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Review Article

Nano-Systems for Micro-Nutrient Delivery in Aquaculture: A Critical Analysis

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- Nanoparticle dynamics
- Hypothetical reactor kinetics
- Aquaculture uncertainties

Abstract

There is growing interest in engineered nanoparticle (NP) based micronutrient delivery systems in aquaculture. However, a comprehensive understanding of the interactions of NPs with its surroundings is required in order to apply these NPs as micronutrient carrier to aquatic animals. A monodisperse and stable NP selection is the first important step to reduce any uncertainty in such delivery systems. Then the NPs should survive during the administration process and get equally distributed among target animals in an aquaculture tank. In case of delivery via feed, the ill-defined raw materials (like fish meal, soybean meal, rapeseed meal, groundnut meal, fish oil, soybean oil etc.) and harsh processing conditions can be a great source of error. Also in the animal body, the NPs should dislodge from the food and survive the gut environment until they get absorbed in the epithelial tissue. Finally, they should be circulated to the target tissues by blood in physiologically significant amount. If the NPs are administered through water, there should be minimal loss of NP due to the myriad of reactions possible in the water column. Rigorous analysis of the fate of NPs in the said delivery steps becomes even more important for some cases (like SeNP) where the window of the effective and toxic dose is very narrow for aquatic animals. Hence, this communication critically examined the mentioned issues and proposed a chemical reactor model to simplify the complex sequence of delivery process of nano-sized micronutrients.

ABBREVIATIONS

NP: Nano Particle; FCR: Feed Conversion Ratio; PDI: Poly-Dispersity Index; AI: Active Ingredi

INTRODUCTION

Continuous depletion of natural fish resources is creating a steep demand for the manmade production system for fish and other aquatic animals [1]. In last four decades, commercial aquaculture has grown magnificently and it is currently contributing almost half of the current global fish consumption [1]. Therefore, traditional pond culture system with an average productivity of few tonnes/hector/year is getting replaced by more intensive fish cultivation systems with productivity up to few hundred tonnes/hector/year to cope up with the growing demand. Also, domestication of new aquatic species for farming is diversifying aquaculture [2]. To support this high productivity and variety, use of technology and adaptation of good fishery management practices are of primary importance [3]. Higher concentration of animals in aquaculture require stringent control over the feed, water quality, and other cultivation conditions to reduce any possible stress on the cultivated animals [4]. In this context, supplying appropriate nutrition is one of the essential and most challenging aspects of intensive aquaculture. Farmers are preferring nutritionally rich but costlier feeds over conventional cheaper feeds. In the first generation of designed feed for aquatic animals, the major emphasize was on delivery of macronutrients or precisely delivery of appropriate quantity and quality of protein and fat content to replace fish meal and fish oil. However, enhanced micronutrients delivery to the cultivated animals is often a good strategy to mitigate stress generated in aquaculture tanks especially at higher stocking densities [5].

Use of nanotechnology has become a ubiquitous tool for solving various problems in aquaculture like water quality control, disease treatment, fish nutrition etc. [6]. For better delivery of micronutrients, engineered nanoparticles (NPs) have been used in food processing, agriculture, animal husbandry, and aquaculture [7,8]. However, nanotechnology is still in its infancy in commercial aquaculture due to lack of understanding of the process involved and its impact on the target animals plus environment. Any physiological role of NPs depends on their structural (size, shape, dispersity etc.) and functional characteristics (surface properties). They can be made up of inorganic (metal, metalloids, metal oxides, chalcogens, carbon) or organic (natural or synthetic polymers and lipids) substances. NPs can be used in powder or dispersion or emulsion form depending on the application [9]. Recently many studies on the

delivery of minerals via metal/metal-oxide NPs and delivery of other organic micronutrients via polymeric nano-carriers to fish or crustaceans have been reported (Table 1). This communication intends to identify the uncertainties in the application of NPs for micronutrients delivery in aquaculture. The discussion is restricted to most studied ones i.e., metal and metal oxide and polymeric NPs. In order to predict physiological efficacy and safety of nanoparticles, the change in quantity or quality of the nanoparticles throughout the entire process of delivery to the tissues of an aquatic animal need to be understood. Also in this report a theoretical frame work has been suggested to assess the uncertainties in the nano-delivery systems for micronutrients in aquaculture.

