Environmental Science
Processes & Impacts

Accepted Manuscript

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Environmental Impact Statement

This review focuses on the release, abundance, and the biological impact of nano-sized plastic. Much attention has been given to plastic in nature and lately micro-sized plastic. However, also nano-sized plastic is released directly into nature, and, perhaps even more important, one can expect that the break-down of plastic continues into the nano-sized range. Nano sized plastic will differ in many ways from larger pieces of plastic due to the potentially very large surface area and the small size. Therefore it is important to focus also on the the effect of nano-sized plastic. We review the small number of exisiting publications of nano-sized plastic in regards of pollution, break-down mechanisms, and the effect on aquatic organisms. We also identify important areas for further studies and describes how these can be performed. Nano-sized plastic may introduce an enourmous plastic surface area into nature. We believe it is very important to high-lght the need for more knowledge of its effects.

Journal Name

ARTICLE

Nano-plastics in the aquatic environment

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

The amount of plastics released to the environment in modern days has increased substantially since the development of modern plastics in the early 1900s. As a result, concerns have been raised by the public about the impact of plastics on nature and on, specifically, aquatic wildlife. Lately, much attention has been given to macro- and micro-sized plastics and their impact on aquatic organisms. However, micro-sized plastics degrade subsequently into nano-sizes whereas nano-sized particles may be released directly into nature. Such particles have a different impact on aquatic organisms than larger pieces of plastic due to their small size, high surface curvature, and large surface area. This review describes the possible sources of nano-sized plastic, its distribution and behavior in nature, the impact of nano-sized plastic on the well-being of aquatic organisms, and the difference of impact between nano- and micro-sized particles. We also identify research areas which urgently need more attention and suggest experimental methods to obtain useful data.

Introduction

Modern plastic was developed in 1907 and mass production of plastic started as early as the 1940s. In 2012, amount of plastic produced reached 280 million tonnes [1] of this 280 million tonnes produced, 90% was low-density polyethylene (LDPE), high-density polyethylene (HDPE), polypropylene(PP), polyvinylchloride (PVC), polystyrene (PS) and polyethylene terephalate (PET) [2]. Over one third of this amount is used for packing products like plastic bags and another third is used for housing components like plastic pipes and vinyl cladding [2]. An astonishing 10% of the produced plastic is expected to end up in the oceans [3]; making plastic a severe and potent pollutant for aquatic organisms [4].

Plastic is the most significant marine litter in the world constituting between 60% to 80% of the total marine debris [5]. Around 80% of the plastic litter in the marine environment is of terrestrial origin [6]; whereas 18% is attributed to the fishing industry [6]. Hence, when plastic is released into the environment, a noticeable amount will end up in the aquatic environment where it is degraded into smaller pieces through UV-radiation, mechanical abrasion, biological degradation and disintegration [7]. A major portion (more than 80%) of the plastic litter accumulates in open-ocean surface waters and is estimated to be between 7.000 and 35.000 tonnes in total [8]. The dominant size fraction of those particles is less than 10 mm in diameter [9]. Over 70% of plastic recovered from beaches in Portugal [10] and 82% of the collected marine debris in the Tamar Estuary (UK) [11] belonged to the size class less than or equal to 5 mm and 17% of the particles collected in Tamar Estuary (UK) were smaller than 1 mm [11]. In freshwater systems most of the detected plastics are small, below 5 mm [12]. Another source of micro-plastic is consumer products, such as facial cleaners, which generally pass through sewage systems and are thereby released directly into lakes and oceans [13]. Released micro-plastics or degraded micro-sized plastics are in the size suitable for ingestion by organisms [14]. Shoreline debris is yet another source of plastic exposure to aquatic organisms and as much as 50-80% of the shoreline debris is plastic [15].

The definition of micro-plastic, or micro-litter, differs between authors, where Arthur et al., as well as The National Oceanic and Atmospheric Administration (NOAA), define micro-plastics as particles smaller than 5 mm in diameter [16]. Costa et al., on the other hand, set the upper limit to 1 mm [17]. The most commonly used definition is that micro-plastic particles have a diameter between 1 and 5 mm, whereas particles larger than 5 mm are referred to as meso-plastic. Nanoparticles are defined as materials with at least two dimensions between 1 and 100 nm [18]. The amount of nano-sized plastic particles in the aquatic environment is not known since most analytical techniques exclude these small particles.

