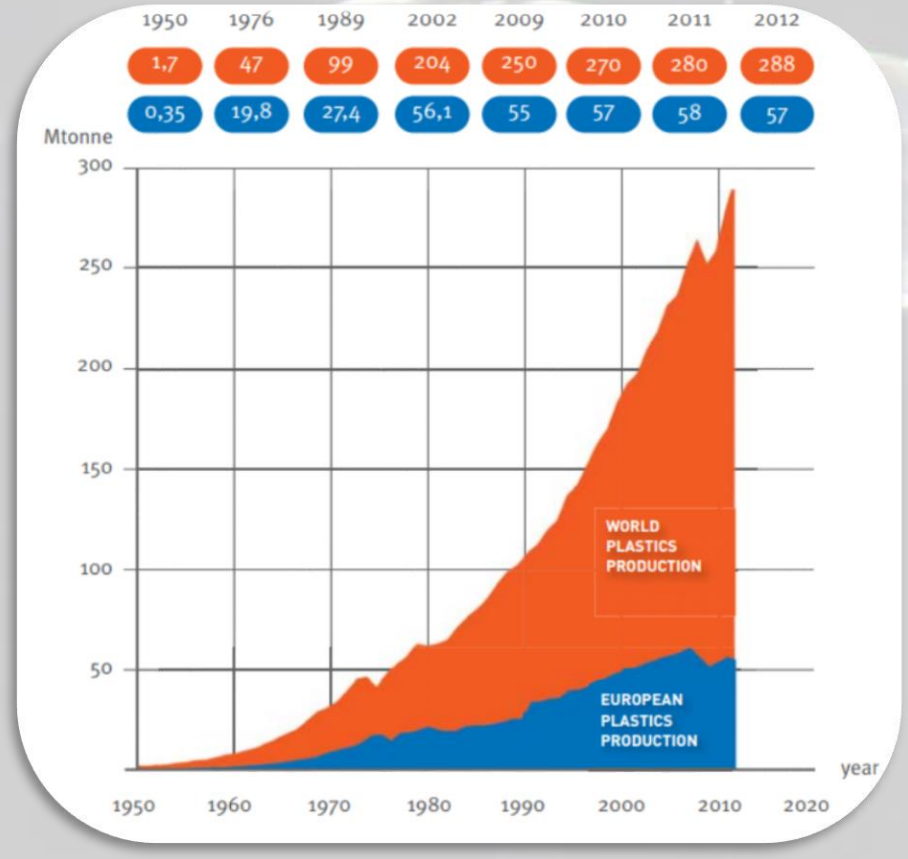


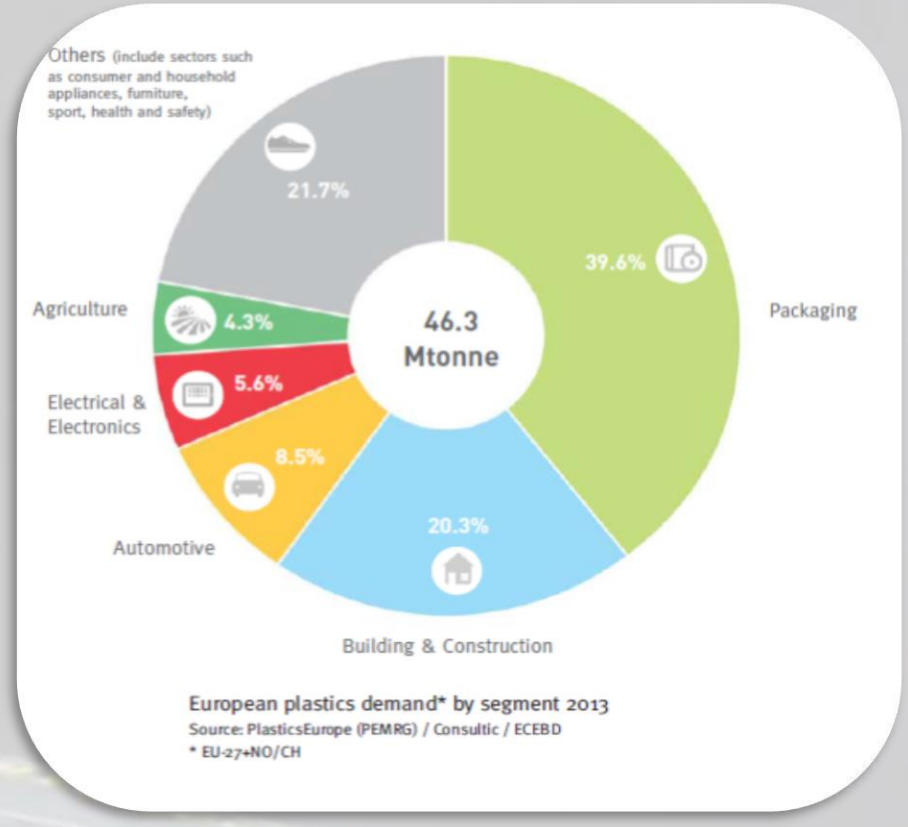




Rise of Plastic Waste:

- Production of plastics rises by ~5% every year since the 1970's
- There is a known pathway of waste of disposed waste through freshwater bodies to the oceans
- There are already large plastic bodies in the ocean's gyres





Primary Nano-plastic

The production from direct industrial usage, such as the creation of plastic-based granulates or pellets found as microbeads in cosmetics, or micro-nano plastics in dissolving dishwasher tablets.

Secondary Nano-plastic

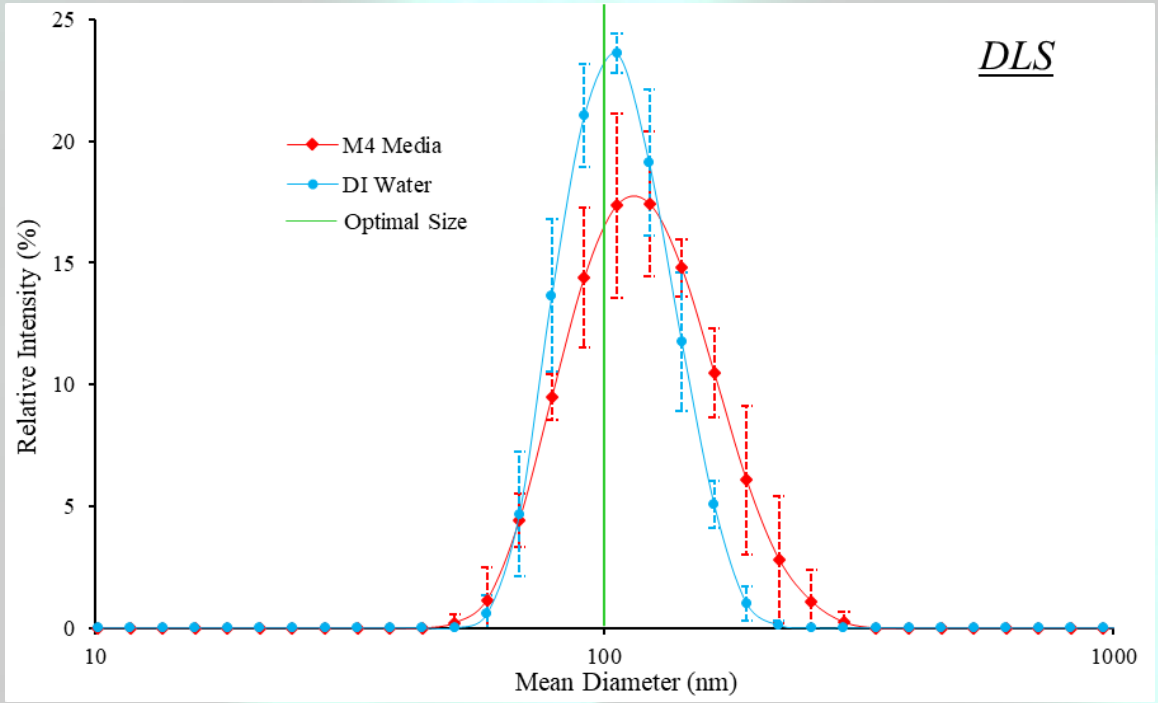
Produced through chemical and physical collapse and degradation actions of bulk plastics (e.g., plastic bags, containers, fishing nets and Styrofoam products).

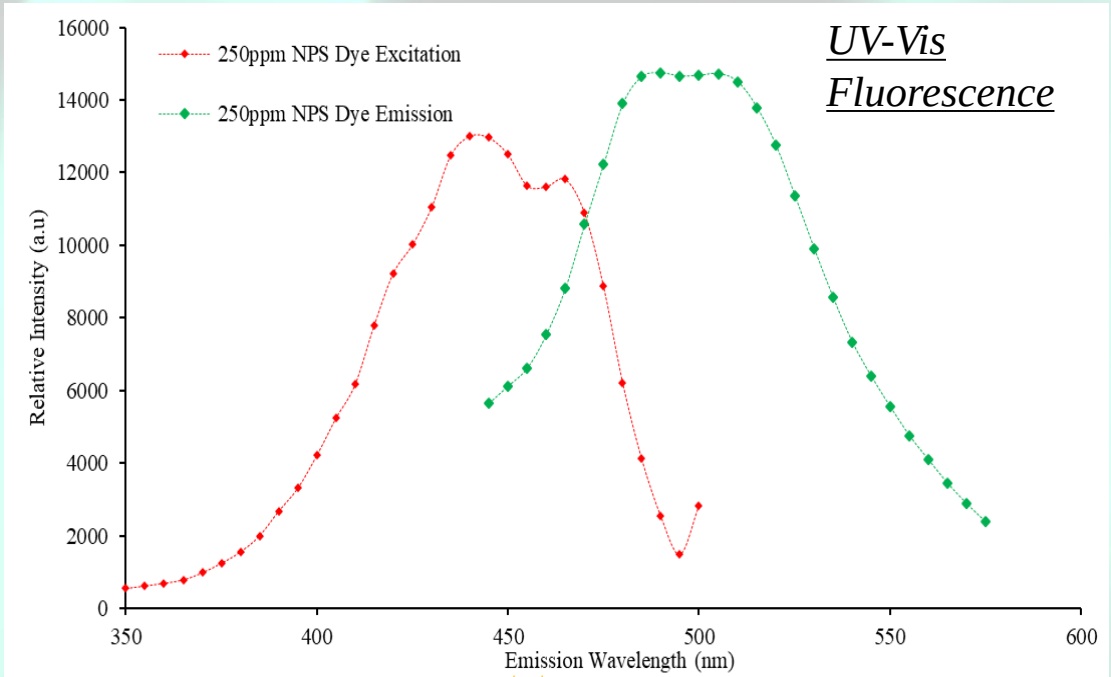
Nano-Polystyrene (NPS)

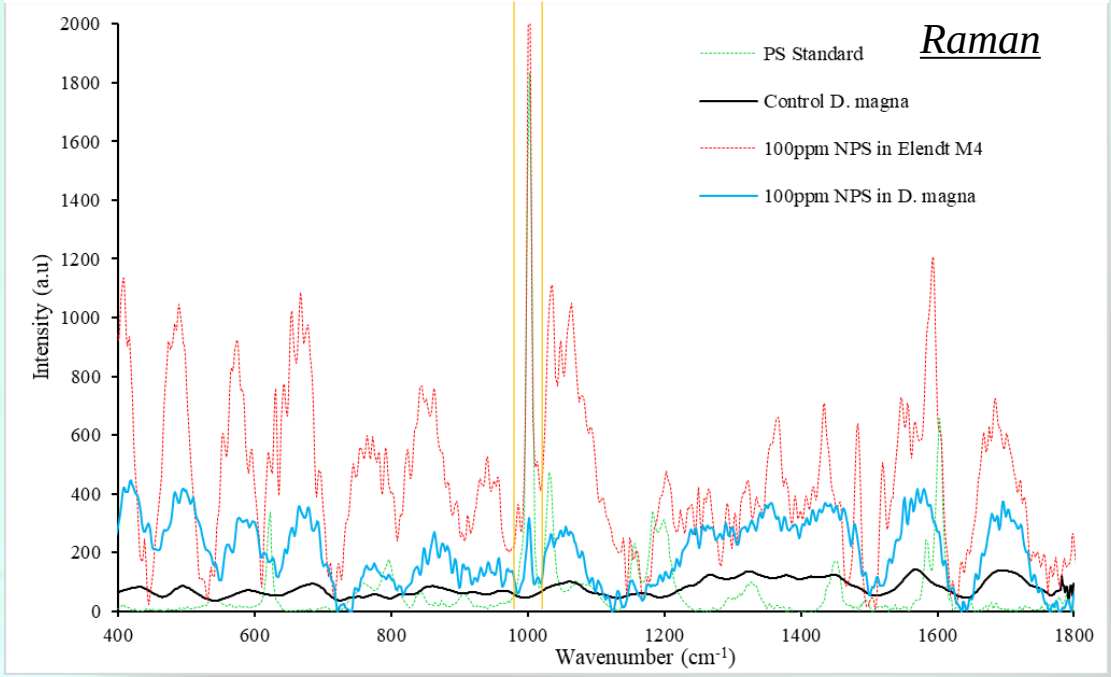
Firefli Polystyrene ~100nm Nano-beads in DI water with fluorescein dye (508nm green) integrated within the styrene chains to avoid dye leaching