Uncertainty in selection of nanoparticles for micronutrient delivery

Nanoparticles exhibit extraordinary functionality including physiological role due to their size, shape, morphology (crystallinity or hierarchical structure) and surface properties (charge and hydrophobicity). Bare inorganic nanoparticles of zero valent metals or metal oxides usually form aggregates in aqueous solution and create a mixture of poly-disperse NPs. A capping agent or stabilizing molecule (polyelectrolyte, protein, surfactant etc.) is necessary to restrict the aggregation and stabilize the inorganic NPs [10]. On the other hand, in polymeric nanoparticles like nano-chitosan one or more polymers appropriately arrange themselves to give rise to thermodynamically stable colloidal structure [9]. Therefore, preparation method is extremely critical to achieve a NP of appropriate quality for an intended use. Also, the NP formulation whether suspension or solid powder should be mono-disperse so that meaningful correlation of NP properties and a specific function can be made. Recently a whole gamut of studies has been devoted on biogenic nanoparticles where a single biomolecule (e.g., enzyme) or crude extract from plants, animals or microbes (e.g., aloe vera extract, fish gill extract etc.) has been used as a reagent plus capping agent for a variety of inorganic nanoparticles. Usually, such synthesis methods are considered as green method due to absence of extreme reaction conditions (e.g., high temperature or pressure) or hazardous reagents (e.g., harsh oxidants or reductants, acid or base, and solvents) [11]. However, in case of crude extracts, care should be taken for controlling properties of the NP. Often the mechanism of such bio-synthesis process remains obscure because of the complexity of composition of the crude extract. Repeatability too is a great concern for these methods of NP synthesis due to the inherent variability of sources of the natural extracts plus sensitivity of the NP production process [12]. A small change in conditions like extract composition, extract pH, the temperature of reaction etc. can influence NP properties drastically. Typically, natural extracts tend to form poly-disperse NPs and fine tuning of the process conditions is essential to produce a homogeneous suspension of NP [12]. Recovery of NPs of a particular type from the biological reaction mixture is very challenging owing to a large number of constituents [13]. For any micronutrient delivery study via NP should start with the detailed physical characterization of the NPs using electron microscopy, X-ray diffraction, Dynamic Light Scattering, infrared absorption spectroscopy, Raman scattering, surface-enhanced Raman scattering etc. Each of these methods has their own advantages and limitations but a detailed discussion is out of the scope of this review. Many updated and well-written references are available for such techniques [14,15]. Once the element to be delivered is decided, the correct type of NP to be rationally chosen. For example, to deliver Fe there is a great pool of iron nanoparticles available commercially with different sizes (less than 5 nm to 100 nm), valency states of the metal (0, II, III, mixed oxide), shapes (irregular, spherical, core-shell structure etc.), functionalizations (*N*-succinimidyl ester, biotin, poly ethylene glycol, streptavidin etc.), and formulations (aqueous and non-aqueous dispersion and powder). Similarly, for an active ingredient (e.g., vitamin C) the Nano matrix to be selected judiciously to provide required stability and better bioavailability. In the application studies, an essential parameter called poly-dispersity index (PDI) is often ignored. PDI indicates the standard deviation of particle size for a NP sample expressed as the percentage of the mean particle size. For a reliable application study of NPs, the PDI should be smaller than 25% [16].

Nanoparticle dynamics in aqueous environment

A great body of literature is available on how the local environment and various nanoparticles can influence each other in dispersed condition [17]. Especially non-specific interaction of proteins/peptides and ions in the aqueous phase are well studied. In a protein-rich aqueous environment, a coating of adsorbed protein over the nanoparticles known as protein-corona occurs in case of both inorganic and organic nanoparticles (Figures 1,2). This corona changes the hydrodynamic radii of individual nanoparticles and in some cases alter their tendency to aggregate [18]. Different ions suspended in the NP containing media regulate pH and ionic strength of the local environment and therefore dictates the surface charge of the NPs [17] (Figure 1). In addition to these interactions different metal NPs exhibit tendency of dissolution to the various degree in an aqueous medium. With the progress of dissolution eventually, the size of the NPs diminishes and the constituting ions release in the medium [19]. This dissolution process depends on various intrinsic parameters (size, shape, morphology, surface character, exposed surface) as well as extrinsic parameters (solution environment viz., pH, ionic strength, the presence of organic matters and temperature) [19]. In case of ZnO NP at low pH (1.5), the half-life of the NP is very small 11s compared to that in neutral pH 12.5 day because of accelerated dissolution in acidic condition [20]. In micronutrient delivery system before employing a NP its dissolution pattern in physiology like conditions (pH, ionic strength etc.) should be known. Figure 1 depicts the idealized conversions of a metal NP occurs in an aqueous environment. For a moderately dissolving NP like ZnO NP where the dissolution is diffusion controlled, a simple model could be equation 1a can describe the process [21]. Whereas for a slow dissolving NP like TiO_2 NP in neutral pH size change effect during dissolution needs to be included. Hence for slow dissolving NPs, [22] proposed the following model (equation 1b).

$$\frac{M_{diss}}{M_{eq}} = e^{(a-kt)} \quad (1a)$$

$$\frac{M_t}{M_0} = xe^{k_{norm}t} \quad (1b)$$

Table 1: Review of some nanoparticle based micronutrient delivery study in aquatic animals.

