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Aqueous fullerene aggregates (nC_{60}) generate minimal reactive oxygen species and are of low toxicity in fish: a revision of previous reports

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This review aims to clarify inconsistencies in previous reports regarding the potential for aqueous aggregates of fullerenes (nC_{60}) to generate reactive oxygen species (ROS) and cause toxicity in fish. Methods for evaluation of ROS production and toxicity of aqueous nC_{60} have evolved over time and limitations in initial studies have led to unintentional erroneous reports of nC_{60} ROS generation and toxicity. Some of these reports continue to lead to misconceptions of the environmental effects of C_{60}. Critical review of the evidence (2007–2011) indicates that aqueous nC_{60} have minimal potential to produce ROS and that oxidative stress in fish is not induced by environmentally relevant exposure to nC_{60}. Future studies should acknowledge that current evidence indicates low toxicity of nC_{60} and refrain from citing articles that attribute toxicity in fish to nC_{60} based on methods shown to be compromised by experimental artifacts. Despite low toxicity of nC_{60} in fish, an emerging environmental issue is that nC_{60} can affect environmental fate, transport, and bioavailability of co-contaminants in aquatic environments in a similar manner to that observed for other anthropogenic particulates (e.g., microplastics).

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Introduction

Advancements in nanoscience have enabled arrangements of atoms into novel configurations and development of new materials with unique properties that offer considerable potential benefits to society. Nanoparticles (NPs; particles with at least one dimension between 1 and 100 nm) and nanomaterials (NMs; materials that contain NPs) [1] are increasingly being incorporated into products, and lifecycle models of NMs predict that many NPs will ultimately be released into the environment [2]. To address concerns of negative environmental effects of NPs, emerging disciplines of nanotoxicology [3] and nanoecotoxicology [4] are evolving rapidly; however, lack of established methods for evaluating toxicity of NPs is a major concern [5]. Some initial experiments have led to erroneous reports that continue to contribute to a misunderstanding of NP toxicity and could impede potential benefits of nanotechnology by creating negative public perceptions [6].

One NP that has perhaps generated the most controversy regarding its toxicity is the C_{60} fullerene. C_{60} is a spherical cage-like molecule (~1 nm in diameter) composed of 60 carbon atoms arranged as a highly stable truncated icosahedron [7]. The ability of C_{60} to both generate and quench reactive oxygen species (ROS) has emerged as a particularly important property for interaction with biological systems [8], and numerous studies have investigated beneficial and toxicological effects of C_{60} [9]. Among those studies, investigations of C_{60} toxicity in fish have generated considerable interest because (i) effects in fish are frequently consistent with the effects in other vertebrates including humans, (ii) fish are important components of ecosystems, and (iii) fish are a primary exposure route of humans to persistent environmental contaminants that bioaccumulate [10]. Reports of ROS generation by aqueous preparations of C_{60} and toxicity in fish have been inconsistent and the objective of this review is to clarify misconceptions and discuss properties of aqueous of C_{60} that are of toxicological importance in fish.

Potential for C_{60} to generate reactive oxygen species (ROS) in water

Generation of ROS by C_{60} is influenced by the medium, functionalization of C_{60}, state of C_{60} aggregation, and presence and type of illumination. Delocalized π double bonds of the fullerene cage can absorb energy from light and produce a triplet excited state sufficiently long lived for high efficiency transfer of energy to molecular oxygen and formation of reactive singlet oxygen [11]. In water, the lifetime of singlet oxygen is only nano or microseconds, but this is sufficient to induce formation of other

ROS species [12] that are also highly reactive with biological molecules. However, C_{60} is extremely insoluble in water [13] and detection of ROS has been inconsistent for aqueous preparations of C_{60} {termed nC_{60}; diameter tens to hundreds of nm (and some exceed nanodimensions >100 nm) [14]}. If functional groups are added to C_{60} [e.g., $C_{60}(OH)_n$], the aqueous fullerene can produce ROS [15], but this review is focused on un-derivatized C_{60}.