Ideal test particle:

- Relatively non-toxic in bulk form
- Good structural integrity and stability in water
- Low size discrepancy range



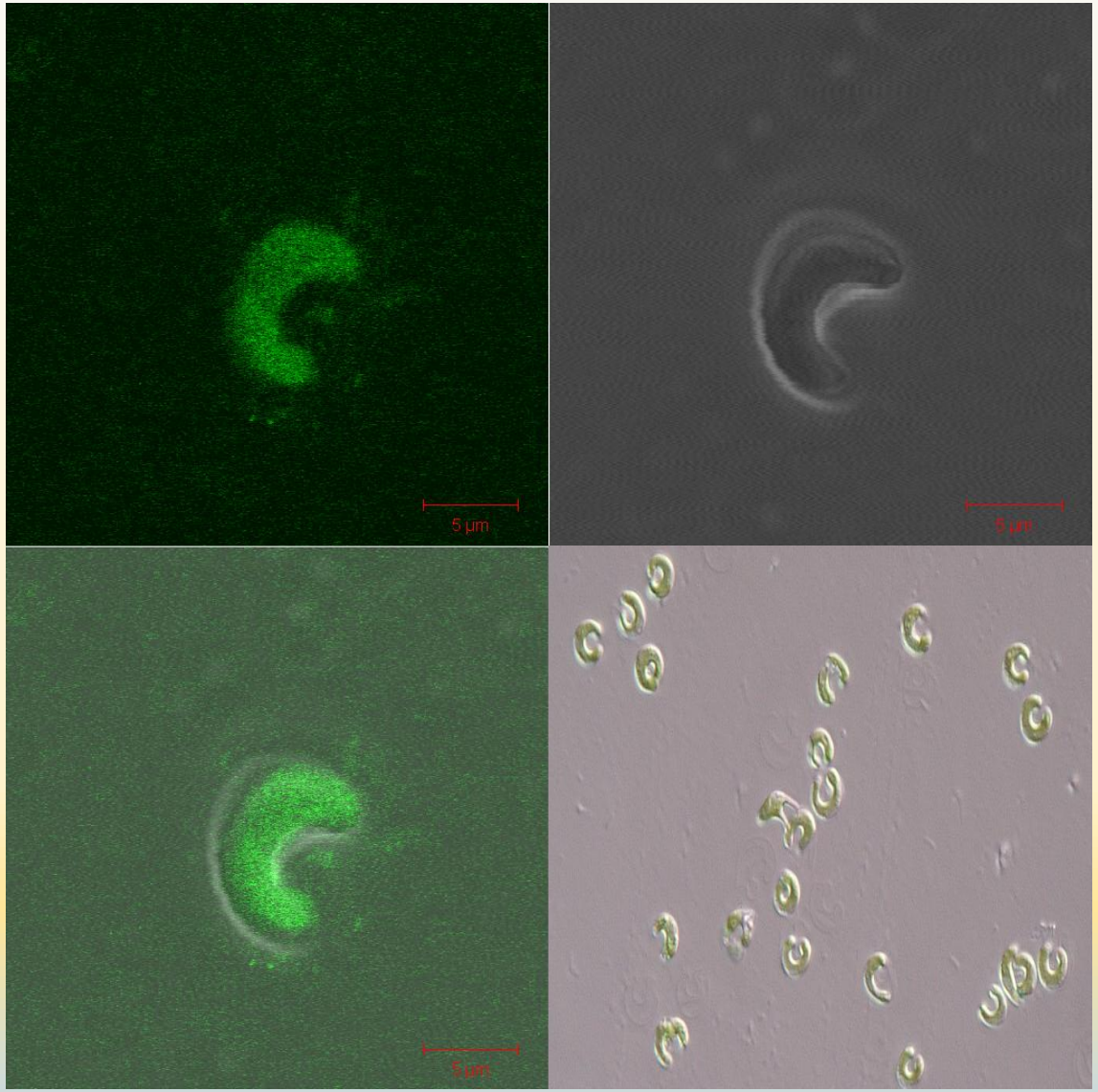




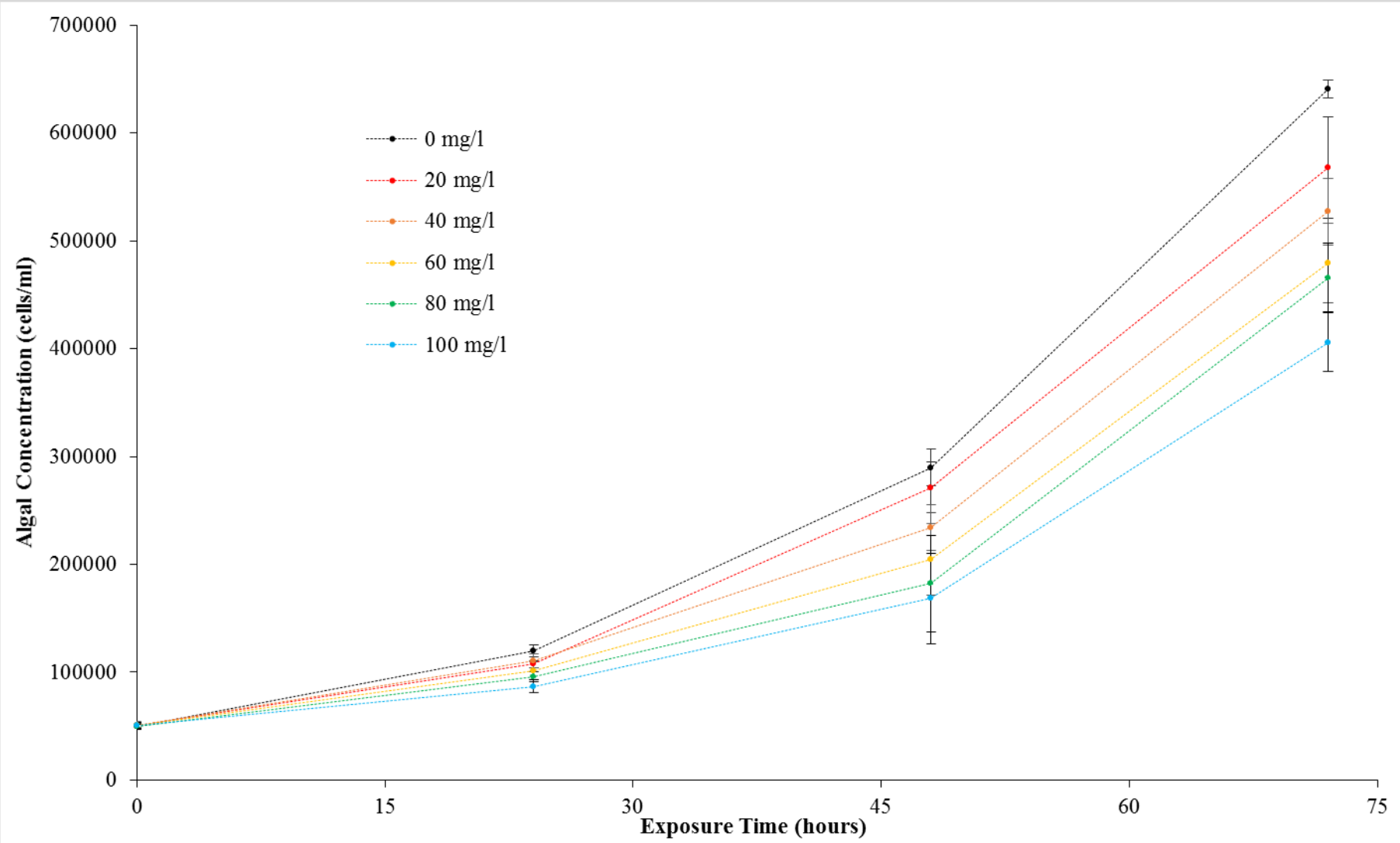
Nano-polystyrene & Algal Interaction Analysis:

Pseudokirchneriella subcapitata

- *Pseudokirchneriella subcapitata (PKS)* are a green freshwater algae, forming a variety of sickle and C-shapes growing from 3-12µm diameter.
- Regularly used in ecotoxicology studies due to their rapid growth rate, easily distinguished under microscopes and commonly avoid agglomeration
- *P. subcapitata* are on the specifically recommended algal cultures advices for toxicology study in OECD No. 201 for algal growth inhibition testing (AGIT).
- A 72-hour NPS concentration range exposure followed OECD guidelines to determine the general affects of both the NPS toxicity and possible side-effects.
- Both the change in cell growth (cells/ml) and the overall physical appearance of the *P. subcapitata* was analysed.



Algal NPS Exposure Toxicological Response

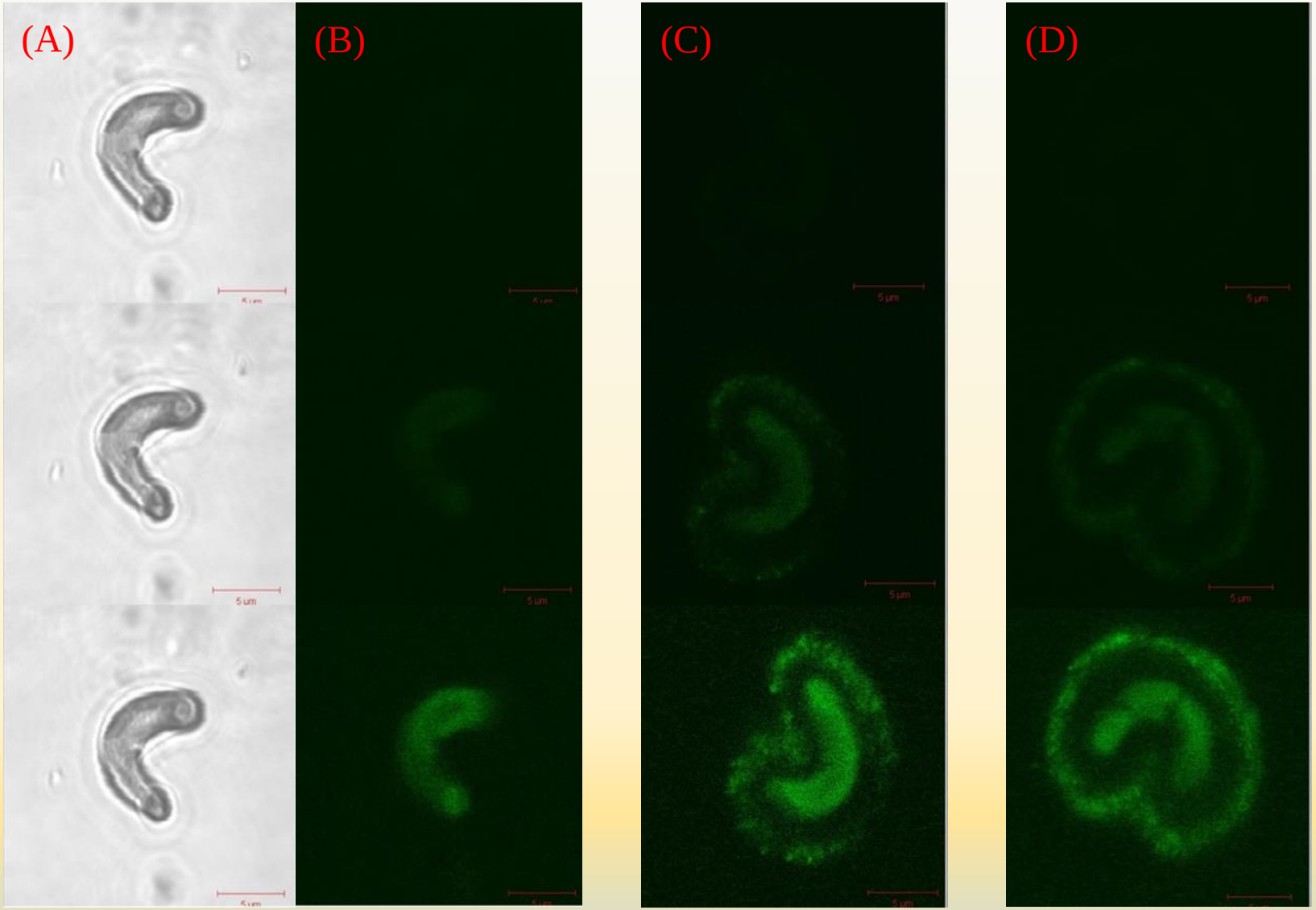


The initial 24 hours showed some growth impact from any samples, only up to 27.6% rate reduction by 100mg/l NPS.