The degradation of plastic into smaller pieces changes the chemical and physical characteristics of the plastic, and thereby its availability to and potentially biological impact to aquatic organisms (Fig 1). The break-down processes are not likely to stop at micro-size, but will continue to produce nano-sized plastic which will differ from both the original material and from micro-plastic. The nano-sized plastic particles will have a high surface curvature and other surface structures will be small compared to biological surfaces and molecules altering the interactions and potentially their biological and chemical impact. Most importantly, however, is that the surface area for a nano-sized particle will be enormous. For example, if a normal plastic shopping bag is totally transformed into

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particles with a diameter of 40 nm, they will theoretically expose a surface of 2600 m^2; enhancing any surface effect.

Nano-materials are of great commercial interest in industrial, technical, medical, pharmaceutical, cosmetic and life science applications [19] and the effects of some nano-materials are well studied in aquatic systems, for example TiO_2 [20] and gold [21]. However, the number of studies including nano-plastics are surprisingly few. Therefore, this review will focus on comparing nano-plastics with micro-plastics with regards to:

1. The course of plastic material into aquatic environments

2. Methods of sampling plastics in nature

3. Plastic degradation

4. Biological effect on aquatic organisms

5. What we believe are the important knowledge gaps where future studies are urgently needed

Plastics in the aquatic environment

Sources of plastic in the aquatic environment

There are two main sources of plastics in the aquatic environment; primary particles, i.e. particles manufactured to be in a size range, and secondary particles, i.e. particles derived from larger particles or other structures (Fig. 1). The plastics enter oceans, lakes and rivers through solid waste disposal, coastal landfill operations and disposal at sea of solid waste from individual vessels. Major inputs of plastics come from ship generated litter, litter carried to the sea by rivers and municipal drainage system and litter left from recreational activities [22], but also from fishing fleets (plastic fishing gear), accidently lost, carelessly handled, or land based sources (packing material). During 2010, 2.5 billion metric tonnes solid waste was produced by 93% of the population. Of this amount 275 million metric tonnes were plastics, and the estimated amount of plastic ending up in the ocean was between 4.8 to 12.7 million metric tonnes [23]. The raw material for production of plastics is resin pellets which often are cylinder- or disk-shaped. These pellet particles are released into the environment both during manufacturing and transport [5] via waste-water discharge from plastic production or plastic processing plants [24]. They are transported to the oceans through surface runoff, streams, and river waters or by accidental spillage during shipping [25]. These particles are widely distributed in the oceans and found on beaches and in surface waters all over the world due to their environmental persistence [25]. The particles generally have a size between 2 and 6 mm [26] and are found both in remote and non-industrialised places and near important industrial centres. The highest concentration of plastic is found offshore in subtropical ocean gyres [27-29] where the bulk of this solid waste consists of material used to package food and other products [24]. The abundance of industrial plastic in surface waters from the North Atlantic subtropical gyre has decreased by almost 75% between 1987 and 2012, while the amount of user plastics is found in around 80% of collected samples without any changes during those years [30]. Plastic particles collected on beaches had a lower density; closer to the density of common consumer products than plastics collected in gyres. This suggests that the plastic is changing as they reside at sea[26].

In European, Asian and North American freshwater systems both primary and secondary micro-plastic of household origin have been reported [11, 31-33]. In some lakes the highest amount of plastics were found close to highly populated areas, industries and tourist areas [33]. Meanwhile, high amounts of plastics have also been detected in remote areas [32] with low population densities; this is likely a result of long water residence time and small surface area of the lake [32].

Also, sediment samples from estuarine shorelines contain high amounts of micro-plastics particles. Of these micro-plastic particles, 65% are fragments originating from larger plastic pieces. Half of macro-plastic found in the estuarine shorelines originated from single use packing items [34].