NP type	Characterization done	Mode of administration	Target aquatic animal	Experimental Design	Findings	Reference
Se NP	Size 30 to 45 nm	Added in feed extruded, and air dried.	Juvenile common carp,	3 levels of SeNP (0.5, 1, 2mg/kg dry diet) were tested for checking growth performance of fish.	1 mg nano-Se per kg diet to improved carp growth but 2mg/kg showed toxicity.	Ashouri et al 2015[46]
SeNP	Unclear	Added in mineral premix. Mixed, ground, Blended, pelletized and air-dried.	Blunt snout bream, Fish	Effect of Na_2SeO_3, and Se NP, at 0.2 mg Se kg^{-1}, Se-yeast at 0.1, 0.2, 0.4 and 0.8 mg Se kg^{-1} supplementation on growth, antioxidant activities, muscle composition and meat quality were tested.	Se-yeast and Nano-Se showed better growth performance, antioxidant activities and enhanced meat quality than Na_2SeO at 0.2 mg Se kg^{-1}.	Liu et al 2017[47]
Se NP (+/-Vit E)	~ 95 nm NP Se	Extruded into pellets	Juvenile Mahseer fish	Vitamin C (100, 200, and 300 mg kg^{-1}) were used in combination with Nano Se dose (0.68 mg kg^{-1}) to see their synergistic effects on growth, feeding, and physiological parameters determined.	At Vitamin C_{300}+Nano Se $_{0.68}$ mg kg^{-1}Blood physico-chemical characteristics Hb, WBC, RBC count improved, liver and muscle protein content increased.	Khan et al 2017[48]
Se NP	Unclear	Artemia sp was grown with nano-Se and fed to fish larva	Artemia sp. (used for fish larvae)	Survival rate, growth parameters, Se retention and glutathione peroxidase enzyme activity were analyzed in fish larvae fed with four enriched Artemia stocks (1, 5, 10, 50 mg/l Se) after 9 Day experiment,	Moderate level of selenium enrichment (~4 mg/kg dry matter) influences the rearing efficiency of fish larvae, but higher dosages could cause adverse effects.	Juhasz et al 2017
SeNP	30 - 45 nm	Added in feed, extruded and dried.	Common carp	SeMet, sodium selenite, NP Se Na_2SeO_3 were added to the basal diet at 0.7 mg Se kg^{-1} diet. Effects on growth, muscle Se concentration, muscle proximate composition, blood enzymes and antioxidant status was measured.	SeNP acts more efficiently on growth performance and antioxidant defence system of common carp than organic and inorganic sources of Se.	Saffari et al 2017[49]
Nano-encapsulate of vitamin C	<300 nm	NPs were prepared by ionotropic gelation and Injected directly in S. senegalensis postmetamorphic larvae	Solea. senegalensis postmetamorphic larvae, Rotifers Brachionus plicatilis	10 µl of solution of FITC-NP solution was administered in larvae and it was analyzed using CLSM to check actual uptake. The rotifers Brachionus plicatilis were fed with a diet of yeast in seawater at 100–600 rotifers/ml. Rotifers were analysed for their vitamin C content by HPLC.	Potential cytotoxicity of the NPs was evaluated in ZFL cells and no decrease in cell viability seen up to 2.5 mg/ml of NP. Two times more uptake of vitamin C was noticed with nano-encapsulated form.	Jiménez-Fernández et al 2014
Se NP	36 nm	Directly added to tank water	*Oryzias latipes,*	Adult fish were administered to a graded series of sodium selenite and Se NP solutions for consecutive 48 h and fasted during the acute exposure. Median lethal concentration (LC50) was calculated	Se NP exhibited strong toxicity for Medaka approximately five fold difference in terms of LC50 compared to selenite due to hyper-accumulation of Se in liver and oxidative stress.	Li et al 2008[50]

SeNP	60 to 80 nm	Se sources were mixed slowly with the diet ingredients part by part in a drum mixer then extruded and air-dried at RT.	Carassius auratus gibelio	3 treatments T-1- (Se NP) T-2, selenomethionine and Control. Relative gain rate and final weight were measured.	Control showed lower Se content in muscle compared to T-1 and T-2 and the highest value of Se content in muscle was observed in Se NP. Survival rate and feed conversion ratio were not affected by the dietary treatments.	Zhou et al 2008[51]
Fe_3O_4 NP	<50 nm	Fe NP and Fe_2SO_4 were added to the basal diets ingredients, then extruded and air-dried.	Rainbow trout, Labeo rohita,	Effects of 0.5 mg of Fe_3O_4 NP (T1) and Fe_2SO_4(T2) per kg dry feed on Hematological parameters, Respiratory burst activity, Serum bactericidal activity were assayed was determined.	Control showed lower Fe content in muscle compared to T1 & T2 and Fe_3O_4 NP showed highest Fe content. RBCs and haemoglobin levels, immune parameters, bactericidal activity and myeloperoxidase activity were higher in FeNP-treated diet.	Behera et al 2014[52]
AgNP,	Unclear	AgNP synthesised by Aloe vera leaf extract was mixed with regular feed	*Catla catla,*	Fish were fed with a commercial pelletized formulated fish feed twice a day in 3 groups 1: control group, 2: supplemented with normal diet feed, 3: supplemented with synthesized NPs mixed with normal diet feed.	Nano diet feed fed fishes showed a gradual increase in weight gain, length gain and specific growth rate of fishes.	Vineela et al 2017[53]
Trypsin NE	147±.25 nm	Chitosan NPs prepared by ionotropic gelation with Trypsin. feed ingredients mixed with water to form dough, pressure cooked. vitamins and minerals and enzymes were added, pelletized and hot air-dried	Labeo rohita	Analysis of NE trypsin (0.01% and 0.02%) along with 0.02% bare trypsin and 0.4% chitosan nanoparticles against a control diet on productive efficiency (growth rate, feed conversion and protein efficiency ratio), organo-somatic indices, nutrient digestibility, tissue enzyme activities, hematic parameters and intestinal histology of the fish.	Enhanced fish productive efficiency using NE trypsin over its bare form was seen, which corresponded with enhanced nutrient digestibility, activity of intestinal protease, liver and muscle tissue transaminases and dehydrogenases serum blood urea nitrogen and serum protein profile.	Kumari et al 2013
ZnO NP	32-57 nm (TEM), XRD	ZnO NP prepared by chemical co-precipitation method and mixed with fish feed.	Talapia (*Oreochromis niloticus*)	Four concentrations (15,30,45 and 60 mg/kg) of ZnO NP and conventional ZnO were added to the feed Fish fed twice daily 3% body weight for 120 days. Growth performance, Growth Hormone, Zn concentrations in muscles, Nonspecific immunity function was determined.	ZnO NP (15mg/kg) achieved specific growth rates like ZnO (60mg/kg). The 60mg/kg ZnO showed the highest of Specific growth rates, growth hormone. The ZnO up-regulated the IL-1β better than the ZnO NP form. Zinc was higher in muscles of fish fed on ZnO NP but within the permissible limits.	Tawfik et al 2017[54]

