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ASSESSMENT OF THE ECOTOXICITY OF NANOPLASTICS

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The presence of micro- and nanoplastics in aquatic environments (including freshwater and marine ecosystems as well as their sediments) is becoming an increasingly serious problem worldwide. A wide range of studies have addressed the ecological effects these particles pose on biota. The main exposure pathway are food chains, e.g. under laboratory conditions these particles accumulate in the brain tissues of fish that feed on zooplankton causing brain damage. These studies, however, report mainly on the physical effects. In order to establish actual ecotoxicological effects, nanoplastics (50 nm in diameter) were assessed using the *Vibrio fischeri* bioluminescence inhibition bioassay (VFBIA). Our results showed that even environmentally relevant concentrations might trigger ecotoxicological effects. This study can be considered to be a first screening, however, results indicate the need for more complex testing on a battery of aquatic test organisms.

Keywords: nanoplastic; ecotoxicity; *Vibrio fischeri*; kinetic assay

1. Introduction

Aquatic environments contaminated by plastic litter are an emerging problem. Remote, pristine mountainous areas are even contaminated by atmospheric microplastic deposition [1]. Polymer particles < 5 mm in diameter are defined as microplastics (MP) and may be derived directly from the use of industrial pellets or indirectly from the degradation and fragmentation of plastic particles [2]. Polystyrene was proven to degrade into micro- and nanoplastics under laboratory conditions [3]. High levels of contamination have been reported in both marine and freshwater habitats [4, 5]. Micro- and nanoplastics (NP) can float freely in bodies of water or be deposited as sediments.

The highest risk associated with these particles is their ingestion, which occurs at different levels in the aquatic food chain. Jabeen *et al.* [6], for example, listed approximately 150 different fish species where ingestion and accumulation have been reported. Particles can also progress upwards in the trophic levels of the food chain, i.e. fish can be exposed to the ingestion of zooplankton which is not able to discriminate between different food sources and consumes micro- and nanoplastics [7]. An experimental study showed that in fish exposed to NPs via the food chain, these particles caused brain damage and behavioural disorders as a result of accumulation in brain tissues [8]. Biomagnification may also affect food safety and human health, though certain knowledge gaps exist in this field [9].

Ingestion may actually lead to starvation and eventually the impairment of their physical condition. Under laboratory conditions, *Daphnia magna* exposed to polystyrene nanoparticles (PS-NP) exhibited reduced body size and severe alterations in terms of reproduction [10]. *D. magna* is a widely studied species due to its key role in the aquatic food chain. It was shown to ingest nano- and microplastics (20 nm to 70 μm in diameter) from water [11]. In a laboratory study by Mattsson *et al.* [8], particles 52 nm in diameter elucidated the most severe effects. Cui *et al.* [12] exposed *D. galeata* to PS-NPs (5 mg/l, 52 nm in diameter) and detected a significant mortality rate after 2 days of exposure until the end of the study which lasted 5 days. Although a standard ecotoxicological test was conducted in this case, the mechanisms of mortality are still unclear: physical contact might have led to a reduction in the survival rate.

In general, most ecotoxicological studies have used relatively high concentrations. Manfra *et al.* [13] investigated the impact of green fluorescently labeled carboxylated polystyrene nanoparticles of 40 nm in diameter with various surface charges on the marine rotifer *Brachionus plicatilis*. It was found that anionic PS-NPs did not elucidate mortality within the range of concentrations tested ($0-50$ μg/ml), while cationic PS-NPs caused mortality at

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concentrations ≥ 2.5 μg/ml. Changes in oxidative stress enzymes were detected within the concentration range of $10-20$ μg/ml in different organisms, e.g. the rotifer *Brachionus koreanus* and the marine copepod *Paracyclopina nana* [14]. The same concentration, 10 μg/ml, was reported to cause 40 % growth inhibition in the green microalga *Dunaliella tertiolecta* [15].