Sampling of plastic in the environment

There are five major methods to sample plastic debris; *beach combing, marine observational surveys, biological sampling, marine trawls* and *sediment sampling* [35]. Beach combing is a sampling method in which an individual searched the shore for marine debris and terrestrial litter. Marine observational surveys are records of visible plastic debris as reported by divers and/or boat occupants. Thereby, particles of micro-meter size or less are not reported because they are not easily traced by the naked eye. When using biological sampling, plastic fragments consumed by marine biota are investigated. This includes marine animals, but also birds that can ingest terrestrial plastic litter. In marine trawling, which is the most common way to study plastic in the aquatic environment, particles are collected from surface water in a moving boat with a net. The mesh size of the net limits which particles can be collected because smaller particles can pass through the net. The speed of the boat also limits which particles size can be collected since some unconfined particles will be forced through the net or pushed forward out of the net. Typical boat speed ranges between 0.5 and 2.5 m/s and typical net sizes are generally between 80 μm (KIMO Sweden) [8] and 1000 μm, whereas the typical mesh size used is around 330 μm. This means that smaller micro-sized or nano-sized particles are normally not collected and consequently there is a lack of data describing the abundance of micro- and nano-sized plastic material in natural systems. Sediment sampling collects benthic material from beaches, estuaries and the sea floor. The most widely used method for detection of micro-plastic in sediments is the extraction method developed by Thompson et al [36]. It relies on the density of a concentrated NaCl solution to separate sediment from micro-plastic particles. When the salt solution is added low-density micro-particles float up to the surface. This method is effective for polymers with a density lower than that of the saturated saline

concentration, i.e. 1.2 g cm^{-3}, but not suitable for extraction of polymers that have a high density, such as PVC or PET. These polymers are, however, common and represent 18% of the plastic products in Europe [37]. Since these particles have a high density they tend to sink more easily. A new technique has been developed by Claessens et al [38] which involves a fluidized sand-bath and a small volume of sodium iodide, NaI. The technique is based on the principal of elutriation, a process that separates lighter particles from heavier when using an upward stream of gas or liquid. The sediment sample is first washed through a 1 mm sieve to remove larger particles. To separate heavier from lighter sand particles, such as micro-plastics, a water flow in combination with aeration is used. The lighter particles are then collected on a 35 μm sieve. After this, the material undergoes a NaI extraction and is manually stirred and centrifuged. In the centrifuged sample the light particles are in the upper layer which is vacuum filtered. This extraction process is repeated two to three times to ensure that all particles are removed from the sediment sample. The methods to sample micro-plastics have, to our knowledge, not been used to sample nano-sized plastics. However, it should be possible to develop these methods for extraction of nano-plastics.

The data of nanoparticle concentration and size distribution in the aquatic environment is limited. The major reasons for this is the lack of suitable methods and that the ones available are generally time consuming and often perturbing to the physiochemical state of the sample. Identification of these small plastic particles is also challenging. However, one of the few quantifications of nanoparticles in natural systems is based on samples from 9 different locations in Sweden [39]. In Sweden, the concentration of nanoparticles in lakes, rivers, coastal areas, as well as waste water and stormwater was assessed, showing that the majority of the particles were between 100 and 250 nm in diameter, occurring in concentrations of 10^7 to 10^9 particles/ml [39].

Degradation of plastics, from bulk through macro- and micro- to nano-size

Manufactured plastic particles in the micro- and nano-size used in consumer products like cosmetics, ship breaking products and industries [19] are introduced directly into the oceans via runoff [6]. It is believed that the majority of micro-plastics in the oceans come from in situ weathering of meso-plastics and larger fragments of plastic litter in the beach environment [6].

Degradation of plastics can be divided into six processes: *thermal degradation, hydrolysis, mechanical/physical degradation, thermo-oxidative degradation, photodegradation and biodegradation* [6]. Thermal degradation describes the commercially heat-generated break down of plastics, i.e. thermal degradation is not an environmental degradation process. Hydrolysis is a bond-breaking reaction brought about by the addition of water and that has been shown to contribute to degradation of plastic marine debris [40]. Mechanical or physical degradation is caused by waves and is very prominent in aquatic environments. Thermo-oxidative degradation is a slow oxidative breakdown at moderate temperatures. Photodegradation is brought about by light i.e. sunlight and is a very efficient mechanism for plastics degradation on land or in air; but in seawater this type of degradation is severely retarded [41]. In biodegradation, living organisms usually microbes, (such as bacteria), break down organic substances. Lower temperature and lower oxygen concentration reduce the rate of degradation in seawater[6]. The degradation processes will produce particles and surface structures of all different sizes. Also, plastic particle surface chemistry will change due to oxidation and photo degradation. Consequently, the aquatic organisms will simultaneously be exposed to a variety of particles and surfaces, each with a potentially different biological impact.

Although data is limited, it is reasonable to assume that the degradation processes produce nano-sized material, thereby potentially generating extremely large surface areas. As mentioned above, a common plastic shopping bag can, if it is totally transformed into the form of 40 nm plastic particles, present a surface area of 2600 m^2. This is unlikely to happen, but given the large amount of plastic bags used and discarded, even a small percent of them will generate enormous surface areas.