SeNP	205 nm (P	Biologically synthesised Se-NPs from fisheries waste, pelletized and oven dried crushed into fine power and fed.	*Pangasius hypophthalmus*	Fish reared under lead (Pb) and high temperature (34 °C) for 72 days. Thermal tolerance and cellular metabolic stress were evaluated.	Thermal tolerance increased with SeNP feeding and also improvements in the oxidative and metabolic enzymes. Incorporation of Se-NPs @ 1 mg/ kg in diet could protect P. hypophthalmus against Pb and thermal stress.	Kumar et al 2017[55]
Se, Zn and Mn NPs	250 - 500 µm	Microdiets prepared by mixing squid powder with water-soluble components, vitamins and gelatin, pelletized, dried, grounded and sieved.	Seabream larvae	Larvae previously fed rotifers enriched with DHA Protein were fed one of the experimental diets (with no minerals, organic and inorganic form of the mineral and nano form of the minerals) every 45 min from at a rate of 2.5–3.5 g/tank for 24 days. Diet acceptance was determined by image analysis.	Feeding with inorganic forms of these minerals was less effective than organic minerals in improving larval weight or bone mineralization in comparison to the non-supplemented diet. Moreover, the larvae were less resistant to stress, and fish showed higher bone anomalies in the pre-hemal region. Adding Zn, Mn and Se in the form of nanometals did not enhance growth, but improved stress resistance and bone mineralization.	Izquierdo et al 2017[56]
SeNP	200-250 nm	Se NP was incorporated into the basal diet. Mixing all ingredients evenly, pelletizing and drying to a moisture level of 10%.	Chinese mitten crab (Eriocheir sinensis)	Se NP at levels of 0.1, 0.2, 0.4, 0.8, and 1.6 mg/kg were fed twice daily at a rate of 3% body weight of crabs then exposed to hypoxic conditions and injected with 4 _ 106 CFU/ kg. Effects of SeNP on crabs in hypoxic condition was studied.	Diet with 0.2 mg/kg Se NP showed increased weight gain rate and reduced feed coefficient compared to those diets with 0, 0.1, 0.4, 0.8, and 1.6 mg/kg SeNP. Dietary supplementation with SeNP offers resistance to hypoxia stress and improves immunity and disease resistance.	Qin et al 2016[57]
Nanochitosan/ zeolite composite	30–40 nm sized chitosan particles loaded on zeolite SEM, TG, XRD done	Nanochitosan was mixed with zeolite followed by ultrasound to get nanocomposite. Added to the diet by spraying 20 ml fish oil per kg feed.	Rainbow trout (Oncorhynchus mykiss)	Prepared composites, zeolite and control diets were fed to rainbow trout in 8 groups for 60 days. Protective effects of experimental diets on digestive enzyme activities and bio-chemical parameters were estimated	Supplemental zeolite could enhance the amylase activity whereas other treatment could increase the pepsin activity besides intestinal alkaline phosphatase, trypsin and amylase activities. Nanochitosan/zeolite composite at 5 g/kg had potential to enhance growth performance, digestive enzyme activities and some biochemical parameters in rainbow trout.	Sheikhzadeh et al 2016[58]
Chitosan NP	62.4–86.5 nm, with the average size 76.2 nm	Chitosan NP added to the feed extruded, and air-dried at room temperature,	Tilapia, Oreochromis nilotica	Effect of dietary chitosan and chitosan NPs at 5 g/ kg on survival rate, growth performance and meat quality of tilapia (Oreochromis nilotica) under laboratory conditions	The addition of chitosan nanoparticles significantly improved ($P < 0.05$) final weight, daily weight gain and feed conversion ratio of the fish	Wang and Li 2010[59]

Zn NP	50 nm	The dough with ZnNP was cooked at 105 °C, cooled mixed with egg albumin and cod liver oil and pelletized.	Macrobrachium rosenbergii	ZnNPs were added at 0, 10, 20, 40, 60 and 80 mg kg−1 with the basal diet, and the level. ZnNP-incorporated diets were fed to M. rosenbergii PL (initial body weight, 0.18±0.02 g) for a period of 90 days.	ZnNP supplementation up to 60 mg kg−1 showed significantly (P<0.05) improved performance in survival, growth and activities of digestive enzymes (protease, amylase and lipase) but at 80 mg kg^{-1}	Muralisankar et al 2014[60]
CuNP	50 nm	Same as Muralisankar et al 2014	Macrobrachium rosenbergii	Cu-NPs were supplemented at 0, 10, 20, 40, 60, and 80 mg kg−1with the basal diets. These Cu-NPs supplemented diets were fed to M. rosenbergii PL for 90 days.	Significant (P < 0.05) improvements were observed in survival, growth, digestive enzyme activities, concentrations of bio-chemical constituents and total and differential haemocytes count of prawns fed with 20 mg (200 nm) Cu-NPs kg^{-1}supplemented feed but 40–80 mg Cu-NPs kg^{-1}supplemented feed showed negative performance.	Muralisankar et al 2016[61]
Mn_3O_4 NP	Spherical with an average size of 40–50 nm synthesized with pineapple peel extract	Added to feed pelletized and dried at 40 ^{0}C	Macrobrachium rosenbergii	Seven groups of M. rosenbergii PL (1.42 ± 0.35 cm length; 0.18 ± 0.02 g weight) were studied with different levels of Mn NP for 90 days.	The experimental study demonstrated that prawns fed with diet supplemented with 3–18 mg Mn-oxide NPs/kg shows enhanced (P < 0.05) growth performance, final weight, FCR.	Asaikkutti et al 2016[62]
Se NP (+/-Vit E)	Unclear	Mixed in the feed dough pelleted and air dried.	Rainbow trout	Effect of different levels of diet studied viz., normal (20 kg m^{-3}; basal diet), Dense (80 kg m^{-3}; basal diet), Vit E (80 kg m^{-3}; 500 mg kg−1 vitamin E-supplemented diet), SeNP (80 kg m^{-3}; 1 mg kg^{-1} SeNP-supplemented diet), and Combination (80 kg m^{-3}; 500mg kg^{-1} vitamin E and 1 mg kg^{-1} Se NP supplemented diet).	vitamin E supplemented diets can exert positive effects on the welfare of chronically stressed rainbow trout subjected to an additional acute stressor.	Naderi et al 2017[63]