By the 48 hour check, NPS had lightly aggregated. The growth rates of all NPS exposed samples further fell, with a 42% drop by 100mg/l NPS.

By the 72 hour check, the NPS was more heavily clumped. However the rate loss remains relatively constant. For example the 20mg/l NPS exposed samples had an 11% reduction in overall growth rate at 48 & 72 hour exposure.

Algal NPS Exposure Confocal Imaging



Confocal Imaging for the *Pseudokirchnirella* based on NPS exposure concentrations.

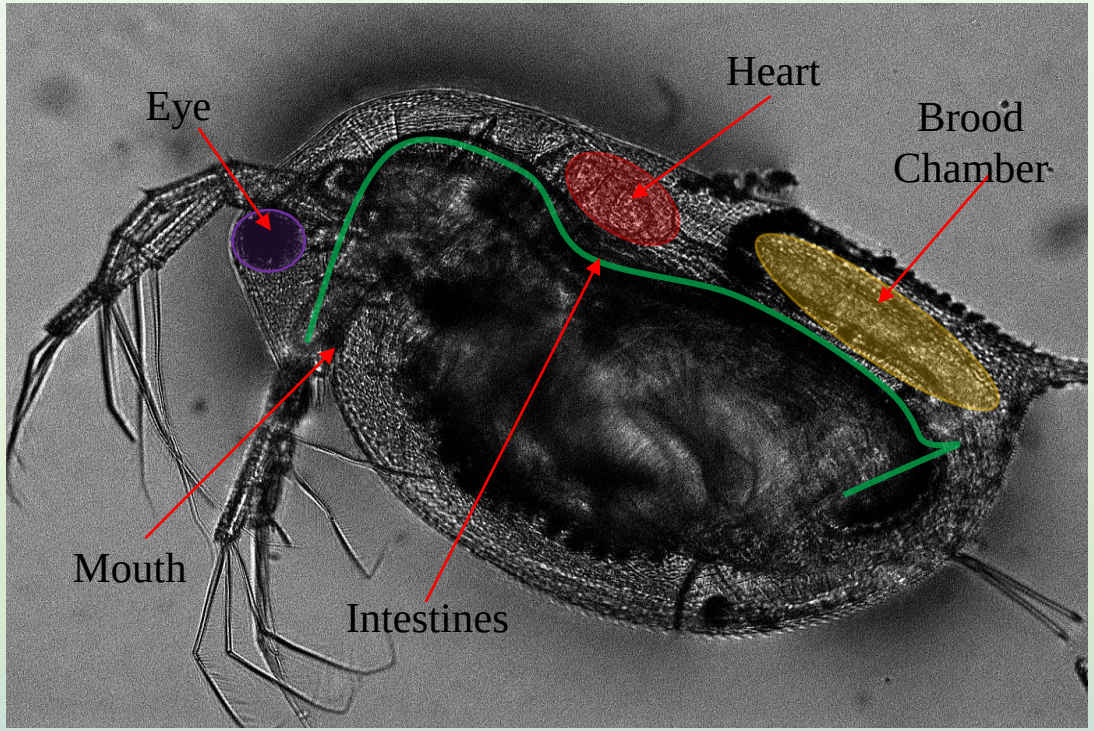
Image set (A) show white light example of the algae, with scale bar of 5µm.

Image sets (B), (C) & (D) show 458nm/513nm Ex/Em fluorescence at increasing Gain (Sensitivity) of 600, 800 & 1000.

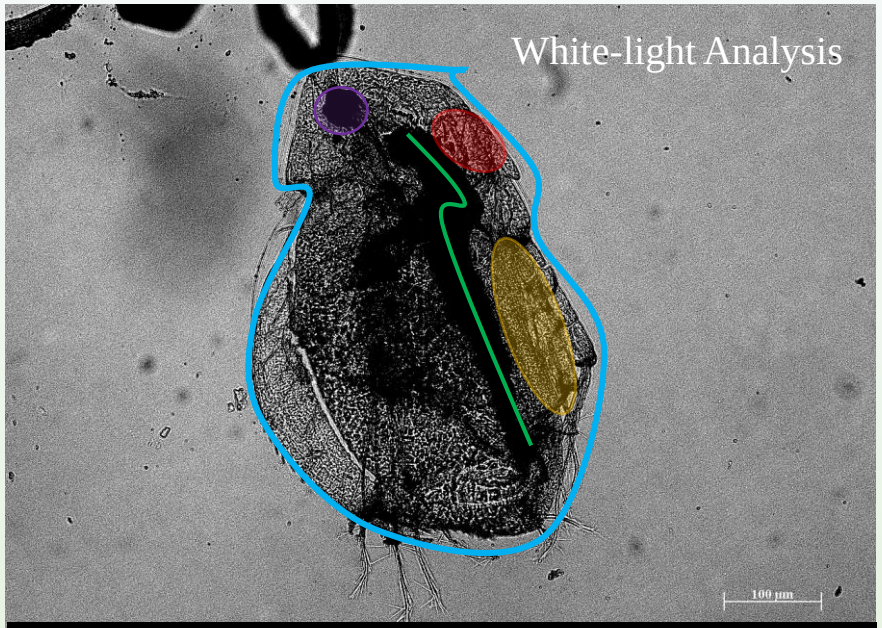
- Image Set (B) represents control PKS samples
- Image Set (C) represents PKS exposed to 20mg/l NPS
- Image Set (D) represents PKS exposed to 40mg/l NPS

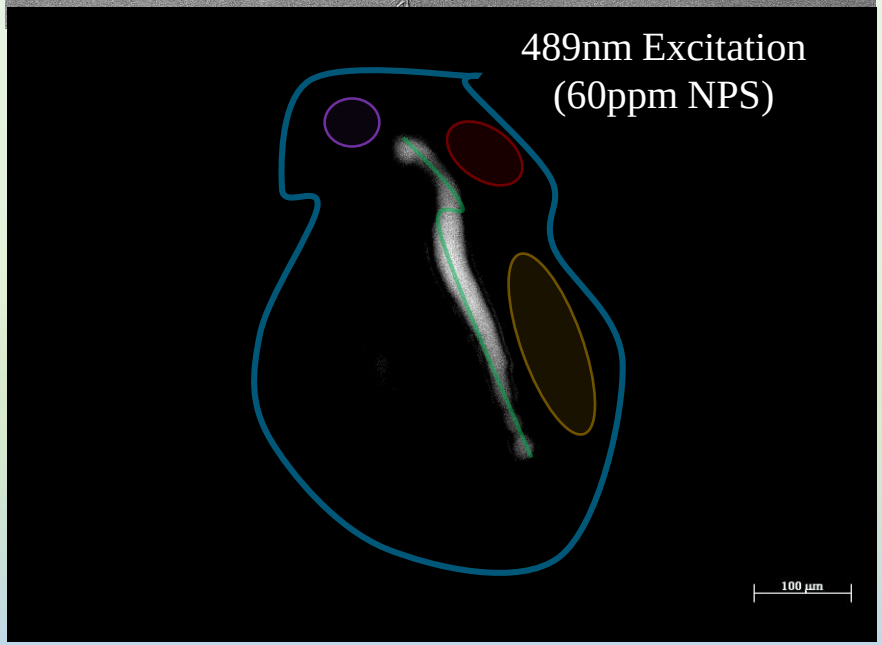
Mobile Primary Consumer: *Daphnia magna*

- Mobile filtering crustacean growing up to 5mm in length found above the north of the equator. Reproduce primarily from cyclic parthenogenesis, “male” *D. magna* can be produced to cope with environmental changes.
- Initial NPS exposure over 24 hours was analysed under epi-fluorescence imaging to determine NPS location within *D. magna* for further analysis
- Clear presence of NPS within *D. magna* intestines, but little or no discernible signal found in heart, eye or brood chamber



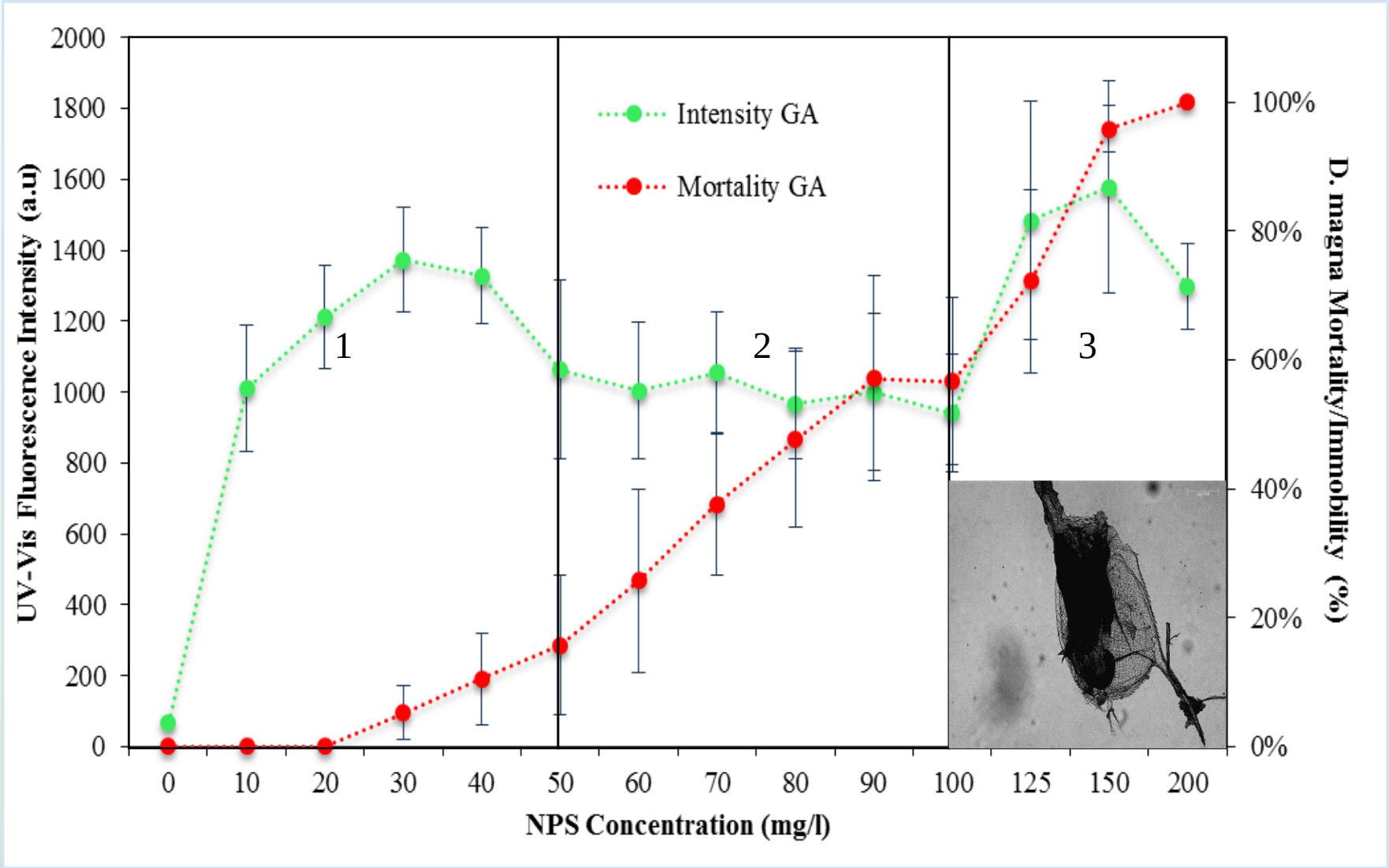
Daphnia magna - Light Microscope (3.7ms)





D. magna Toxicity/ Emission Analysis:

- Toxicity testing run with NPS to OECD Testing Chemical Guidelines No. 202 for Immobility/Mortality
- Data compared with UV-Vis fluorimetry emission to the same concentration of NPS
- Scans occurred on 12 *Daphnia magna* with a triplicate run
- Results appeared to indicate three phases of response:
    1. Detrimental Phase – Low MI responses from neonates to NPS exposure
    2. Hazardous Phase – Rapid rise in MI effects and reduced activity from neonates
    3. False Positive Phase – Excess NPS aggregate blocked accurate reading