What happens with nano-sized particles in the aquatic environment?

Nano-sized particles in the environment will interact with their surroundings and their biological fate, mobility and bioavailability will depend on their size, shape, charge and other properties. Their large surface to volume ratio, high surface curvature, high surface reactivity and small size, enable different uptake rates and biodistribution of nanoparticles. This makes them highly dynamic in the environment. The particles can undergo transformations while present in the environment or while in the biological systems. These transformations can be divided into four groups; including *macromolecular interactions*, *physical, chemical* and *biological transformations* [42, 43]. Physical transformation, or aggregation, can occur as homo-aggregation, i.e. aggregation between the same type of particles or hetero-aggregation, i.e. aggregations between different nanoparticles or nanomaterials. Particle aggregation leads to reduced surface area to volume ratio and new surface structures. Macromolecule interactions occur when particles interact with natural organic materials. This is described as the formation of a bimolecular corona [44, 45] as it creates a new surface around the particle. This new surface or corona, which differs in surface structured from the naked plastic particle, has different biological implications on the effected organism. The corona will also change the particle aggregation tendency. Biological transformation, i.e. reactions between particles and living tissues, both intracellular and extracellular occur in the core of the nanomaterial and the coatings. These affect the behaviour of the material, including surface charge, aggregation state and reactivity, thereby affecting transport, bioavailability and toxicity. Chemical transformation means that particles in the environment can undergo reduction or oxidation. Photooxidation and photoreduction affect coatings,

oxidation state, generation of reactive oxygen species and persistence. The transitions depend on the condition of the solution, but also on the history of the nanomaterial; since this will affect its properties, state and the numbers of transitions that will occur [42]. The number of transitions a particle will undergo is almost impossible to assess or predict since some transitions will have a time scale of months or years.

Plastic particles as carriers of other materials

Two mechanisms account for the fact that plastic particles can act as carriers of chemicals and other materials. Plastics can adsorb chemicals because of the low polarity of their surfaces and they can consist of different chemicals. Plastics have different surface structures depending of what polymer they consist of and thereby they contain different additives [25].

Plastics may also absorb hydrophobic compounds, such as persistent organic pollutants, POPs, which include for example PCB [46]. The absorbed pollutants may add to the risk that nanoparticles impose to aquatic organisms. For example, PP can accumulate PCBs, as well as nonylphenol and pesticides [47]. Many papers suggest that plastic particles that carry POPs are likely to be ingested by organisms [36, 46]. Ryan et al reported an indirect evidence for contaminant transports by plastics based on the correlations between ingested plastic particles in seabirds with the concentrations of PCB in seabird tissue [46]. It is likely that smaller plastic particles increase the uptake and transport of hydrophobic compounds because of their large surface area [48]. Small plastic particles have a high capacity to absorb phenantrene and thereby they are likely important for the transfer of contaminants to organisms [48]. POPs are present in low concentrations in seawater all over the world and are picked up by meso and microplastic via partitioning. The concentration of POPs in meso and micro litter is many magnitudes higher than in seawater since the POPs are hydrophobic. POPs enter the food web when these contaminated plastics are ingested by aquatic organisms. Small plastics thereby increase the bioavailability of POPs to the biota and it is therefore likely that the carrier effect for nano-sized plastic is further enhanced due to the large surface area of the particles. However, the bio-magnification in food webs has not been studied in detail [6].

How do plastic particles affect organisms?

Plastic waste has been shown to affect over 660 marine spices worldwide [4]. Plastics are lightweight, strong, durable, and cheap; and these characteristics make them suitable for manufacturing of many different products. However, the same properties are the reason plastics act as environmental hazards [49]. The most pronounced threat of plastic to aquatic life occurs from ingestion or entanglement. Entanglement in fishing gear is a serious threat for aquatic animals like gannets, sea turtles and marine mammals like seals [5]. Entanglement can lead to drowning, decreased ability to catch food or to avoid predators. It can also reduce fitness; as the entanglement increase so does the energetic cost of traveling [50]. Fulmars (*Fulmarus glacialis*) are one of the first seabirds reported to ingest plastic marine debris and the amount of plastic found in them are well studied. From mid-1980s to mid-1990 the amount of plastic in Fulmars increased both in mass and number. This was followed by a decrease of mass until the new century but not of the number of ingested particles [30]. The amount of plastics in seabirds has increased considerably [51], and many aquatic species select specific plastic shapes and colours since they mistake them for prey items [52]. The same behaviour has been reported for fish [53] and sea turtles [54]. The ingestion of plastic particles also affects the formation of fat in birds [55, 56]. Harmful effects of ingested plastics have been reported for seabirds, turtles, fish and cetaceans [5]. Macro-plastics have been shown to affect at least 267 marine species by ingestion or entanglement [5, 57]. These plastic particles cause injury and death of marine birds, mammals, fish and reptiles resulting from plastic entanglement and ingestion [35].