Abbreviations: AgNP: Siver nanoparticle; CLSM: Confocal Laser Scanning Microscopy; DHA: Docosahexaenoic acid; FCR: Feed Conversion Ratio; FITC-NP: Fluorescein Isothiocyanate Labeled Nano Particles; Hb: Haemoglobin; HPLC: High-Performance Liquid Chromatography; LC: Lethal Concentration; NE trypsin: Nanoencapsulated trypsin; NP: Nano Particle; RBC: Red Blood Cell; SEM: Scanning Electron Microscope; SeMet: Selenomethionine; TEM: Transmission Electron Microscopy; TG: Thermo Gravimetric Analysis; WBC: White Blood Cell; XRD: X-ray powder diffraction; ZFL: Zebrafish liver cell-Line

Where $M_{0,}$ M_t, $M_{eq,}$ M_{diss} are the concentration of total Zn NP initially present, remained after time t, Zn^{2+} at equilibrium and Zn^{2+} at any time t of the dissolution process. Parameter 'a' separation from equilibrium and k approach towards equilibrium. 'x' is the mass fraction of the dissolved Zn^{2+} and k_{norm} is the specific rate of dissolution.

In case of organic NPs or precisely solid polymeric NPs, the active ingredient can exist in either of the three basic forms [23]. First one is reservoir type (where the active ingredient (AI) is located in the core and slowly diffuses through the outer polymeric matrix layer. In the second case if the AI is soluble it can be uniformly distributed throughout the matrix phase and dissolve in the water. The third kind is rare one where the AI is locked at the core but does not dissolve in the external medium but requires matrix dissolution to be released in the external medium (Figure 2). Organic NPs are also amenable to aggregation [24], corona formation and electrolyte interaction in aqueous solution and additionally, the polymeric matrices can undergo unfolding/dissociation of the chains to lose its structure [25]. These multiple changes of a NP are difficult to predict because of their interdependence. At a higher temperature, these reaction rates can increase in different degree and their impact on the physico-chemical status of the nanoparticle can be profound.

Incorporation of nanoparticles in feed: possible alterations

Most of the studies on micronutrient delivery attempted to incorporate different forms of inorganic and organic NP in the feed of the aquatic animals (Table 1). Indeed, the feed is the most convenient means of delivery of the NPs through the

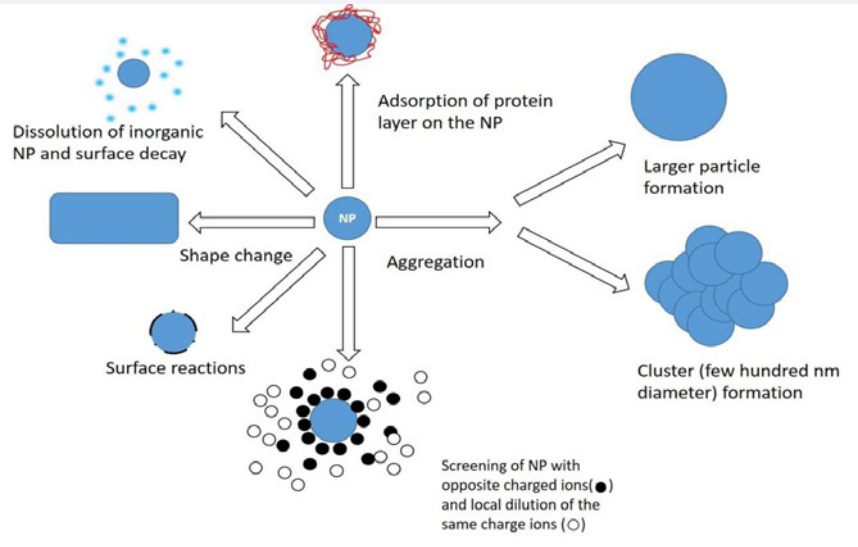

Figure 1 Interaction of inorganic nanoparticle with aqueous phase constituents.

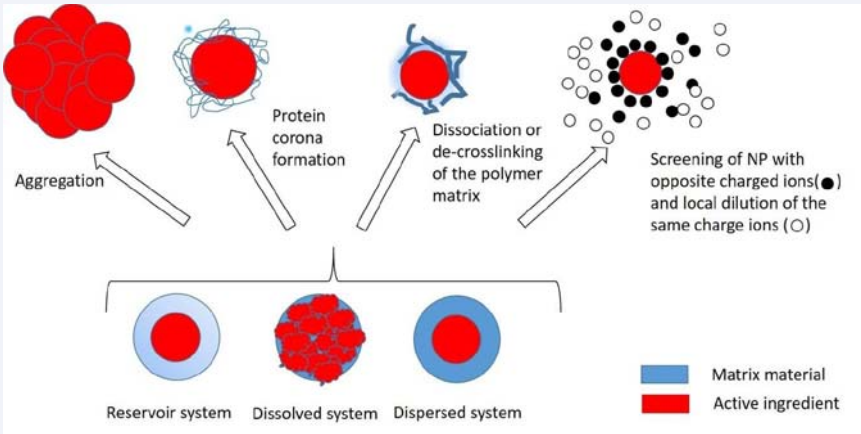

Figure 2 Interaction of organic nanoparticle with aqueous phase constituents.