In order to distinguish real (eco)toxicological effects from physical damage, a test based on the bioluminescence inhibition of the marine bacterium *Vibrio fischeri* was selected. The species has been reclassified as *Aliivibrio fischeri* [16], however, as most standards and even recent papers from the literature still use the name *V. fischeri*, it will be used hereinafter.

Bioluminescence is regulated by the enzyme system NAD(P)H:FMN oxidoreductase-luciferase. In toxic environments, enzyme inhibition is reflected by a rapid decrease in the luminous emittance of the bacterium. The reduction in light intensity is easy to measure as it is proportional to the strength of the toxicant, therefore, provides a quantifiable endpoint. This test has been used in various environmental matrices [17–19].

Lappalainen *et al.* [20,21] developed a special version of the test which was later standardised (ISO 21338:2010: Water quality - Kinetic determination of the inhibitory effects of sediment, other solids and coloured samples on the light emission of *Vibrio fischeri* (kinetic luminescent bacteria test)) in which bacteria are kept in suspension in direct contact with potentially toxic solid particles. Luminescence readings were taken when the test commenced and the light intensity continuously monitored over the first 30 secs after the sample had been mixed with the bacteria. The light output pattern, therefore, might already provide some indication of the expected toxicity of the sample [22]. The light intensity was measured once more after the pre-set exposure time (5, 15 or 30 mins as per standard). Toxicity values are normally expressed as EC50 and EC20, i.e. concentrations causing luminescence inhibitions of 50 and 20 % in this assay, respectively.

2. Materials and Methods

In our experiments, the Ascent luminometer (Flash system, marketed by Aboatox, Finland) was used. A suspension of the test bacteria (NRRL B-11177) was prepared in accordance with manufacturer instructions (Hach Lange GmbH).

Polystyrene particles with a nominal diameter of 50 nm were used as a sample (supplier Thermo Fisher Scientific). As no comparative data were available on the potentially toxic concentration, a range-finding concentration series was set [23]. Three initial sample concentrations were selected (1 g/l, 1 μg/l and 1 ng/l), which were further diluted, the number of dilutions was 11 (the number of concentrations the 96-multiwell plate permits) and the dilution ratio 1 : 2.

Table 1: The measured EC20 values of the polystyrene nanoparticles.

Concentration	1 g/l	1 μg/l	1 ng/l
EC20	5.2	17.31	30.51

The *Vibrio fischeri* strain NRRL B-11177 was reconstituted by adding the contents of one vial of $+4$ °C 1243-551 Reagent Diluent. The reconstituted reagent was equilibrated at $+4$ °C for 30 min. Then the reagent was stabilised at $+15$ °C for 30 mins before being pipetted into the wells.

Luminescence readings were taken when the test commenced, Time0, and after the pre-set exposure time of 30 mins, Time30. The luminescence inhibition of each sample was calculated as follows:

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CF = IC30/IC0
INH \% = 100 $-$ 100 x (IT30 / CF x IT0)
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where

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CF = correction factor
IC30 = luminescence intensity of the control
    sample after the contact time (30 mins) in
    the RLU
IC0 = initial luminescence intensity of the
    control sample in the RLU
IT30 = luminescence intensity of the test
    sample after the contact time (30 mins) in
    the RLU
IT0 = initial luminescence intensity of the
    test sample in the RLU
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EC20 values were calculated using the Ascent software, also developed by Aboatox Oy.

3. Results and Discussion

Table 1 shows the ecotoxicity expressed in EC20, i.e. the calculated concentration of the sample that caused 20 % bioluminescence inhibition. Fig. 1 illustrates the bioluminescence inhibition during the first 30 secs for the samples of 1 g/l and 1 ng/l in concentration.

EC20 (or in some cases, EC10) are considered thresholds for the estimation of the lowest observed effective concentration [24], i.e. the sample is normally considered (eco)toxic if the elucidated effect exceeds 20 %.