ROS generation by aqueous nC_{60} has been related to preparation method, presence of associated substances in water, and exposure to light. Preparation methods for aqueous nC_{60} have been reviewed extensively [8] and can be divided into three categories as follows: solvent extraction ($nC_{60(solvent)}$ [16]); use of micellar solutions ($nC_{60(micelle)}$ [17]); and water stirred ($nC_{60(stirred)}$) [18]. ROS have been detected in aqueous $nC_{60(solvent)}$ preparations [notably tetrahydrofuran (THF); e.g. [19,20,21]; however, these reports have been confounded by evidence that solvent can reside between individual C_{60} molecules within $nC_{60(solvent)}$ [22], and that degradation products of solvents (e.g., THF) can remain in the water [23,24,25]. Zhang *et al.* [26•] demonstrated that $nC_{60(solvent/THF)}$ preparations contained oxidizing agents (THF degradation products) that explained ROS activity and that vigorous washing of $nC_{60(solvent/THF)}$ preparation was necessary to eliminate ROS activity. Results of experiments that did not appropriately control for solvent effects should no longer be used as evidence that aqueous nC_{60} can produce ROS.

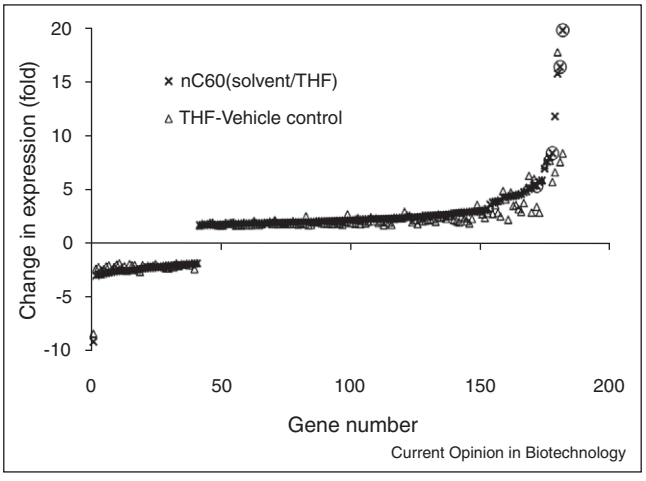
Potential for ROS generation in aqueous nC_{60} preparations that are not compromised by solvents depends on aggregate structure and interactions among C_{60} molecules. Aqueous nC_{60} preparations absorb light at wavelengths (near 450 nm [15,27]) expected to excite C_{60} to the triplet state; however, lack of photoreactivity and minimal generation of ROS have now been consistently reported for aqueous $nC_{60(solvent)}$ and $nC_{60(stirred)}$ preparations [15,28,29,30,26•,31]. An exception is the studies by Hou and Jafvert [32,33•] that report detectable ROS production after longer periods of solar irradiation, but investigators note that ROS is 'drastically' less than expected for C_{60}. Selfquenching (interactions among C_{60} within nC_{60}) is suggested to resolve higher C_{60} energy states induced by light absorption [29,32], a process influenced by aggregate size (based on theoretical models [34]), and also expected with bulk C_{60} [27]. Overall, present evidence indicates that ROS production by aqueous nC_{60} is minimal.

Appreciable amounts of ROS can be generated by micellar solutions of aqueous $nC_{60(micelle)}$ depending on arrangement of C_{60} molecules and associated substances. Numerous studies have reported ROS generation in $nC_{60(micelle)}$ preparations [15,28,29,35,36,27,17], and ROS production can occur when a surfactant (e.g., Triton X100, TX) is applied at above the critical micelle concentration (cmc) [27]. Above the cmc, C_{60} may behave as if dissolved in an organic solvent; however, when nC_{60} aggregates are present within micelles, selfquenching is likely (as described above) and ROS are minimal [15,27,34]. Environmental relevance of $nC_{60(micelle)}$ is debatable, but behavior of C_{60} within micelles may be similar to what could happen if C_{60} is able to reside within a lipid bi-layer of a cell membrane [15].