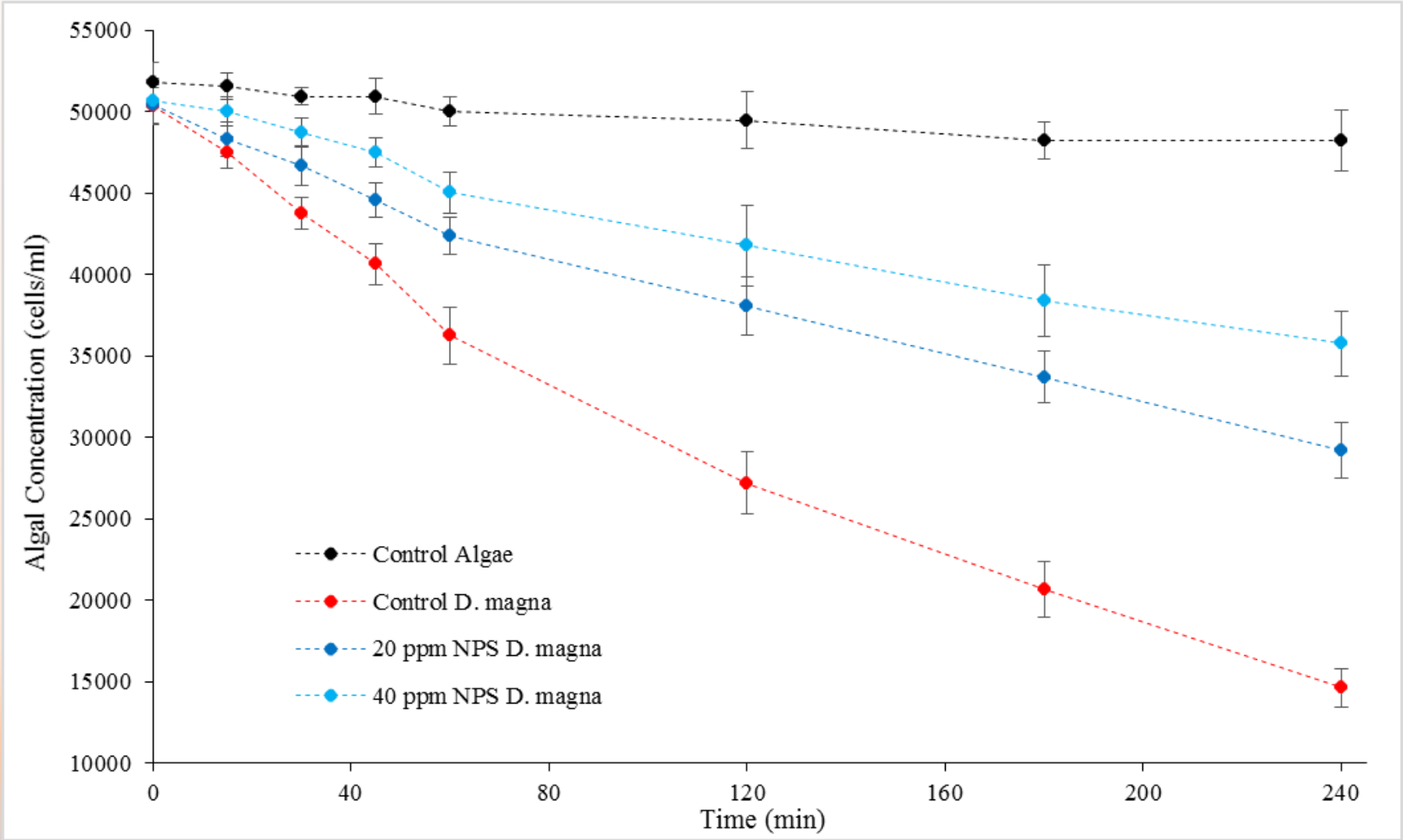


D. magna Consumption Rate Analysis:

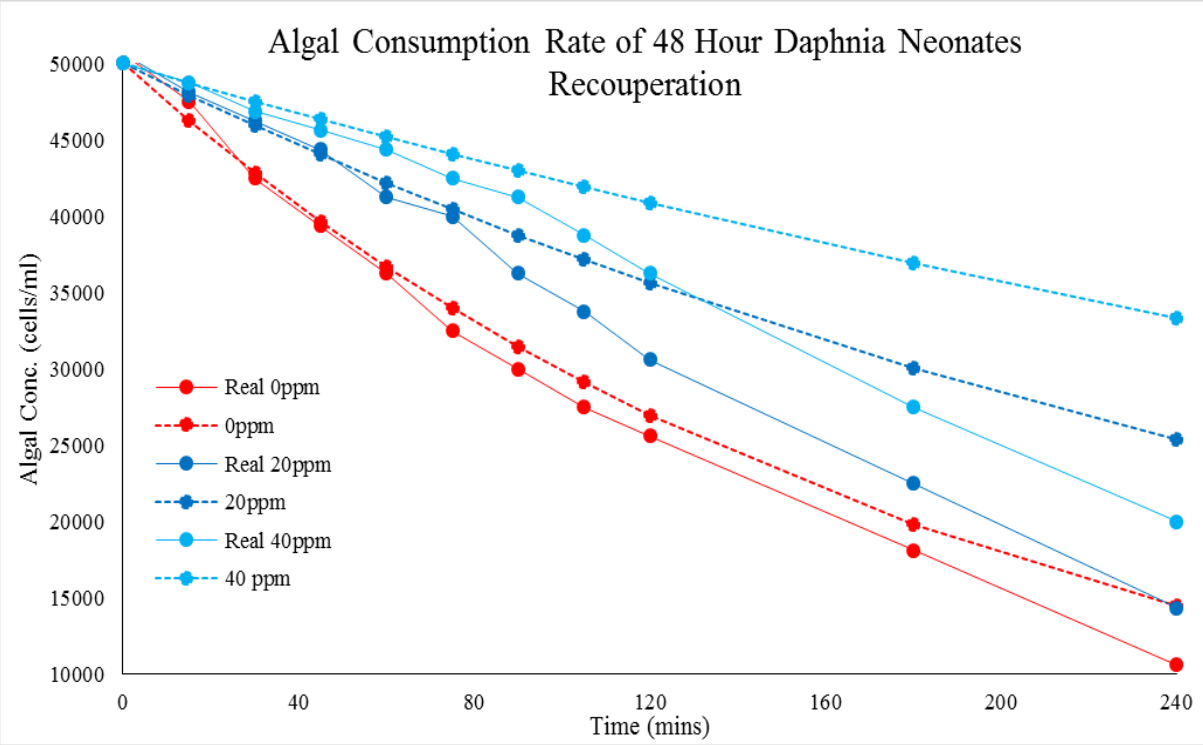
- Four runs on D. magna neonates using 3 NPS concentrations & 1 control
- 12 *Daphnia magna* per concentration
- Samples run over 4 hours using ~50’000 cells/ml *Chlorella* algae and checked using haemocytometer
- Calculated using rate equation:

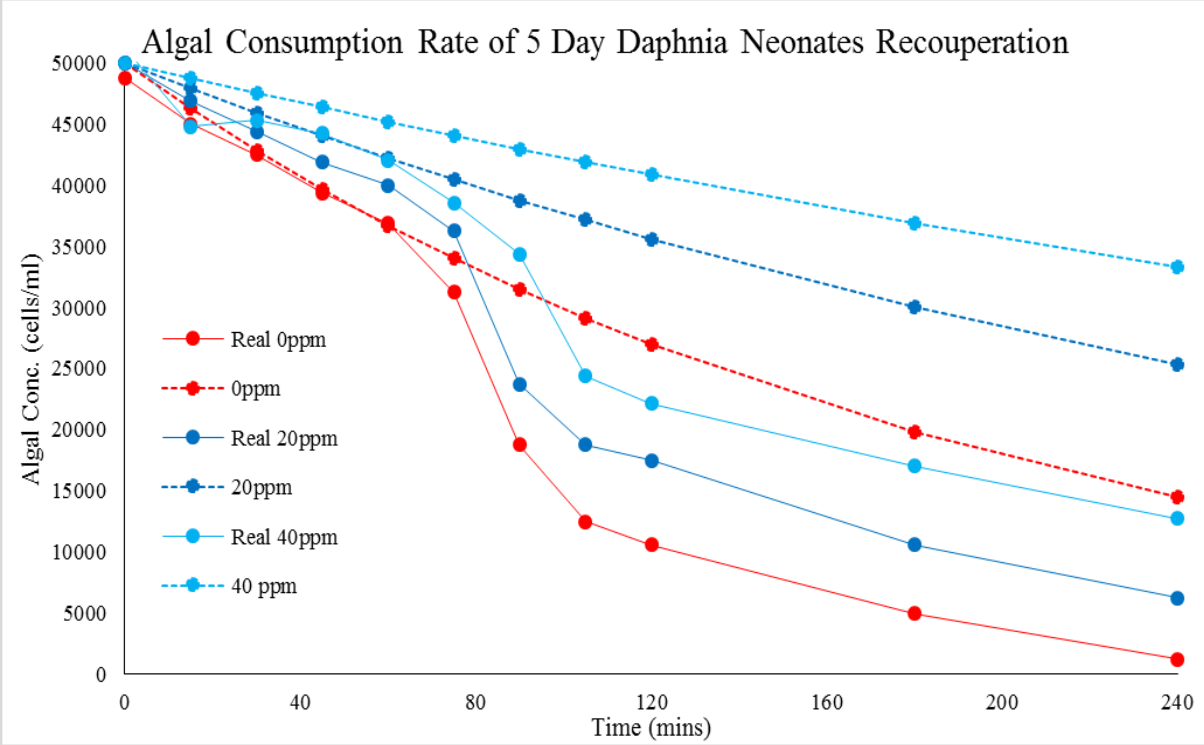
  $dA/dt = A \times \exp(R \times T)$
- Rates Constant Calculated in two divisions:

  1st Hour & 2-4 Hours



D. magna Recuperation Analysis:

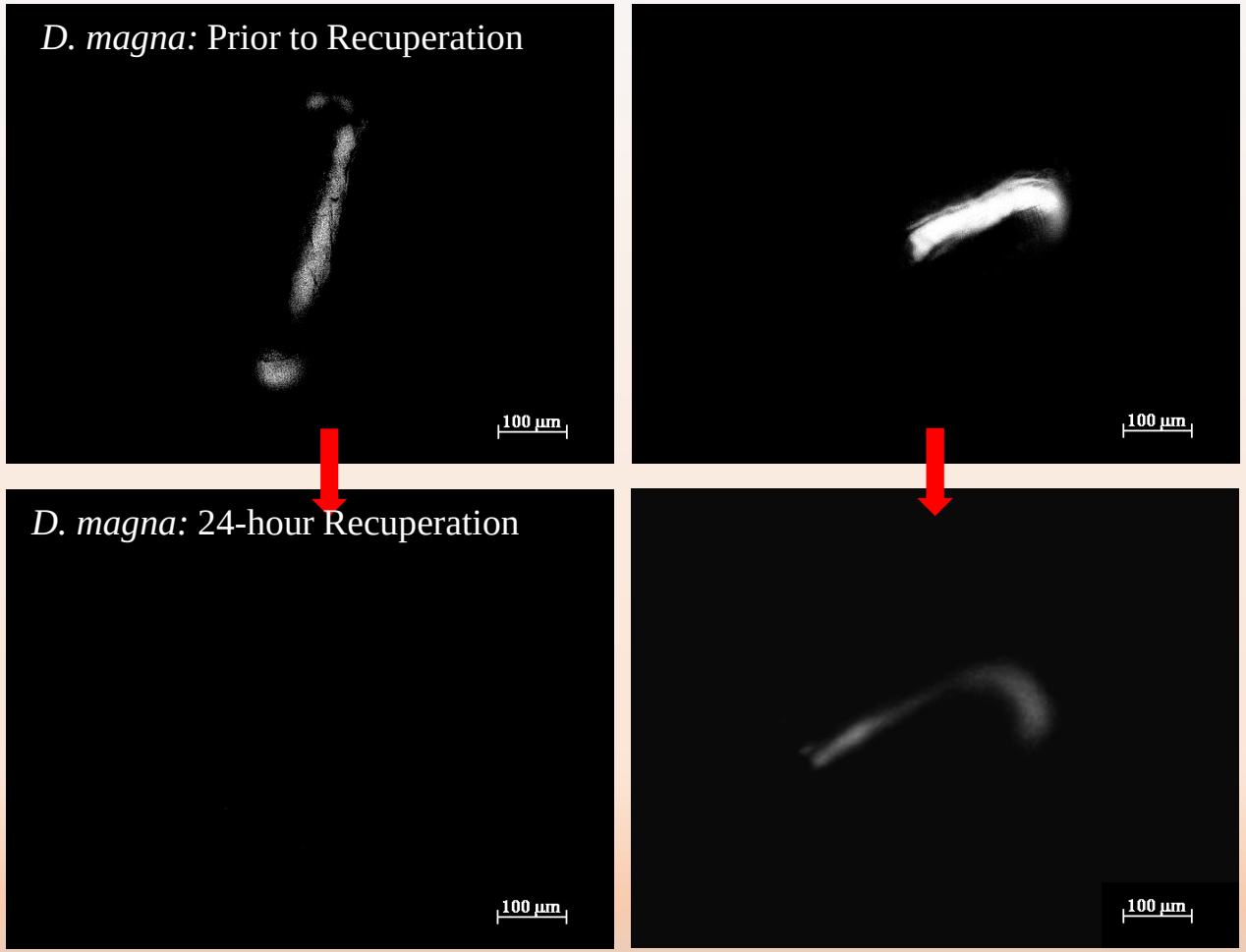




- Examination of the effects of leaving Daphnia in “refreshed” water to simulate the clearing of contamination.
- Determine whether low level concentration exposures can be resolved if conditions in water systems improve.
- Samples checked over 24 hours & 96 hours extra in refreshed M4 media & algae, before being cleaned out and re-examined for consumption rates.
- Samples compared to existing consumption rate models for comparison.