These results show that the *V. fischeri* bioassay detected a measurable degree of toxicity even at a concentration of 1 ng/l. Booth *et al.* [25] used the non-kinetic version of this bioassay (Microtox®), however, in their study, the calculated toxic concentration exceeded the range of concentrations studied ($0.001-1000$ mg/l). The same negative effect was reported by Casado *et al.* [26]. The higher degree of (detectable) toxicity in our study might be explained by the differences in the test system used. While Microtox® is a non-kinetic test, the Flash system (Ascent luminometer) was especially developed to test the toxicity of different suspensions or samples containing solid particles. The Ascent luminometer uses a 96-multiwell microplate. A specific feature of it is that

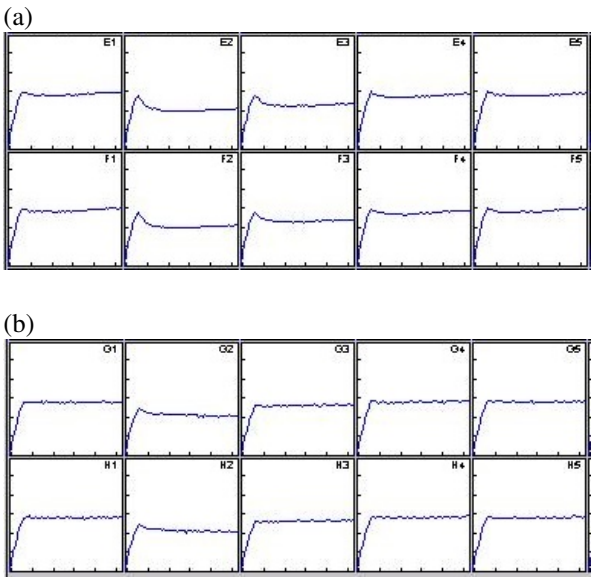

Figure 1: Kinetic diagram of the 1 g/l (a) and 1 ng/l (b) samples. The light output is recorded over the first 30 seconds. After the peak, toxicity causes a rapid reduction in the light output, on the other hand, it remains constant during the control. The two columns show the two replicates. E1-F1/G1-H1 (left): control. E2-F2/G2-H2 (right): sample, maximum concentration.

during luminescence readings, the microplate is continuously shaken by the instrument, resulting in the resuspension of particles.

According to our results, environmentally relevant concentrations might already pose ecotoxic effects. Actual environmental concentrations are relatively difficult to compare and assess, mostly due to difficulties in sampling and the lack of standardized sampling methodologies [27,28]. Indicative data are available: e.g. microplastic concentrations of $0.4 - 34$ ng/l in bodies of freshwater in the USA [29] or 0.51 mg/l in marine environments [10].

However, in real-world environments, even higher levels of toxicity can be expected as particles might absorb organic pollutants from the surrounding water [30], including highly toxic pesticides or polychlorinated biphenyls (PCBs) [31]. Though their bioavailability is still questionable [32], Batel *et al.* [33] conducted a laboratory study on microplastics and one polycyclic aromatic hydrocarbon (PAH), benzo[a]pyrene (BaP). It was demonstrated that BaP adsorbed on microplastics and was transferred via an artificial food chain. These particles might also possess inherent toxicity due to the use of additives during manufacturing processes [34].

4. Conclusions

It is a well-known paradigm in ecotoxicology that the sensitivity of different test organisms to a particular chemical varies, therefore, the *V. fischeri* test can be regarded as a first screening. The bioluminescence inhibition assay is an acute test that uses a maximum exposure of only 30 minutes. Naturally, chronic effects cannot be extrapolated from these results. However, the fact that the tested nanoplastics have already elucidated ecotoxicological effects in environmentally relevant concentrations emphasises the need for more complex ecotoxicological testing involving a properly selected battery of test organisms. In addition to widely used aquatic test organisms such as the aforementioned *Daphnia magna*, an ideal candidate could be the *Caenorhabditis elegans* test. It is a standardised bioassay using a sediment-dwelling, widely distributed nematode. However, in order to distinguish physical damage from toxic effects, the measurement of changes in oxidative stress enzymes can be useful no matter which test organism is applied.

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