Micro-sized plastics

Ingestion of micro-sized plastics is less studied than macro-sized. This is mostly because micro-sized plastics are comparable in size to sand grains and planktonic organisms; all of which are difficult to quantify due to their small size. Thereby, these particles are available to a wide range of invertebrates near the base of food chains. There are four potential dangers with particles in this size: the potential toxicity from ingesting the particle in itself, contaminants leaching from the micro-plastics, pollutants following the micro-plastics and accumulation of these particles in organisms.

Many organisms, such as amphipods (detritivores), barnacles (filter feeders) lungworms (deposit feeders) [36], zooplankton, echinoderm larvae [35], mussels, fish and seabirds can ingest plastic particles in this microscopic size. Micro-sized particles (0.2 mm to 4.8 mm) were found in 5 of 7 species in North Sea fish [58]. The mean value of the examined fish contained 2.6% and the highest content, 13%, were found in cod [58]. Polystyrene microspheres of 10 μm can be ingested by filter feeders like polychaetes, echinoderms, bryzoans and bivalves. These ingested particles can translocate through the epithelial membrane of the gut into tissue, or they can be egested (the process of discharging undigested or waste material from an organism) through defection [59]. In zooplankton collected from Portuguese coastal waters, micro-plastics were found in 61% of the samples [60]. These particles can have a negative impact on the function and health of animals [61]. The mussel, *Mytilus edulis*, can ingest particles of 3 μm and 9.6 μm and the particles end up in the hemolymph and the circulatory system. The particles can also disintegrate into smaller fragments and potentially accumulate in tissue [59]. The crab, *Uca rapax,* can accumulate micro-sized polystyrene fragments in their hepatopancreas, stomach and gills[62].

Nano-sized plastics

The behaviour and interaction of nanoparticles with living organisms distinctly differs from larger pieces of bulk material [63, 64]. This is because of their unique physiochemical nano-scaled

properties. Their small size makes it possible for them to pass biological barriers, to penetrate tissue [65] and to accumulate in organs [66]. The high surface area increases their potentially enhanced reactivity and it has been shown that the surface area impacts the biotoxicity of polystyrene nanoparticles [67]. Other examples of important parameters are: chemical composition, purity, doping, hydrodynamic size, morphology, size heterogeneity, redox parameters, tendency for aggregation, nature and composition of the shell or coating material, surface modifications and surfactants, chemical and colloidal stability, solubility, biodegrability, concentration, duration of exposure and unknown interactions with other nanoparticles or behaviour under electromagnetic field exposure.

Nano-plastic effects on algae

Algae are aquatic primary producers (i.e. plants) occurring in many different shapes and sizes. The smallest are just a few micrometers and the largest are several meters long. Cellulose is a major component in cell walls of green plants and algae serve as a starting point for particle introduction into the food web [68]. Although the literature provides many examples of nanoparticles interacting with algae, there is only a few studies focusing on plastic nanoparticles and all of them have used PS.

Bhattacharya and co-workers investigated the effect of algal morphology on plastic adsorption for the single cell algal species *Chlorella* and multi celled *Scenedesmus* [69], both of which contain cellulose in their cell walls. Bhattachary used positively charged amidine PS and negatively charged carboxyl PS in the size of 20 nm. The two different particles were delivered to algae both as non-aggregated and aggregated particles to mimic natural conditions. Positively charged particles have a higher binding affinity compared to negatively charged particles and more positively charged particles are adsorbed to the algae, possibly because of the electrostatic interaction between the particles and the cellulose content in algal cell wall. This implies that cellulose plays an essential role in initiating the binding between the algae and the particles. *Chorella* has a higher adsorption for the negatively charged particles than *Scenedesmus*. The adsorption interferes with algal photosynthesis and promotes their reactive oxygen species (ROS) production [69], which may affect their viability. This is further exemplified by exposure of *Scenedesmus obliquus* to 70 nm PS particles, which inhibited growth and reduced the chlorophyll concentration in the cells [70]. The few studies on how plastic nanoparticles affect algae, and the fact that only PS is investigated emphasize the strong need for future studies to be able to evaluate possible negative effects of nano-sized plastics on algae.