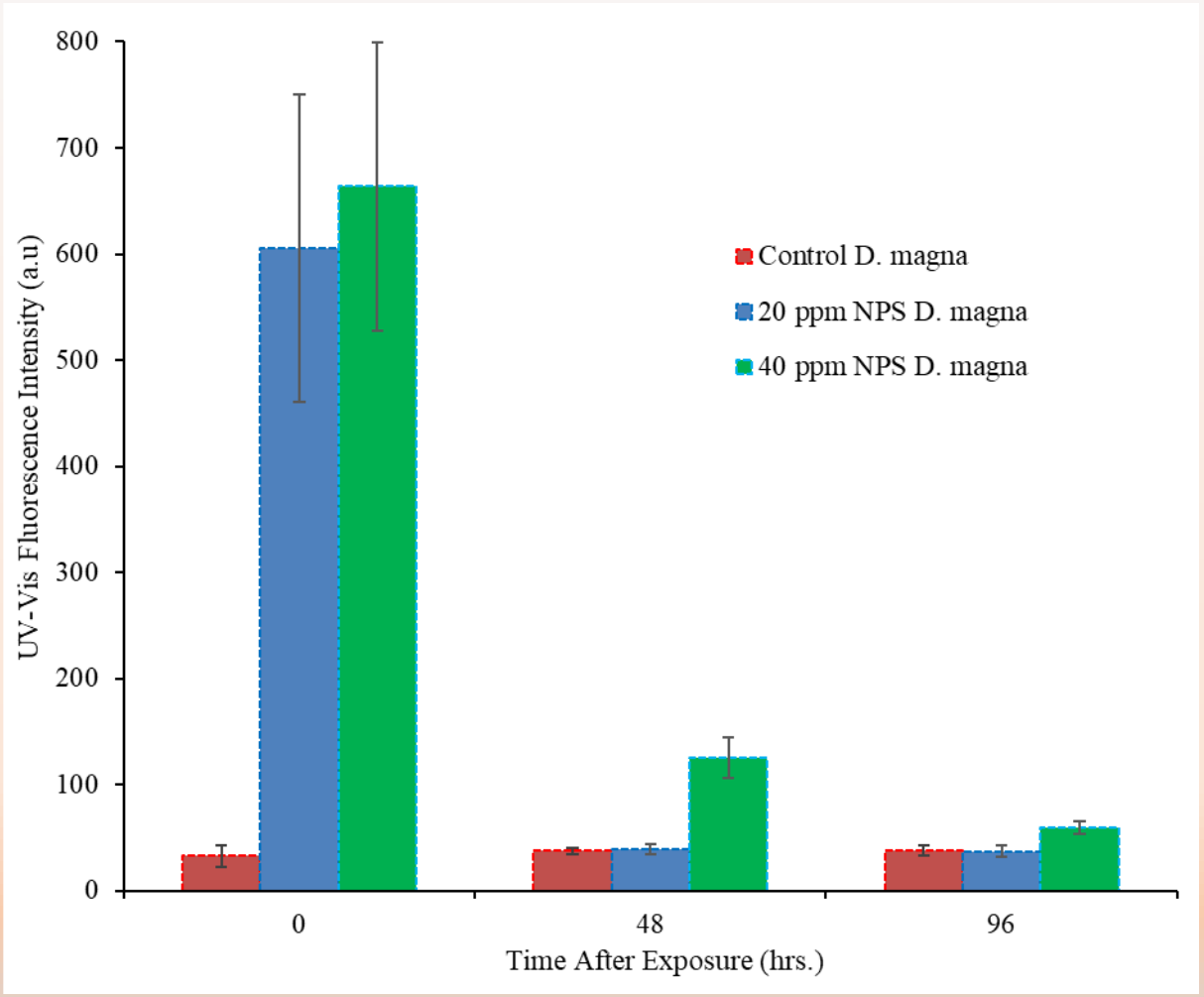
D. magna Fluorescent Imaging Analysis:

