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**A SCREENING STUDY ON THE FATE OF FULLERENES (nC<sub>60</sub>) AND  
THEIR TOXIC IMPLICATIONS IN NATURAL FRESHWATERS**

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Running title: Fate and toxicity of nC<sub>60</sub> by varying water characters

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**Abstract**—Increasing usage of fullerenes (C<sub>60</sub>) increases their opportunities to be released into the environment. For risk assessment, it is important to understand the environmental fate and ecotoxicological effects of C<sub>60</sub>. In this study, fullerene settling was measured during a one-year period with four different lake waters and an artificial freshwater, and *D. magna* immobilization and fullerene accumulation was also measured in each of the lake waters. Depending on the characteristics of the lake waters, fullerenes exhibited either extended water stability or settled rapidly, and in all waters, there was a fraction that remained stable after one year. Water stability was affected by quality and the molecular size distribution of dissolved natural organic matter (DNOM). Increasing DNOM molecular sizes with high aromatic content enhanced water stability. *D. magna* immobilization was generally quite low (under 20%) and highly variable after 24 and 48 h at initial fullerene concentrations up to 10 mg/L. Substantial settling occurred during the time period for acute toxicity assays (i.e., 48 h) which should be planned for when conducting toxicity assays. There were no significant differences in the quantity of accumulated fullerenes among the different lake waters at fullerene concentrations of 0.5 mg/L but there were differences at 2 mg/L.

**Keywords**—nanoparticles, nanotoxicology, water chemistry

## INTRODUCTION

Increasing usage of fullerenes ( $C_{60}$ ) in commercial products such as for cosmetics, optics, medical and electrical applications [1, 2] increases the opportunities for fullerenes to be released into the environment. Some applications of fullerenes, such as cosmetics, provide a direct route to aquatic ecosystems [2]. Water treatment systems have been shown not to remove all of the nanoparticles entering the plants [3]. Moreover, fullerenes have been detected in the effluents of wastewater treatment plants with the highest observed concentrations being at the  $\mu\text{g/L}$  level [4]. As such, it is important to measure their fate and ecotoxicity in natural water bodies and to assess how the characteristics of those natural waters may influence those results.

The fate of traditional hydrophobic organic chemicals (HOCs) in aquatic environments is often estimated using their octanol-water distribution coefficient  $\log K_{ow}$  [5]. Poorly water soluble pollutants with high  $\log K_{ow}$  values are expected to accumulate in sediments and the lipid fraction of aquatic organisms. In molecular form, fullerenes exhibit a highly lipophilic character with a water solubility of  $8 \text{ ng/L}$ , and their  $\log K_{ow}$  was measured to be  $6.67$  [6], which would suggest an affinity toward lipids and sediments if fullerenes act similarly to HOCs. However, it is unclear if fullerenes would follow this model given that an octanol-water distribution approach was recently shown not to predict the accumulation of carbon nanotubes, another carbonaceous nanoparticle [7].

Fullerenes form colloidal clusters ( $nC_{60}$ ) of many single molecules via mixing in water [8]. In the agglomerated form, they can remain in the water phase for periods up to several months depending on the properties of water chemistry such as the ionic strength [9]. The chemistry of natural waters is typically complex with many important characteristics such as the quantity and quality of dissolved natural organic matter (DNOM), water hardness, pH, and ion concentrations. These factors may profoundly impact water stability of  $C_{60}$  agglomerates and affect their mobility [e.g. 10, 11, 12, 13]. Enhanced water stability increases the period that aquatic organisms in the water column could be exposed to  $nC_{60}$ . The stability of fullerenes in the water phase for extended

time periods also allows for sunlight to interact with them to form water-soluble derivatives [14] with unknown ecological effects [15, 16]. To enable a scientifically robust risk assessment, it is important to understand fullerenes' water stability in environmentally relevant conditions.