Nano-plastic effects on filter feeders

Filter feeders feed by straining suspended aquatic matter and food particles over a specialized filtering structure. They play an important role in clearing water and are a natural entering point for nanoparticles into food webs. Nanoparticles in the environment can be ingested by filter feeders as primary particles but out commas around more likely they are ingested aggregated with other matters [63]. It is not well known whether or not aggregated or primary nanoparticles have the same physiological impact on aquatic biota [6]. As for algae, there are numerous studies of the effect of nanoparticles but only a few in which plastic nanoparticles are used.

Daphnia magna, a freshwater invertebrate commonly found in lakes and ponds, can ingest nano- and micro-sized (20 nm to 70 μm) particles from surrounding water [71, 72]. Besseling et al. exposed *Daphnia magna* to 70 nm PS particles under four different conditions. First they used a mixture of nanoparticles and algae given directly to the *Daphnia*. The second mixture was aged algae, a mixture of algae (*Scenedesmus obliquus*) and nanoparticles where the algae were allowed to ingest the nanoparticles for 5 days before the *Daphnia* received the mixture. The third condition was the same as the second but the algae were filtered away before the *Daphnia* received the mixture. The last was a mixture of algae, nanoparticles and fish kairomones given directly to *Daphnia*. Fish kairomones from the predator perch (*Perca fluviatilis)* were used because of their capability to induce life history responses in *Daphnia* [73]. Compared to the control group, authors reported a six times higher mortality for aged algae compared to fresh algae, a lower reproduction rate and a more pronounced reduction in body size for *Daphnia* receiving fish kairomones. The pre-exposed algae likely absorbed nanoparticles to a higher extent and therefore the uptake by *Daphnia* was higher. It is also believed that aging may enhance the transfer of styrene monomers from the particles to the algae, thereby increasing the bioavailability of styrene. Reduction in body size is explained by the difference in survival strategy with and without predator presence [70].

Bivalve molluscs capture 6 μm particles with an efficiency of 90% while smaller particles are captured with an efficiency that decreases asymptotically with decreasing size. Thereby, the expected risk to accumulate significant amounts of free nanoparticles is very low. However, nanoparticles are, as mentioned above, likely to aggregate with other matter in nature. Ward et al. fed the mussel *Mytilus edulis* and oyster (*Crassostrea virginica*) with 100 nm PS particles, aggregated 100 nm PS and 10 μm PS particles [63]. Both animals ingested nanoparticles at a significantly higher rate when those were aggregated. Furthermore, mussels ingested the aggregated particles at a higher rate than the oyster. The egestion was higher in the beginning for the biggest particles (10 μm) than for aggregated nanoparticles. However, the egestion of aggregated nanoparticles increased with time. It was much easier for the animals to ingest aggregated particles than nanoparticles. A higher gut retention time was seen for animals fed with nanoparticles. Nanoparticles can accumulate in mussels and oysters and a large fraction is ingested and not excreted as pseudofeces. The aggregates are likely broken down by the action of cilia on the gills and labial palps and the constituent particles ingested [63].

In another study, the blue mussel, *Mytilus edulis,* was exposed to PS nanoparticles both as dispersed particles with a size of 30 nm and

as large aggregates with a size of 968 nm [74]. In one media *Mytilus edulis* was exposed with algae and in a second media *Mytilus edulis* was exposed to nanoparticles without algae. Both exposures resulted in reduced filtering activity and in increased production of pseudofeces. This suggests that the particles were adsorbed to the gills and identified as low nutritional food [74]. These two studies imply an important feature of nanoparticles in nature as there is a nano-size effect despite that the particles were aggregated.