Fluorescent Imaging of NPS Exposed *D. magna* with 24-hours Recuperation

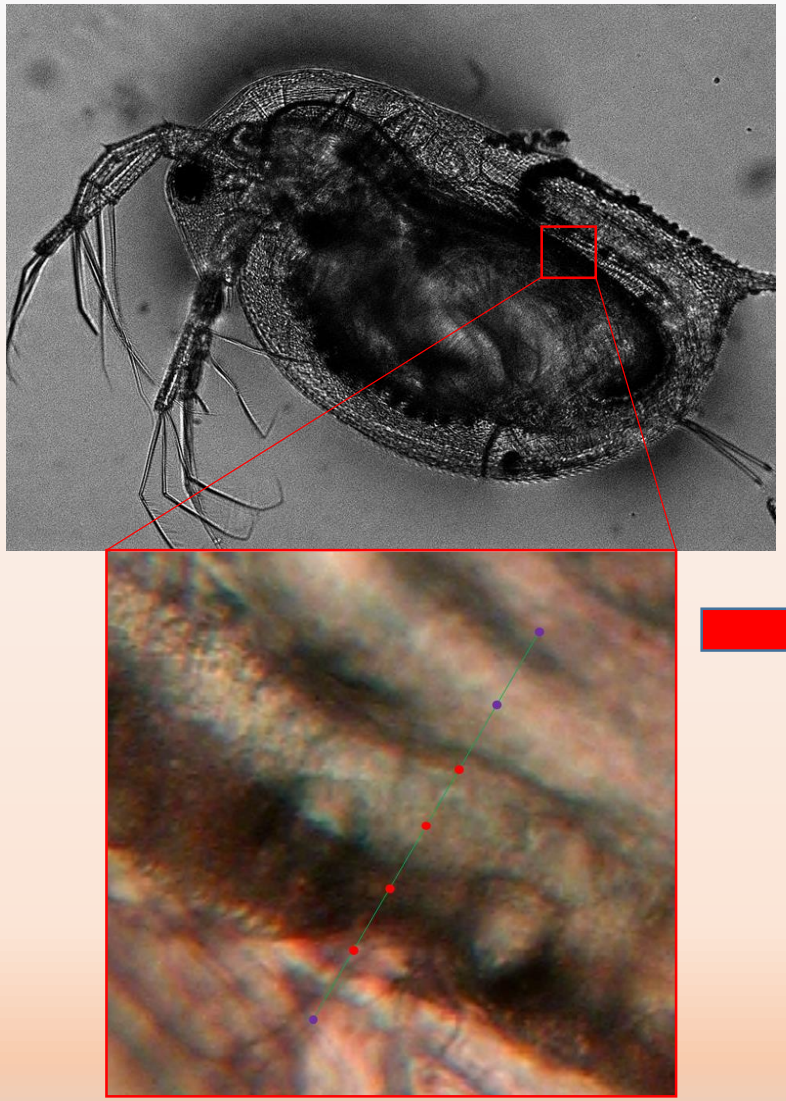


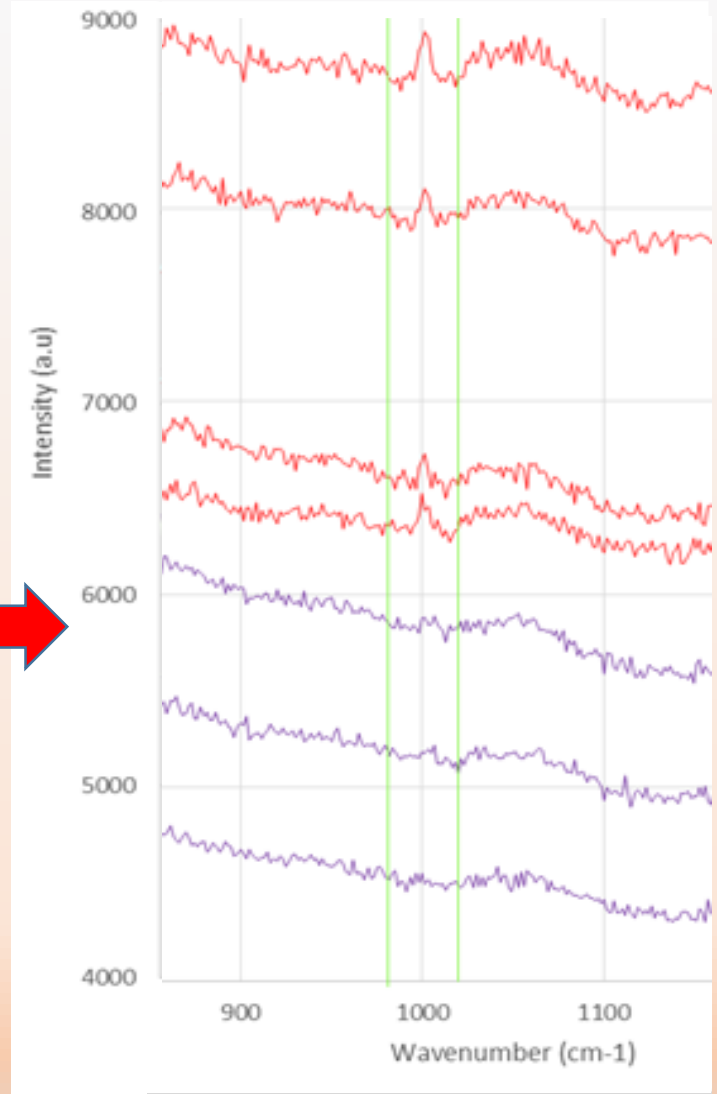
20 mg/l NPS Exposed *D. magna*

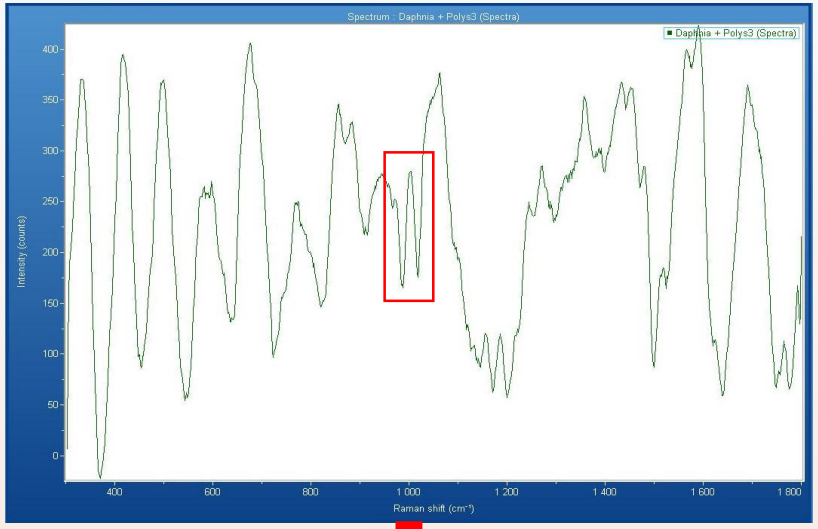
40 mg/l NPS Exposed *D. magna*

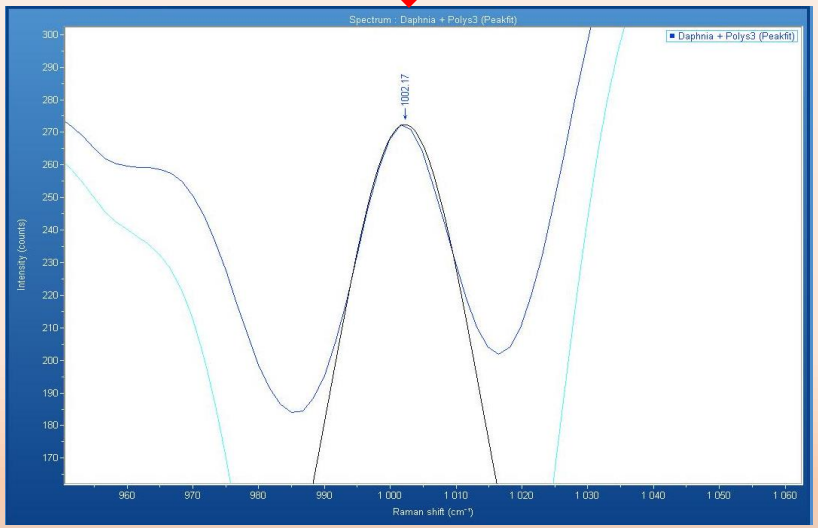


D. Magna & NPS Raman Analysis:







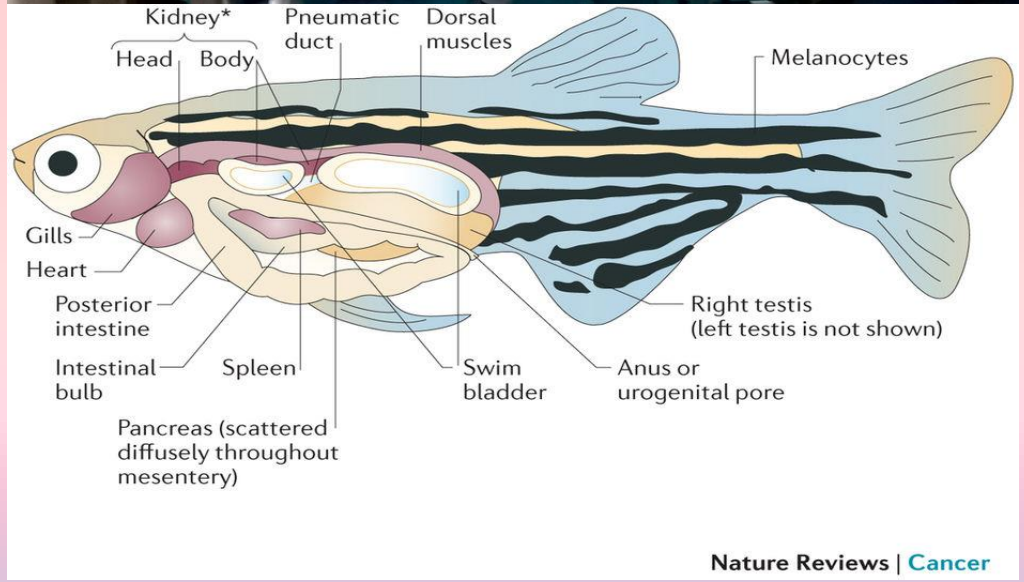


Raman spectra analysis of 40mg/l NPS *Daphnia magna* samples with N-Polystyrene produced distinct peaks at ~1001cm^{-1}

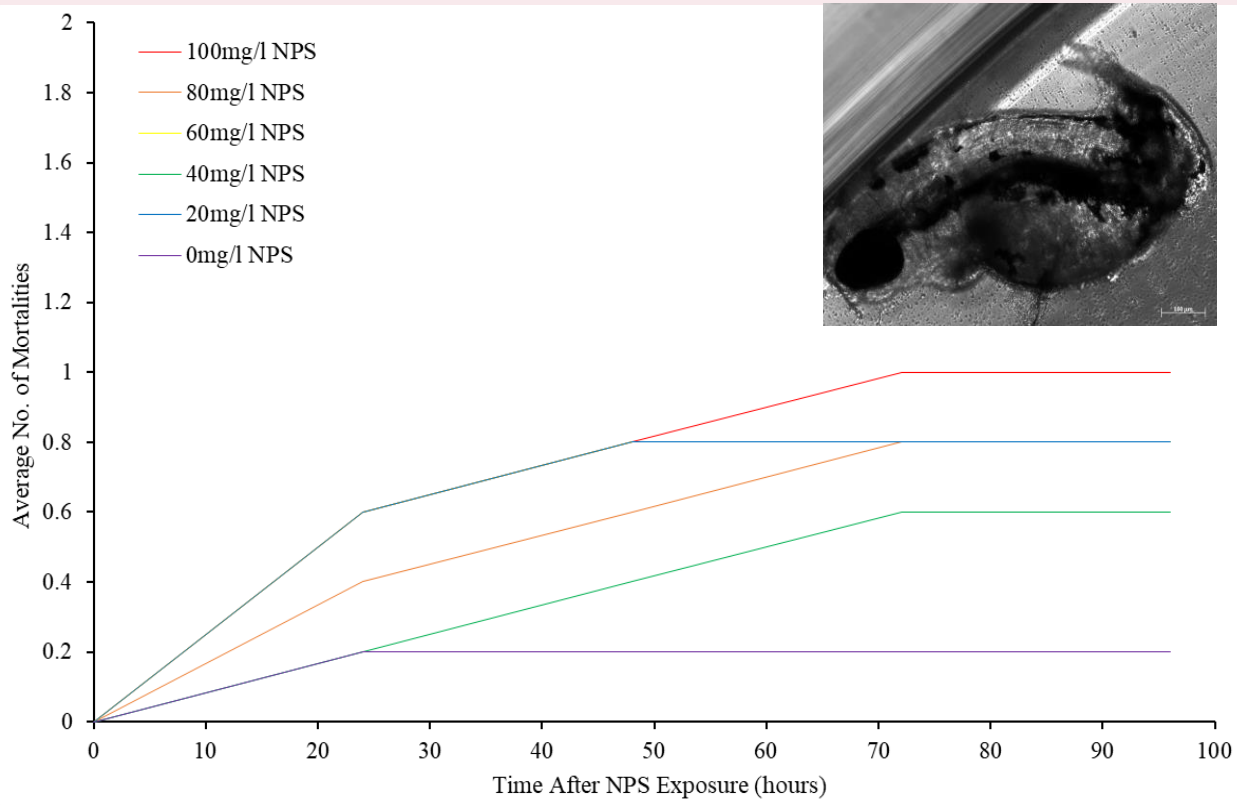
Embryo Phase Secondary Consumer:

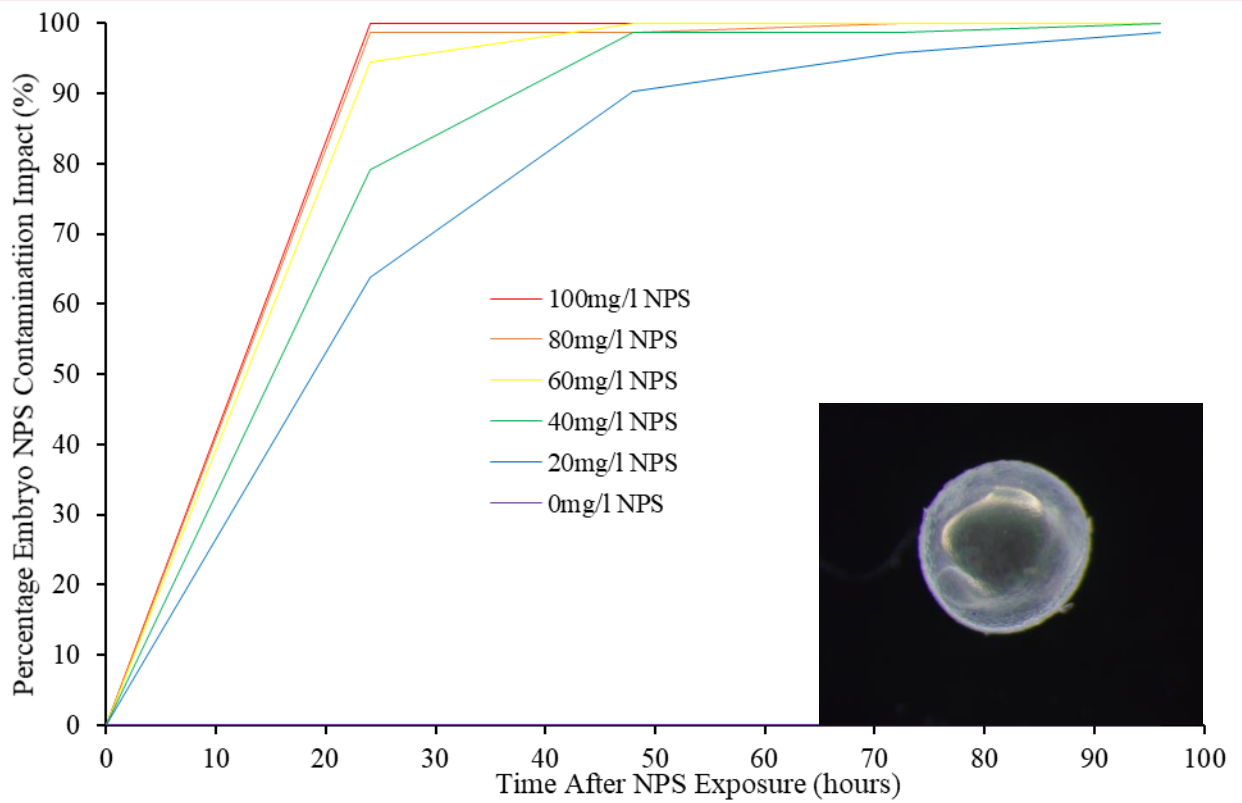
Danio rerio (Zebrafish)

- *Danio rerio* are small secondary consumer fish growing up to 5cm in length originating from India
- Analytically significant, as they reproduced readily and produce relatively transparent chorion protected embryos. The *D. rerio* embryo also commonly respond to stresses by physiological alteration in development
- *D. rerio* already have numerous scientific studies for toxicology and genetic manipulation, including OECD No. 236 for fish embryo toxicity (FET). This protocol will be utilized for a 72 hour NPS exposure to primarily observe nay morphological impact.
- After the 72 hour NPS exposure testing is complete, embryos can be preserved and run under UV-Vis fluorimetry and Raman spectroscopy. This will determine permeability and impact location of any NPS to breach the chorion



Zebrafish Toxicity & Morphology Responses





Zebrafish Mortalities (graph left) were examined by checking if specific physical deformation occurred such as a lack of heartbeat, however non-lethal NPS contamination changes (graph right) were also accounted for

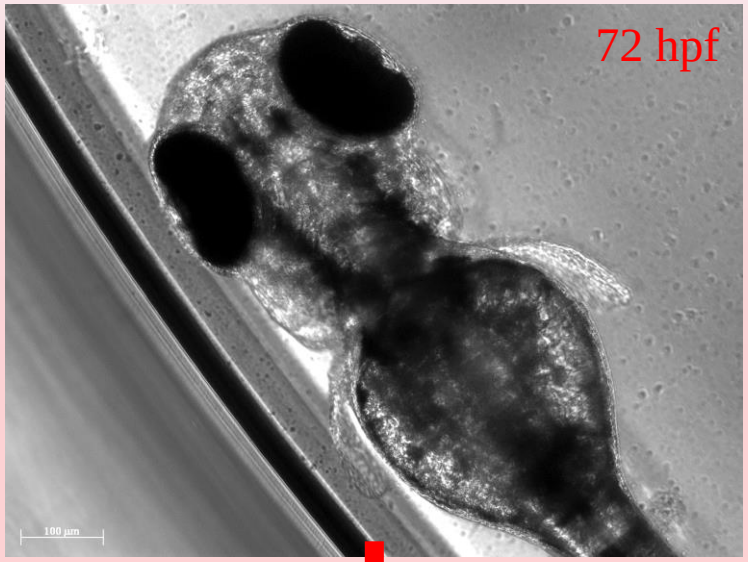
Test run on a total of 60 Zebrafish embryos per NPS exposure concentration (300 total tested)

