Visualization of labeled nanoplastics in algae, using STED microscopy and Fluorescence Lifetime Imaging (FLIM)

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The potential ecological impact of nanoplastics (NPs, size < 1 µm) is assumed to differ from microplastics (MPs), due to their small sizes and increased reactivity which is mainly attributed to higher surface-to-volume ratios. In understanding the impact of nanoplastics on marine ecosystems, we need to elucidate processes such as their possible bio-accumulation in the foodweb, and their vertical transport in the water column. The extent of these processes is determined by the interaction between nanoplastics and marine primary producers and consumers, including processes like adsorption and absorption, uptake and retention, and possible bio-accumulation. In order to elucidate these processes, this project aimed to develop a labelling and visualization methodology for nanoplastics in their interaction with marine organisms. This includes the finetuning of a highly advanced super-resolution microscopy technique, that allows for tracking single nanoparticles with sizes below the diffraction limit of 200 nm with an acceptable resolution, allowing visualization of such particles in the interaction with algal cells. Furthermore, the use of the exponential decay rate of the fluorescent label ('fluorescent lifetime') allowed for the distinction of nanoplastics from other autofluorescent biological material in complex samples. The developed techniques could be of high value in nanoplastic-impact experiments, regarding more sensitive end-points beyond mortality.

The method development consisted of several steps. First, the labeling technique based on absorptive swelling as described by Karakolis *et al.*, (2019), with the commercial dye 'IDye' (ex. 669 nm, em. 550 nm), was optimized. This dye showed high stability for fluorescence imaging and is compatible with the STED (Stimulated Emission Depletion) microscope. Subsequently, the suitability of this labeling for toxicity testing was checked by assessing the acute toxicity of the dye to marine algae, using OECD protocols. A dose-response curve was modeled around the assumed exposure concentration (1.4×10^{-8} mg ml^{-1}). Based on the modeled dose response curve, an EC_{50} (0.0277 mg ml^{-1}) and EC_{10} (0.00836 mg ml^{-1}) for marine algae was calculated. These values are well above the applied concentration, indicating no expected toxic effects of the dye. In addition, the leaching of the dye from the particles and the fluorescence lifetime of the particles were analyzed as a function of time to account for false positives and negatives in the image analysis. The effects of the labeling on plastic particle properties were compared to the unlabeled plastics, using FTIR, Single Particle Tracking (SPT) and a Tecan plate reader.

Finally, visualization methods were optimized since this research focused on the smallest nanoparticles (smaller than 200 nm) which become indistinguishable due to the resolution limit of optical microscopes. To enable the visualization of these particles, the super-resolution STED (Stimulated Emission Depletion) microscope was used, which resulted in significant increases in resolution. To visualize the labeled nanoplastics in interaction with the autofluorescent phytoplankton species, FLIM (Fluorescence Lifetime Imaging Microscopy) was used in an addition to STED imaging. This technique made it possible to distinguish between phytoplankton species and the plastics due to differences in the exponential decay rate of the photon emission.

In conclusion, based on this research project, we were able to not only optimize a staining method for nanoplastic particles to be used in experimental settings but we were also able to pinpoint and optimize appropriate visualization methods to support new research on the interactions between nanoplastics and marine primary producers and consumers.

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