Clarification of C_{60} toxicity in fish

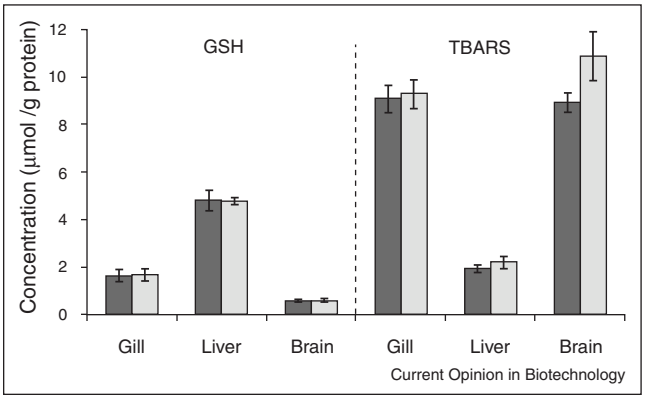
Oxidative stress has been reported in fish exposed to aqueous $nC_{60(solvent)}$ preparations, but recent studies indicate that some results must be revised. Oxidative stress reported in $nC_{60(solvent/THF)}$ exposures that did not control for solvent effects [e.g. [19,37,38]] is no longer an appropriate evidence of nC_{60} toxicity because effects have been convincingly linked to THF decomposition products (Figure 1) rather than nC_{60} [23,24,25,26•]. Investigations with $nC_{60[solvent/dimethyl\ sulfoxide(DMSO)]}$ in zebrafish and Japanese medaka *Oryzias latipes* reported significant embryo mortality and deformity [39,31], induction of antioxidant defense genes [40], and induction of GSH [31]; and similar effects have been reported for $nC_{60(solvent/toluene)}$ in Japanese medaka embryos [31]. However, although solvents are known to reside within

Figure 1



Fold changes in expression of larval zebrafish *Danio rerio* genes (182) that differed significantly ($P < 0.05$) relative to the control (normal zebrafish husbandry water) after 72-h exposure to aqueous $nC_{60(solvent/THF)}$ or to the tetrahydrofuran (THF) vehicle control. Gene expression was evaluated by the Affymetrix GeneChip® Zebrafish Genome Array designed to interrogate expression of approximately 14,900 *D. rerio* gene transcripts. Expression profiles were essentially identical for $nC_{60(solvent/THF)}$ and THF vehicle treatments, which had only four genes (indicated by circle around ×) that differed significantly in expression from each other (all up-regulated). In the same experiment, no toxicity was observed in *D. rerio* larvae exposed to water-stirred nC_{60} [$nC_{60(stirred)}$]. Only ten genes of fish exposed to $nC_{60(stirred)}$ were differentially expressed (from 2.10 to −6.3 fold) relative to control, eight of these genes were down-regulated, and none were associated with any known toxicological response (no evidence of oxidative stress; further details in Henry *et al.* [23]).

Figure 2



Total glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) in the gills, liver, and brain of juvenile rainbow trout *Oncorhynchus mykiss* fed 500 mg C_{60}/kg food (shaded bars) compared to control fed fish (light bars) after six weeks exposure. No significant differences in GSH or TBARS were detected and there were also no significant effects on any other toxicological endpoint measured (including: survival, growth, haematology, tissue ion concentration, histopathology, osmoregulation, or biochemistry). No dietary toxicity of C_{60} was observed; details in Fraser *et al*. [45•].

nC_{60} [22], the effects of residual solvents on these fish embryo toxicity test results are not well understood. Perhaps solvent generated aqueous nC_{60} will become industrially important such that $nC_{60(solvent)}$ exposure in fish becomes environmentally relevant, but it is inappropriate to attribute toxicity to C_{60} until effects of the solvent are more adequately understood. Preparation of nC_{60} without solvents (i.e., $nC_{60(stirred)}$) is recognized as most environmentally relevant and numerous studies have now investigated toxicity of these preparations [e.g., [41,23,42,31]].