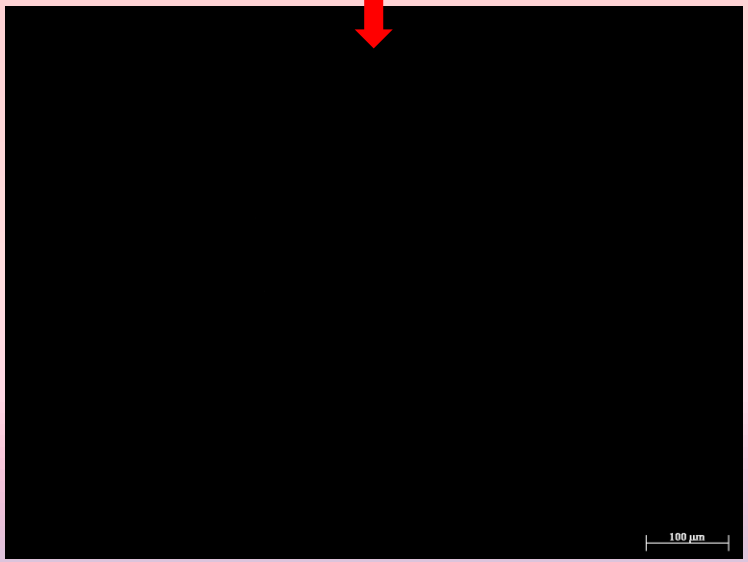
Control Zebrafish embryos showed no significant mortalities, NPS exposed samples suffered mild rise in mortality as NPS concentrations rose. NPC contamination issues, however, rose significantly at all NPS exposure concentration

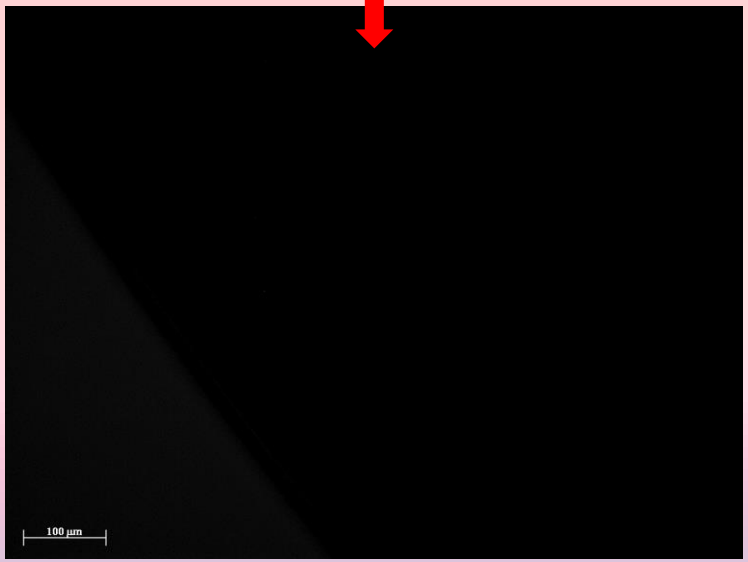
Zebrafish Embryo Epi-fluorescence Images

White Light Imaging

Fluorescence Imaging





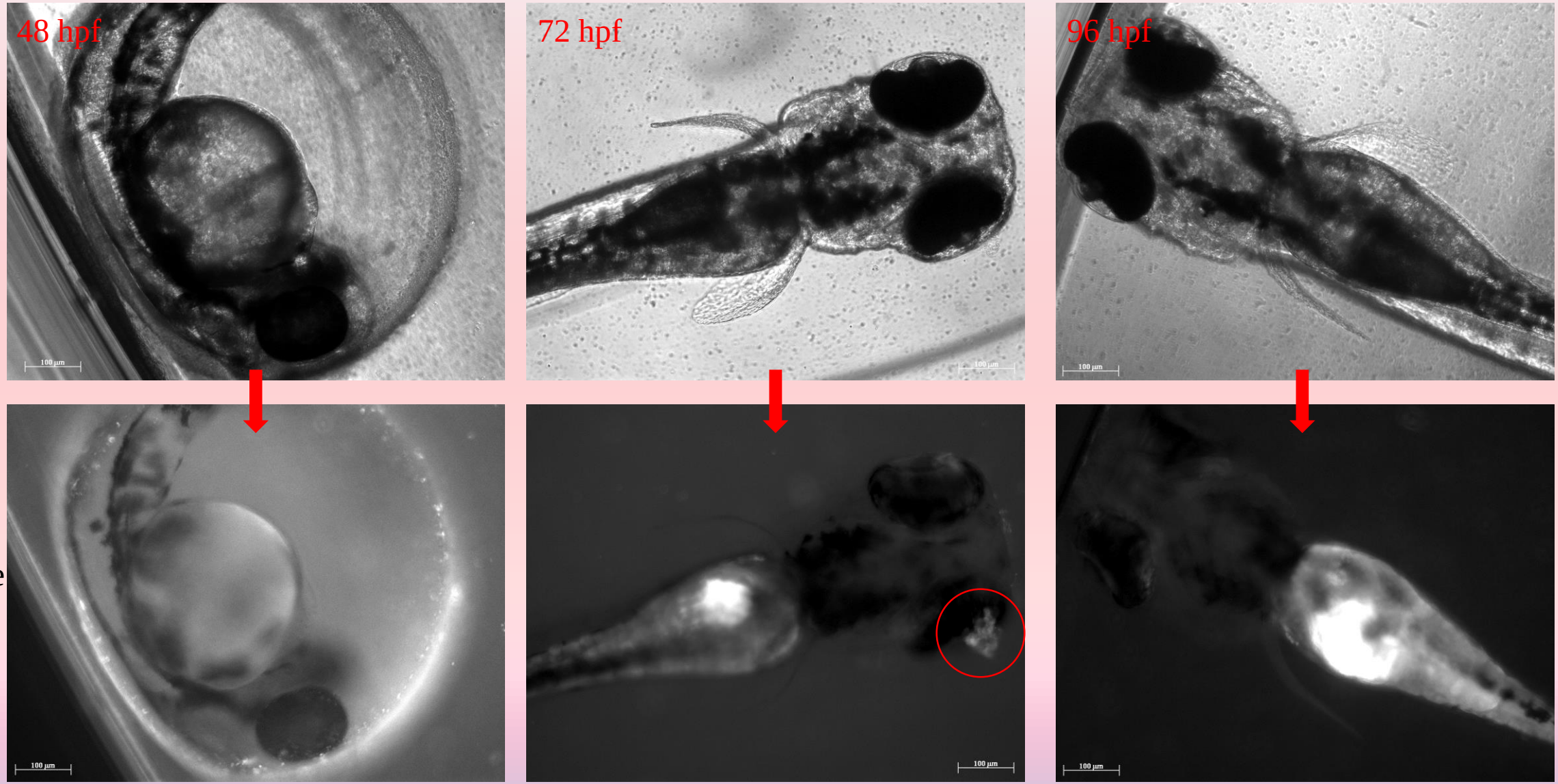


- Epi-fluorescence Imaging run at 489nm excitation and analysing above 505nm emission
- Zebrafish embryos showed a distinct body structure and development over 96 hours
- Control embryos (Images left) showed no signs of fluorescence at any point during development
- Zebrafish embryos exposed to both 20mg/l & 40mg/l (next slide) displayed clear fluorescence emissions within intestinal regions
- Further fluorescence could be seen from aggregate NPS on the chorion and later the eyes of the zebrafish

NPS (40mg/l) Exposed Zebrafish Fluorescence Images

White Light Imaging

Fluorescence Imaging



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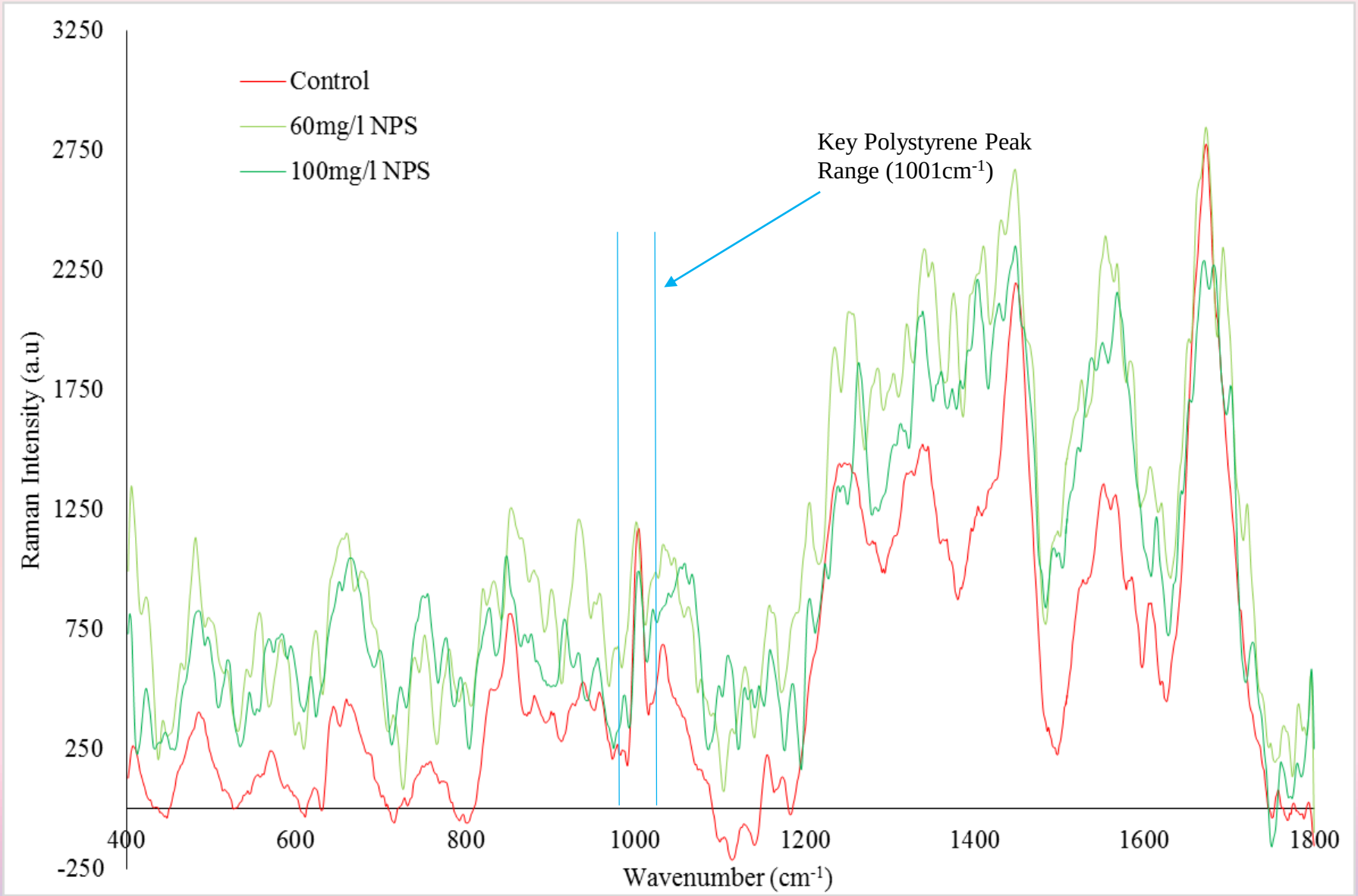
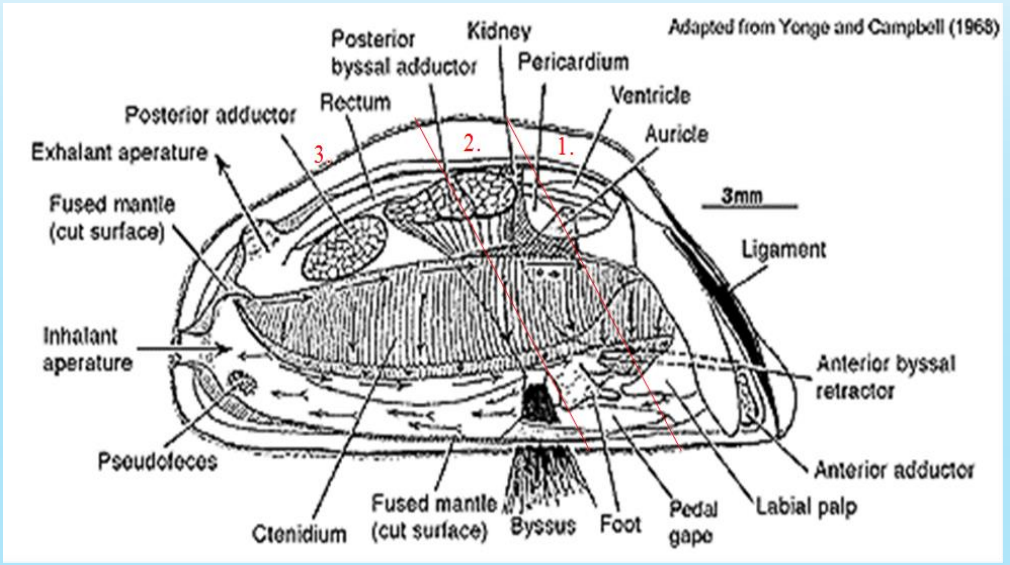
NPS Exposed Zebrafish Epi-fluorescence Images