Nano-plastic effects on consumers

Bioaccumulation of plastic nanoparticles may be very prominent, especially in long food webs. In two different studies, the top consumer, Crucian Carp (*Carssius carassius*), was exposed to PS nanoparticles (24 and 27 nm) through a natural food chain, from algae through zooplankton to fish [68, 75]. The experiments were designed as a three-day cycle and on day one, nanoparticles were given to algae which were then grown for 24 h. On day two, *Daphnia* were allowed to feed on the algae for 24 h. They showed no change in behaviour during this time. On day three, the *Daphnia* were fed to the fish. After two months, the fish behaviour during feeding was recorded and analysed. Behavioural changes were found, including: feeding time, activity and shoaling behaviour. Moreover, metabolic changes were reported for fish receiving nanoparticles through the food chain, including: such as disturbed fat metabolism, increased ethanol concentration in the liver, and increased levels of inosine/adenosine and lysine in muscles were reported for fish receiving nanoparticles through the food chain [68].

In another study the transparent fish medaka (*Oryzias latipes*) embryos and larvae were exposed to four different PS particles, non-functionalized 50 nm and 500 nm, and carboxylated c-50 nm and c-500 nm. When examining the uptake, excretion and survival rate for the larvae, the uptake rate was found to be much higher for the smaller particles (50 nm and c-50 nm), and highest for 50 nm, whereas the uptake was lowest for c-500 nm particles. The smaller particles were more difficult to excrete than the bigger ones [76].

Torre et al. (2014) exposed sea urchin embryos, *Paracentrotus lividus*, to two differently charged PS nanoparticles, carboxyl modified negatively charged or amine modified, positively charged. The carboxylated particles were more aggregated but showed no toxicity and accumulated inside the digestive tract of the embryos. In contrast, the nanometer-sized amine PS particles, caused severe developmental defects and the particles were more dispersed inside the embryo. The different effects depended on the different surface charges and aggregation [77].

Future challenges

The experimental data describing the effects of plastic nano-particles on the aquatic wildlife is limited. However, the few studies performed indicate severe and particle size-specific effects. This, together with the large amount of plastic and the expected breakdown of plastic material, highlights the urgent need for more information on the impact of nano-plastics in nature. Along with more research, several experimental methods need to be fine-tuned in order to obtain more information. We identify the most important needs and experimental challenges to be:

1. Sampling, quantification, detection, and the fate of nano-plastics in nature

2. Characterization of the degradation process and rates of plastics into the nano-size range

3. Nano-size specific effects on aquatic organisms

Detection of nano-plastic in the environment and their fate

There are several challenges regarding sampling of plastic material in the environment. For example surface sampling, where the sampling mesh size limits the detection size and detection of the particles in tissues, as the carbon based plastic particles resemble the surrounding tissues. Therefore new approaches and methods are needed for detection of nanoparticles in surface waters and in tissues.

We see two possibilities to improve our knowledge of nanoparticle abundances and distribution in the aquatic environment. First, we suggest a method for collection and characterization of nanoparticles in surfaces, as well as from deeper waters. Sampled water will be filtered with a mesh, similar to sampling micro-sized particles[28] but in this case to exclude larger particles and retrieve small plastic and organic particles in the sample that has passed through the mesh. Further filtration using 400 or 200 nm filters can be done but then particles associated with biological materials may be lost. Preferably samples from different filtration steps should be analysed to differentiate free and aggregated particles. The nanoplastic in the samples are thereafter separated from organic matter by degradation with specific enzymes, for example cellulases and chitinases to break down outher structures, and protteinaeses, DNAses and RNAses to brake down cellular structures. We believe specific degradation by enzymes is better than chemical degradation to ensure that the plastic polymers will be less affected. Strong acids and organic solvents will probably affect the plastic as well as the biological materials. The nano-plastic can thereafter be separated from the degraded biological matter by size and density based methods and characterized for size and concentration as described below.

Second, we suggest laboratory experiments to enlighten the fate of nano-plastics. There are three possible fates for the particles in nature. They can be unaffected, i.e. remain in a monodispersion, by the surrounding matter, they can aggregate with other plastic particles, or they can interact with other matter in the environment. How nanoparticles in the environment will interact with other present substances that are naturally occurring in the environment, for example macromolecules and chemicals, can be studied under controlled conditions. The interactions between these particles can

be observed in the laboratory where degraded and primary particles are added to water from the environment. The size of the particles can be measured, as well as their chemical composition. When plastic degrade the surface area increases and thereby also the potential binding to chemicals. The surface chemistry changes may affect which chemicals that will bind to the plastic. The question if nano-plastic will carry more and different chemicals than macro-plastic needs to be addressed and answered to allow for evaluation of the impact of nano-plastics. Further knowledge about what happens with the chemicals over time is needed as there are likely some conditions that will make the chemicals leave the particles.