Oxidative stress has been reported in fish exposed to aqueous $nC_{60(stirred)}$, but critical review indicates that these results are more likely a consequence of the assay technique rather than nC_{60}. Indications of oxidative stress (enzyme induction, lipid peroxidation) in fish attributed to $nC_{60(stirred)}$ was reported in one study [37], but the same investigators reported no effect on these endpoints in a separate study [41]. Chronic (32 d) exposure to $nC_{60(stirred)}$ had a subtle reduction in growth of goldfish *Crassius auratus* and some changes in antioxidant enzyme activity, but effects were not related to C_{60} concentration (0.04, 0.2, 1.0 mg/L) [43]. Some results of oxidative stress reported in the literature are likely to be false positives. Shinohara *et al*. [44•] demonstrated lipid peroxidation assays are vulnerable to false positives when nC_{60} is present, and when conditions (light intensity) were properly controlled, no effects were observed. Similar false positives could explain inconsistencies in oxidative stress indicators reported in Zhu *et al*. [43] and Blickley and McClellan-Green [42]. It is noteworthy that 72-h exposure to 6 mg/L $nC_{60(stirred)}$ did not cause significant changes in global gene expression in larval zebrafish *Danio rerio* assessed by the Affymetrix GeneChip® Zebrafish Genome array (≈15,000 gene transcripts [23]). The only dietary exposure (500 mg C_{60}/kg food) in fish (juvenile rainbow trout *Oncorhynchus mykiss*) did not report any oxidative stress or other toxicological effects of C_{60} during or after 6-week exposure (Figure 2) [45•]. Taken together these studies indicate $nC_{60(stirred)}$ is of minimal toxicity in fish for the endpoints that have been assessed.

Ability of nC_{60} to affect the environmental fate and bioavailability of co-contaminants

Emerging concerns about release of C_{60} into the environment include interactions between aqueous nC_{60} and other substances (e.g., toxicants, termed here 'co-contaminants') and consequent effects on co-contaminant fate, transport, and bioavailability. Changes in environmental behavior of co-contaminants by nC_{60} could be similar to that recognized as an important component of the presence of other anthropogenic particulates, such as microplastics, in aquatic environments [46]. Co-contaminants can accumulate in aqueous nC_{60} and accumulation appears to be related to physicochemistry of both nC_{60} and the co-contaminant [47,48]. Some co-contaminants appear to associate strongly within nC_{60}, and there is some evidence that co-contaminants [e.g., 17α-ethinylestradiol (EE2)] adsorb to aggregate surfaces before absorption within nC_{60} and then become considerably more difficult to disassociate [48,49•].

Effects of nC_{60}-co-contaminant associations on co-contaminant bioavailability are largely unknown. Only one study has effectively tested environmentally relevant bioavailability of a co-contaminant (EE2) associated with $nC_{60(stirred)}$ and demonstrated by assessment of vitellogenin gene (*vtg*) expression that nC_{60} reduced bioavailability of EE2 in fish [49•]. The association between EE2 and nC_{60} led to a greater propensity for aggregates to sediment out of the water column over time, and supports the hypothesis that accumulation of settled aggregates by filter feeding and sediment dwelling organisms as a first step into the aquatic food chain [50]. Filter feeding invertebrates can accumulate nC_{60} with associated EE2 [51]; however, the EE2 did not become bioavailable (*vtg* not induced) when invertebrates were fed to fish suggesting that aggregate integrity and nC_{60}-EE2 association were sufficiently robust to withstand fish digestive processes [51]. Co-contaminants held within nC_{60} may be less vulnerable to degradation processes and lead to enhanced persistence and transport of co-contaminants in the environment although perhaps with decreased co-contaminant bioavailability.

Conclusions

Techniques for evaluation of ROS production and toxicity of aqueous preparations of nC_{60} have evolved

over time, and current understanding of fullerene toxicity must recognize that limitations in some initial techniques have led to unintentional erroneous reports of nC$_{60}$ ROS generation and toxicity. Minimal ROS production by aqueous nC$_{60}$ based on current evidence and revisions of early reports of oxidative stress induced by nC$_{60}$ in fish leads to the conclusion that nC$_{60}$ is of minimal toxicity in fish when appropriate experimental controls have been employed to eliminate artefacts (i.e., solvent effects and controlling light). An emerging environmental issue is that nC$_{60}$ may have important effects on environmental fate, transport, and bioavailability of co-contaminants in aquatic environments similar to that observed for microplastics.

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Certain commercial equipment, instruments, and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the NIST nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

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Dietary exposure to carbon nanotubes and C_{60} (500 mg/kg food) in fish was conducted to determine if differences in shape of these NPs could be related to toxicity. Results indicate no overt toxicity or evidence of shape effects from these NPs. Dietary exposure is recognized as a particularly relevant route for fish, but lack of toxicity after six-week exposure to rather high levels of these NPs suggests they are of minimal toxicity.

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