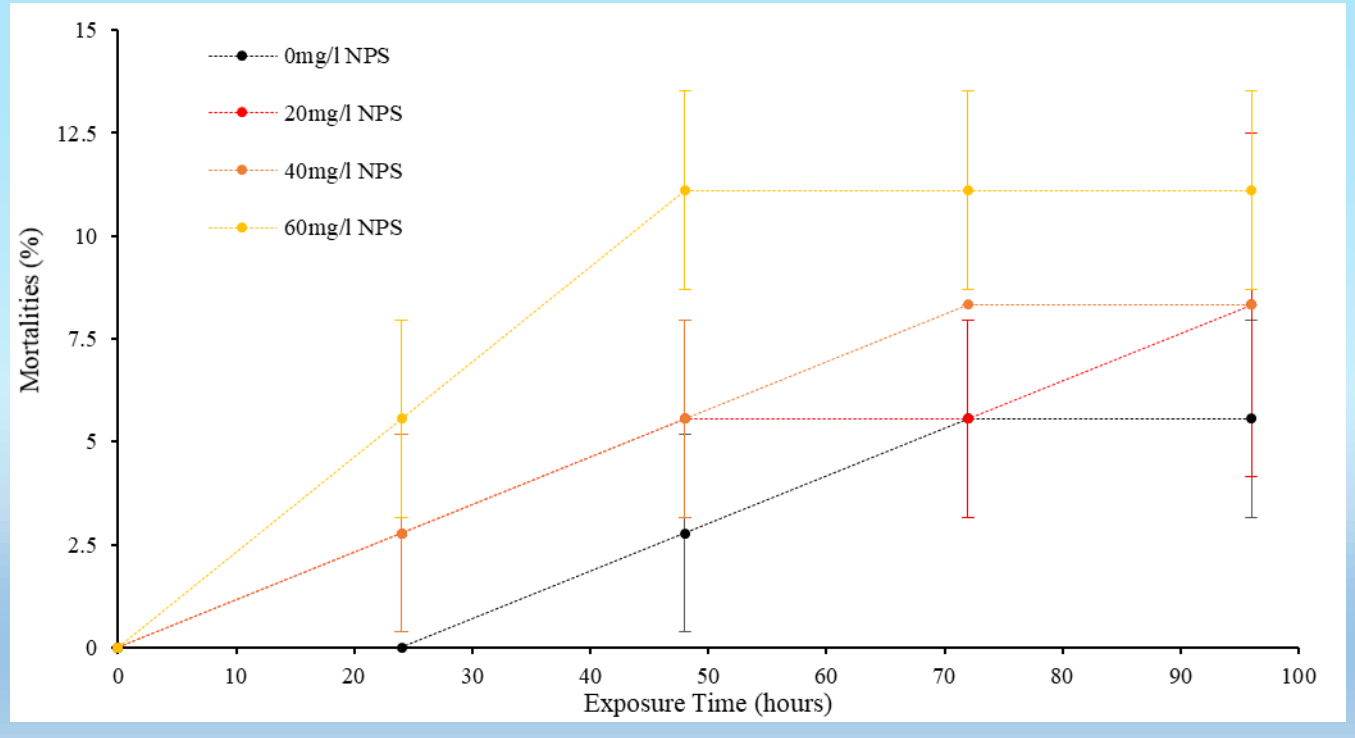


- Zebrafish Embryos produce a distinct Raman spectra
- Recurring issue occurs related to crucial polystyrene 1001cm^{-1} emission blocked, likely by Phenylaniline (amino acid) emission (~1002cm^{-1})
- Two other key polystyrene peaks (1032cm^{-1} & 1608cm^{-1}) failed to appear in any NPS exposed Zebrafish embryo Raman spectra
- Clear issues in utilizing non-homogenate samples containing significant numbers of phenylaniline

Static Primary Consumer: Zebramussel *(D. polymorpha)*

- *D. polymorpha* are parasitic freshwater bivalve mussels that grow up to 5cm in length. They are resilience to most chemical and physiological stresses, offering an ideal species to study chemicals impact from bioassay
- Mussels exposed to NPS over 24 & 96 hour to examine molecular level damage or bio-responses from NPS. Tissue samples will also be analysed using Raman spectroscopy. This will determine NPS permeability and impact locations





- *D. polymorpha* were exposed to various NPS concentrations and run under conditions set from quagga mussel test (OECD 121)
- Mortalities following 96 hours NPS exposure (see left) show clear casualties in all samples including control
- There is little consistency between exposure concentration and mortality. indicates that only 60 mg/l NPS exposed samples showed a distinct rate rise compared to controls.

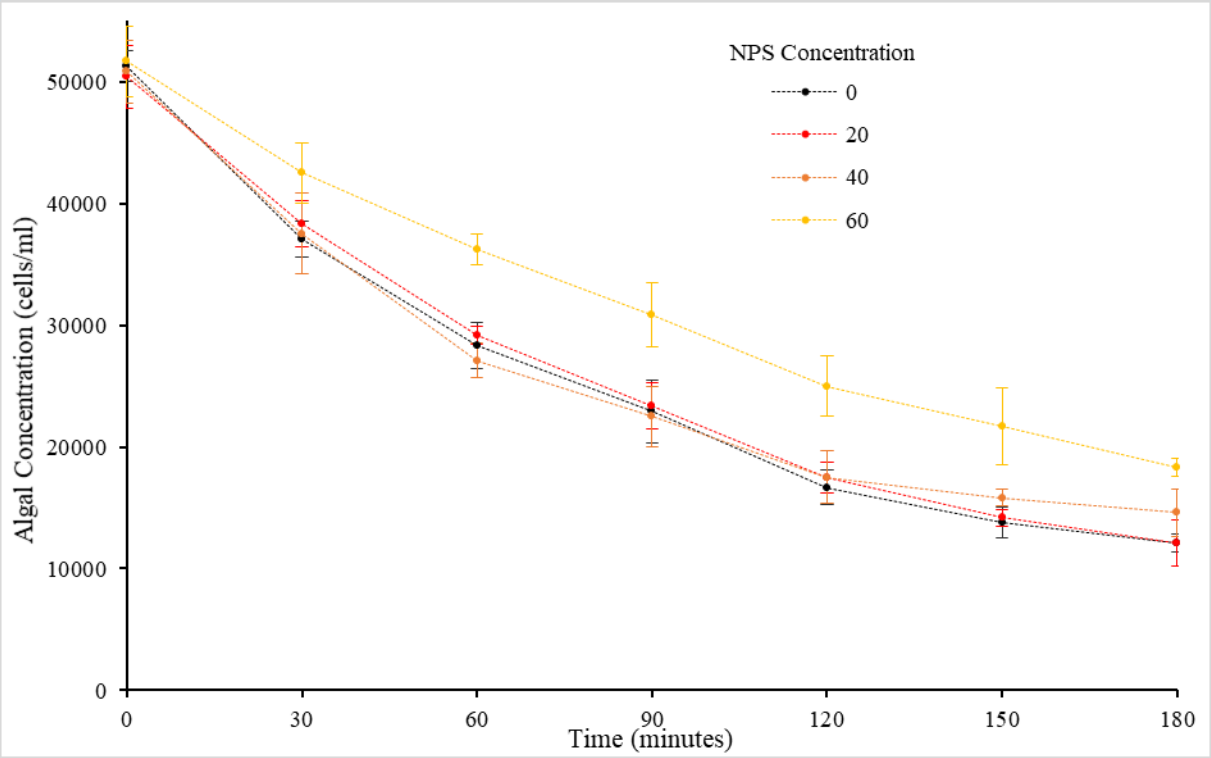
Dreissena polymorpha Exposure Response Testing

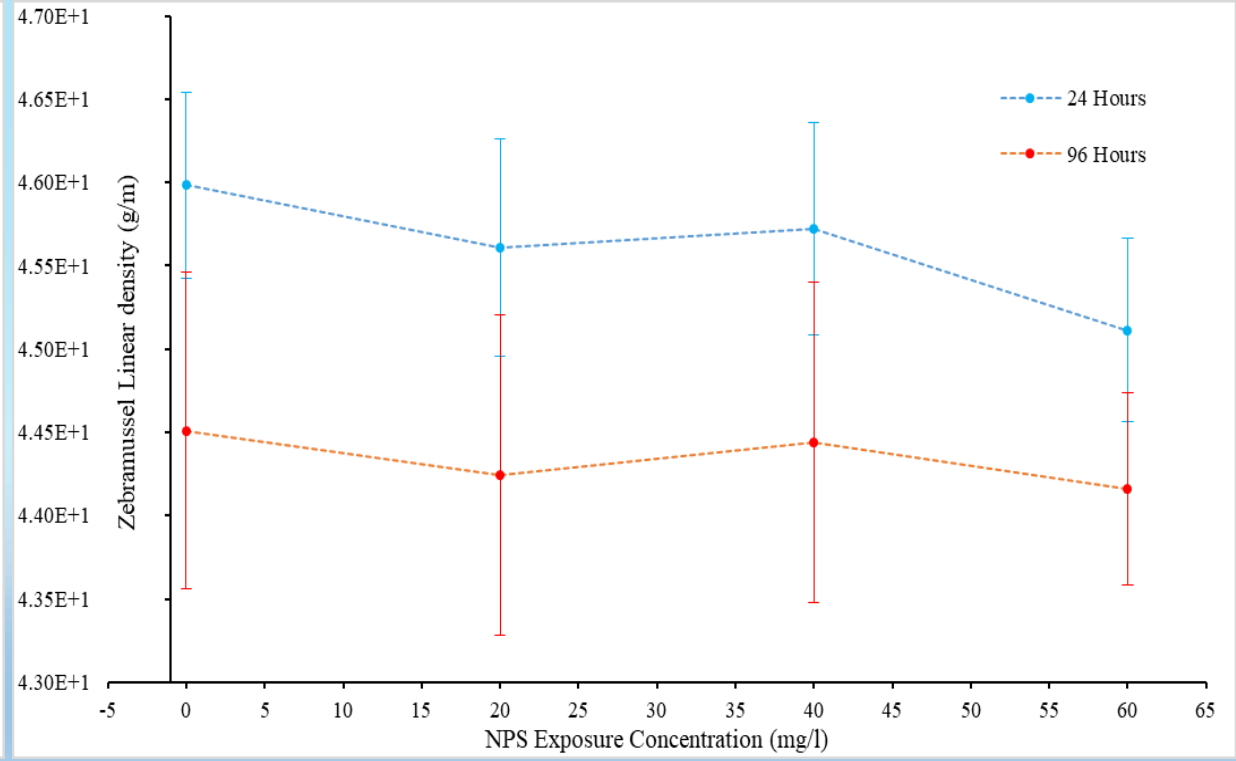
Consumption Rate analysis:

- *D. polymorpha* consumption rate testing showed little alteration in 20mg/l & 40mg/l NPS exposed samples compared to controls
- Clear reduction in 60mg/l NPS exposed mussels consumption rate (~32%) compared to control samples

Linear Density analysis:

- *D. polymorpha* following 24 hours NPS exposure had an indiscernible differences between NPS exposure concentration to their linear density
- Following 96 hours exposure, 60mg/l NPS mussels showed a ~35% reduction in linear density change compares to control samples





Conclusion

- Lack of effective toxicology analysis of nano-scaled particles within environment replicating tests for accurate regulation
- Fluorescent Imaging backed by Raman distinguished position from polystyrene nano-spheres (NPS) absorption routes within *Daphnia magna*
- Mortality/immobility results compared to fluorescence showed a concentration of ~50mg/l where acute effects by NPS could be clearly seen
- *P. subcapitata* testing showed a clear correspondence in inhibition of growth rate to rising NPS concentration exposure, with NPS causing surface coating under Confocal imaging
- Zebrafish demonstrate clear contamination of NPS on protective chorion, and showing a build-up within the intestinal region, but didn't demonstrate discernible rise in morphological or toxicological impacts.
- Further research on Zebramussels with demonstrate bio-assay based results of whether NPS induces toxic responses from known bio-markers
- All information will be compared to accepted polystyrene concentrations levels from current legislation to determine if existing techniques can address the risks from the nano-scale