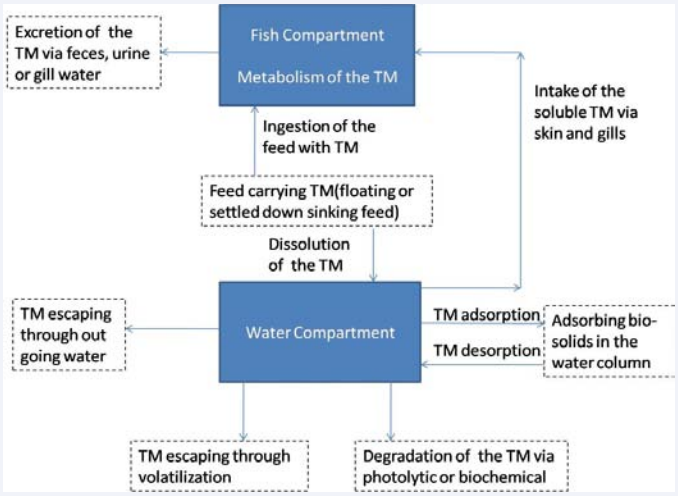

Figure 3 Fate of a particular nanoparticle. (TM or target material represents the so called most effective form of nanoparticle that has been applied for the micronutrient delivery purpose and it is not any other derivative which is formed in the aquaculture tank.).

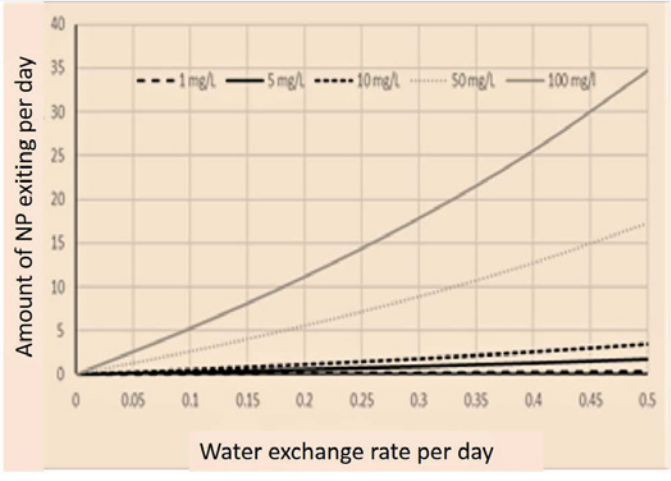

Figure 4 Amount of nanoparticles exiting from an aquaculture tank for various concentration of nanoparticles in the water at the different water exchange rates.

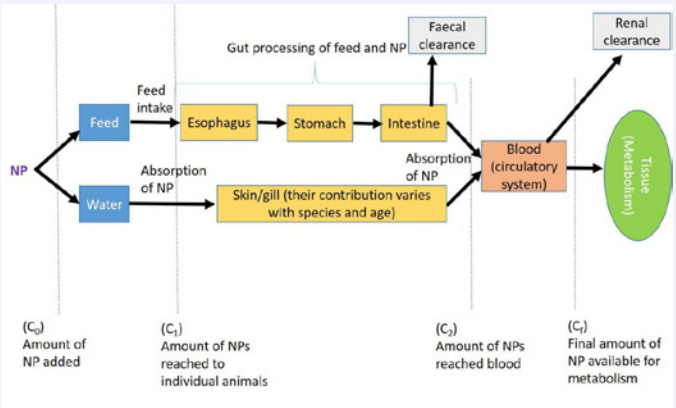

Figure 5 Schematic representation of transport and processing of nanoparticles during micronutrient delivery in physiological phase of an animal in aquaculture.

oral route that eliminates any dilution in the water phase of the aquaculture tank/pond or unwanted absorption through skin or gills. Commonly the NPs are mixed into the feed ingredients preferably in a blender. Water is added to form dough out of the ingredients and it is then extruded, cooked, baked, or simply dried in pellet form [26]. A typical commercial feed formulation example is fishmeal (15 %), meat meal (5 %), soybean meal (20 %), groundnut meal (10 %), rice bran (10 %), wheat middling's (15 %), corn/broken rice/cassava (15 %), vegetable/fish oil (4 %), dicalcium phosphate (2 %), vitamin premix (2 %) and mineral premix (2 %) (www.fao.org). The ingredients in dry feed preparation would start with mixing powders. So the first uncertainty arises in proper mixing of micronutrients in bulk solid ingredients which is always difficult to mix compared to liquid blending [27]. Commonly the solid mixing is done in stages of 1:10 weight ratio i.e., the amount of NP is dispensed in a smaller quantity of ingredient mix and the premix obtained is added to a ten times larger quantity of the bulk ingredients. The operation continues until the entire quantity is mixed. Here the aim is to distribute the NPs in such a way that each of the final pellets formed should have almost the same amount of NP within. A salt solution is easy to mix but a batch of NP having very different physical property than rest of the ingredients is a challenging task. Hence, the mixing process should be standardized (i.e., proper settings of the speed of rotation, mixing time etc. to be decided) for a given mixing device (double cone blender, ribbon blender etc.) [28]. A convenient mixing index (MI) can be used to find optimal conditions for mixing. For two components MI can be defined as $(s_o^2 - s_t^2)/s_o^2$. Where S is the standard deviation of the concentration of a target component (here NP) among various sample tested at any given time of mixing. 0 and t indicate the beginning and after t-time of mixing progress respectively [29]. MI should increase from 0 to 1 theoretically during mixing but a value near 1 is practically acceptable.