Degradation of plastic in natural aquatic systems

Although the available literature is limited it suggests that there are good reasons to believe that nano-sized plastic have different effects on aquatic organisms compared to larger plastic pieces [59, 63]. It may be difficult to sample nano-sized plastic directly from oceans, lakes, and waterways. So instead plastics can be broken down and studied in controlled experiments that mimic natural conditions. We suggest that well defined micro-sized plastic particles, as well as daily used plastics are exposed to some of the natural forces that exist in aquatic environments under controlled laboratory experiments. Mechanical forces, temperature, salinity, pH, and UV radiation are conditions that are easily applied and controlled in the laboratory. After degradation the products need to be separated by size. Possible size segregating methods are filtration, size exclusion chromatography, and sedimentation. Dynamic light scattering, analytical sedimentation centrifugation, and nanoparticle tracking analysis can determine size in nanoscale. The degraded plastic pieces will most likely be heterogeneous not only in their size distribution but also in form and surface chemistry. This will require further analysis by, for example, electron microscopy, RAMAN spectroscopy, fourier transform infrared spectroscopy and surface charge characterization. Once the degraded plastic particles are separated and characterized meaningful tests on how the different pieces affect the aquatic organisms can be performed. It will also be possible to evaluate how much of each size a certain amount of plastic will degrade into and thereby approximate its biological impact.

Effects on organisms

We have pointed out that data on the effects of nano-plastics on aquatic organisms is scarce and there is an urgent need for more thorough studies. There are however three important factors that have to be considered. Firstly, the origin of the plastics is important. As we have described nano-plastic can originate from direct release of nano-plastic in products or from the degradation of discarded plastic (Fig 1). Therefore, plastic used in experiments preferably should come from these sources or be a relevant model plastic. In any case the material needs to be well characterized for its size and surface chemistry. Secondly, the size of the nanoparticles are important for its biological impact. Therefore experimental set ups should include differently sized plastics in the nano size range and preferably reference material in the micro size range. Thirdly, although nano-plastic is expected to aggregate with itself or with other biological matter in the surrounding environment the available data suggest that size dependent properties remain after the particles have entered the organisms. Therefore, testing of nano-plastics should include, when possible, the monodispersed particle, aggregated particles and aggregates with different biological matters. One way to achieve this in consumers is to expose them to the nano-plastics through a food web (Fig 1). For example, model plastics, or break down plastics in "pure" water or water with relevant biological compounds, are characterized for size and surface properties and exposed to the first trophic level, in this case algae, or directly to the second or third trophic level (Fig 1). Although the exposure route through algae may represent the most relevant scenario, the other two routes may reveal the importance of aggregation and biological process of the particles. The nano-plastic incubated algae are thereafter exposed to zooplankton which are exposed to fish. The organisms are then characterized for survival rate, and behavioural and biochemical changes. Factors that need to be taken into account is exposure time in each trophic level and how to avoid free nano-plastic in the next trophic level. The nano-plastic dose is another factor that is difficult to determine. It will be depending on the exposure times and number of exposure events. We have seen that the nano-plastic effects on the top consumer in a food web were manifested after two to three months of repeated exposure [68, 75]. When using food webs it is important to document how the size and composition of the particles affect each organism, since a chemical effect can be manifested as a behavioural or morphological change. This might induce a secondary effect on the next trophic level in the food web without a direct effect of the plastic.

We see two main ways to study the effects of nano-plastic on organisms, laboratory experiments or sampling organisms from the environment. Although both are needed, the possibility to detect nano-plastic effects in organisms in their natural environment is limited, and the few available techniques are time consuming and expensive at least for organisms larger than a few mm. Therefore, we conclude that for the time being the most achievable way forward is to create realistic laboratory conditions.

Acknowledgements

We would like to thank the Centre for Environmental and Climate Research, the NanoLund at Lund University, the Swedish Research Council (vr) and the Crafoord Foundation. We would also like to thank Simonne Jocic for comments on the manuscript.

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Figure 1: Plastic nanoparticles can enter the aquatic food web either as plastic waste degraded to nano-size, or as manufactured nano-sized plastic particles. Nano-sized particles from both those pathways will mainly enter the aquatic food chain via algae and bacteria, which are then consumed by filter feeders (black arrows), or taken up directly through filter feeders (grey arrows) and then eaten by fish.