Next level of uncertainty is during mixing where the feed ingredients are moistened to get the consistency of a dough. The aqueous environment in such dough provides the NPs opportunity to change structurally as discussed in the previous section. Subsequently, when the dough is processed (steamed or extruded) the major constituents the two major components of the feed starch and protein undergo drastic chemical change.

Starch molecules hydrate and gelatinize whereas protein molecules denature on thermal treatment. Depending on the exact composition these two reactions cause plasticization of the feed mass. The microstructural changes of the feed during different processing condition are not much studied barring few case of feed extrusion [30]. However, examples from food processing suggest, the apparent continuum mass of gelatinized starch possess significant heterogeneity in microscale (< 100 μm level) in food extrusion [31]. NPs in such microenvironment at high temperature can undergo dynamic changes. In case of Se NP, a study revealed thermal treatment of 1 h at 90°C converted spherical particles of the mean diameter of 80 nm into not only larger but also into rod-shaped particles. Therefore, thermal processing of NP embedded in dough creates the possibility of heterogeneous transformation of the nanoparticles. Perhaps addition of NP in post thermal processing stage would help to restrict any unwanted changes in feed. Otherwise at least an assessment of the changes occurring to the NPs might be done so that additional NP can be added to compensate for the loss of the nanoparticles.

Once the NPs are successfully incorporated into the feed another challenge is to reduce fish to fish variation and temporal variation of fish intake in a particular aquaculture system. Average parameter values like weight increase, mean feed conversion ratio (FCR) may not provide the actual physiological impact of the NP [32]. For a given system (tank, fish species, fish age, fish/animal density, water flow, aeration etc.) feeding protocol to be standardized to reduce competition and other causes of intake variation [33]. Also, incorporation of NP can often change the feed palatability compared to the control feed. Therefore, real-time monitoring of feed intake can eliminate any such variations. Image analysis is a technique which is costly but effective method of feed monitoring [26].

Fate of NPs in the water compartment of an aquaculture tank

In case of NP delivery via feed, if proper feed quality (feed stability > 4 h; good palatability) [26] and feeding management (appropriate feeding dose and frequency) is maintained leaching of NP from the uneaten feed can be minimized. However, if the uneaten feed is significant the NP leaching in the water column of the aquaculture tank cannot be ignored. Also the excreted NP form the cultured animal would contribute NPs in the water. Often NPs are delivered directly for better absorption by the animal [34,35]. Figure 3 gives a general scheme of dynamics of NPs in water column of an aquaculture tank. As discussed earlier NPs would involve in multiple transport- and reactive-processes in the water column. There would be structural changes, dissolution of constituents (ion dissolution from inorganic NPs, unfolded polymers from polymeric NPs) and secondary degradations like photocatalytic or biochemical degradation.

Direct addition of NPs has two major uncertainties; first error in predicting the effective NPs delivered to the animals due to the multiple processes involved in it (Figure 3). Secondly, the amount of NP waste generated because of the water exchange. Assuming well stirred condition in a flow through aquaculture system the amount of NP waste generated in a 500 L tank per day has been simulated with various water exchange rate (Figure 4). Water exchange rate depends on the cultured animal/fish species, stocking density, required self-cleaning of the tank, aeration etc. [36]. Hence, for 100 mg/L water phase concentration of NP and for 30 % per day water exchange rate, 18 g of the NP would exit from the tank. Both the issues to be considered before using micronutrient NPs directly in a commercial aquaculture.

Interaction of the Nanoparticles with the physiological phase

The modes for micronutrient delivery via NPs to aquatic animals can be orally (both directly or with feed) or through the water medium of aquaculture tank (Table 1). The water phase NPs below a particular size can theoretically enter via skin, gill, gut epithelial tissue and reach the blood circulation system. In case of inorganic NPs, it is more likely both the constituent ions

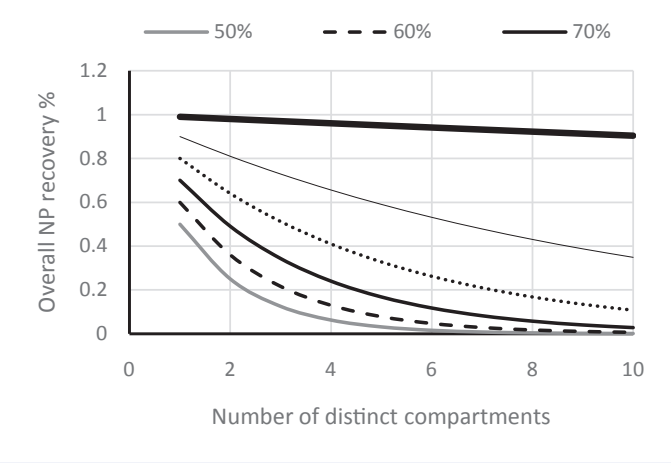

Figure 6 Variation of overall recovery ratio of nanoparticles w.r.t number of functionally distinct compartments (or required hypothetical reactors) and percentage recovery in each step.

plus NPs will enter the circulation system. Assesment of any possible health benefit should consider the contribution of both entities of a metal. It is generally believed the nanosized mineral or nanoencapsulated micronutrient (like a vitamin) augments bioavailability of the micronutrient. Often this generalization can be misleading because bioavailability is a multifarious process consisting of five basic steps; the liberation of the NP, absorption through the gut epithelial tissue, systemic circulation and distribution in other tissues, metabolism at various locations for maintaining (or often augmenting) physiological activity and finally clearance from the body [37]. This chain of the event varies with species as well as with life stages of a given species. Figure 5 exhibit a cartoon of the assimilation of the NPs (or the active micronutrient) through various routes (skin, gill, gut). For increased biovailability, at least one step of the first four steps should have more flux of the micronutrients. While delivering through diet, the liberation of NPs from the feed matrix can be tested *in vitro* whereas survival of the NPs in the aqueous environment of the gut is often difficult to assess. Nevertheless the earlier discussion on the interaction of the NPs with the aqueous environment can provide some theoretical insight. For better bioaccessability (absorption and pre-systemic processing) if the bowel movement and/or excretion rate is slower it can increase the gut adsorption. Here the changing environment of the gut (e.g., local pH) and digestibility of the medicated feed needs to be evaluated *in vitro*. Understanding of microstructure changes during gut processing of food and its influence in micronutrient liberation, conversion, and absorption in other animal systems, can help in visualizing bioaccessability of micronutrients in aquatic animals and designing appropriate *in vitro*, *in vivo* and *ex vivo* studies [38]. However, even in an aquaculture tank, the animals may show non-uniform bowel transit time which alters the bioavailability of micronutrients [39,40].

Internalization of NPs in cells happens through endocytosis depending on the size of the particles. NPs diffuse through the stagnant fluid layer adjacent to the epithelial tissue but there is a wide variation in the rate of internalization among different NPs and different tissues [41]. Recently, some *in vitro* studies have been done to varify cellular uptake of NPs using tissue culture [42,43]. However, such studies should be seen in conjugation with the behaviour of the target NP in an aqueous environment. Systemic circulation, distribution in various tissues, and metabolism of the micronutrient i.e, the element (e.g., Se) or molecular (e.g., vitamin C) species should be known for a target animal before initiating any NP-based delivery system design. In different tissues, accumulation and clearance of a particular nutrient happen differently so for NPs also similar tissue specific dynamics can be expected. Unfortunately, such dynamics of NPs in the aquatic animal systems is still less studied. Considering the large variety of aquatic animals are being cultivated (> 200 species) there is a pressing need to develop theoretical models by plugging in the data available from numerous discrete work on a particular species for a given family of NPs.

Framework of assessing uncertainty in the micronutrient delivery chain

A nanosized particle to exhibit any advantage over its non-nanoform (e.g., metallic nanoparticles over its constituting ion or nanoencapsulated vitamin over bare vitamin molecule), it should reach the site of physiological activity or specific tissue in intact form. The entire chain of events in nano-sized micronutrient delivery as discussed in the earlier sections is quite complex. Hence the decision of the optimal quantity of NP for a physiological activity is prone to error. However, using lumping of parameter principle of mathematical modelling, the events can be simplified into a manageable number of distinct compartments. In each of these compartments, the dynamics of NPs is quite different. For example, conjugation of NPs with proteins in blood would be quite different than that in low pH semisolid undigestible diet in the stomach. We propose a simplification of the entire chain of delivery of NPs with three minimum steps; NP reaching the epithelial tissue of the aquatic animal (via feed or water), entering the blood through absorption from gut or gill/skin and distributed to one or more target tissues through blood. In this three steps, the target NP is either transforming into some other entity or transporting out of the delivery system. It can be assumed that the three steps occurring in three hypothetical chemical reactors connected in series. Reactor analogy of biological processes is not new. There are studies where gut has been considered as a plug flow reactor [44], and the blood circulation system as a continuous flow stirred tank reactor [45], to analyze a diseased condition or healthy physiological status. In case of animals, such reactor model can help in designing an optimal micronutrient delivery system.

For an aquaculture tank, this idealism is assuming steady state w.r.t NP concentration and no significant difference between individual animals. Therefore, the NPs are distributed in four compartments *viz*., the abiotic compartment (feed or water column), gut phase, blood phase, and tissue (*cf*. Figure 5). For estimating the overall percentage of available NPs for physiological activity individual recovery ratio can be used. Therefore, the percentage of bioavailable NP or overall recovery ratio = $C_f/C_0 = (C_f/C_2) \times (C_2/C_1) \times (C_1/C_0)$ where C_0, C_1, C_2, and C_f are the amount of NP entering into the abiotic compartment (feed or water column), gut phase, blood phase, and tissue respectively. Hence, (C_1/C_0), (C_2/C_1), and (C_f/C_2) are the percentage recovery of NPs after it passes the abiotic compartment, gut phase, and blood phase respectively. Such analogy gives the idea that the overall recovery of NPs would be always lesser than the smallest recovery ratio among the three steps. The recovery ratio can varify if a particular NP delivery system is at all suitable for an animal. Also, the ratios would indicate the phase in which maximum loss of NPs is taking place to take remedial measure. It is worth mentioning for a more complex delivery system the number of dynamically distinct phases might be more in such case the overall recovery of NP would be very sensitive to the recovery ratio of individual steps (Figure 6).

CONCLUSION

The entire sequence of micronutrient delivery to the cultured aquatic animals is a complex process. It involves uncertainties in each step; the administration, processing in the gut, adsorption, distribution to tissues etc. Reactivity or instability of NPs makes the use of nanoparticles for micronutrient delivery much more complex. Due to which often discrepancies among the findings from different trials can be seen. For assessing physiological

advantage of the nanosized micronutrient delivery (e.g, increased bioavailability of nano selenium over selenium salt) or any suggestion of dosage requires more detailed study and single aspect (e.g., growth rate or FCR) based predictions are often misleading. The unique ability of the NPs in this context can only be harvested if a NP is properly chosen and administered in such way that maximum amount of the NP can be delivered in intact form to the site of metabolism. This communication has identified the possible sources of uncertainty and suggested a chemical reactor model to asses the efficacy of a NP delivery system.

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