The risk caused by fullerenes has been related to their capacity to generate reactive oxygen species in cytotoxicity studies [17] and to cause damage to the alimentary canal cells of the aquatic crustacean *Daphnia magna* [18]. However, studies investigating fullerene toxicity to aquatic organisms using fullerene suspensions prepared without organic solvents have typically indicated only minor or no effects [19, 20, 21, 22]. Lower toxicity than expected based upon fullerenes' molecular properties may be due to their tendency to form agglomerates [19]. Nevertheless, agglomerated fullerenes have caused population level effects with earthworms [23] and their potential for trophic transfer in aquatic species has been discussed [21, 22, 24]. Given the potential for chronic effects and trophic transfer, the environmental risks and fate of fullerenes need to be understood.

Several studies on the behaviors of fullerenes in water have been conducted by mimicking natural conditions by adding humic or fulvic acids to artificial water [13, 14], but not by using unmodified natural freshwaters. Characteristics of DNOM in natural freshwaters vary among different sources [25]. Mostly, DNOM consists of humic substances formed from the decomposition of biomaterials and organic molecules such as carbohydrates, lipids, and organic acids [26]. These components give DNOM its chemical character, which can affect its binding potential with fullerenes and subsequent impacts on fullerene settling behaviors.

The aim of the current study was to elucidate how different natural water characteristics affect fullerenes' stability in water and their ecotoxicity in the aqueous phase (Table 1). One particularly important ramification of determining the environmental fate of fullerenes in different natural water bodies is that this information can guide future ecotoxicological experiments such as the relevant concentrations to test and the most probable target organisms for exposure in natural freshwaters. This screening study clarifies fullerenes long-term behavior in four natural waters and

offers insight to their toxicological effects and accumulation potential using the aquatic crustacean *D. magna*. Additionally, limitations of common toxicity test methods with fullerenes are discussed.

## **MATERIALS AND METHODS**

### *Chemicals and experimental waters*

Crystalline C<sub>60</sub>-fullerene (98 %) was purchased from Sigma-Aldrich (USA); extensive characterization of these materials including thermogravimetric analysis and quantification of metal impurities was recently described [27]. Natural freshwaters were collected from four Finnish lakes near the city of Joensuu: Lake Höytiäinen, Lake Kuorinka, Lake Mekrijärvi, and Lake Kontiolampi. All sample natural waters were from clean areas and were fairly pristine. These waters were filtered using Whatman 0.45 µm pore size membrane filters to remove large particles. Artificial freshwater (AFW) without DNOM was made by adding inorganic salts (analytical grade, Baker, CaCl<sub>2</sub> x 2H<sub>2</sub>O 58.8 mg L<sup>-1</sup>, MgSO<sub>4</sub> x 2 H<sub>2</sub>O 24.7 mg L<sup>-1</sup>, NaHCO<sub>3</sub> 13.0 mg L<sup>-1</sup>, and KCl 1.2 mg L<sup>-1</sup>; hardness [Ca<sup>+2</sup>]+[Mg<sup>+2</sup>]=0.5 mmol\*L<sup>-1</sup>) to Millipore-water (at least 18.2 mΩ) and adjusting the pH to 6.8 with NaHCO<sub>3</sub> and HCl . This artificial freshwater corresponded to Finnish lakes with its pH and (Ca+Mg)-hardness. Toluene used in extractions was purchased from Baker, and NaCl was from WVR International; both were analytical grade.

### *Preparation and characterization of the nC<sub>60</sub> suspension*

Suspensions of nC<sub>60</sub> were made by mixing fullerenes at nominal initial concentration of 100 mg/L (i.e., assuming 100% fullerene suspension) in filtered natural freshwaters and AFW using magnetic stirring for 4 weeks at 104.72 rad/s (1000 rpm) at 20 ± 2°C in amber bottles covered in aluminum foil to prevent light exposure. An antimicrobial agent was not used because of the antimicrobial activity and non-biodegradability of nC<sub>60</sub> [28]. To measure fullerene concentrations in suspension, 1.5 ml of 2 % NaCl was added to water samples and nC<sub>60</sub> was extracted two or three times to 3 ml toluene in capped glass test vials by sonicating for 5 minutes in a bath sonicator (Sonorex Super RK 106, Bandelin). In toluene, fullerenes have characteristic peaks at 335 nm and 407 nm. Absorbances at 335 nm in the toluene phase were recorded by Cary 50 bio UV-VIS

spectrophotometer (Varian), and a calibration curve at 335 nm was used for fullerene quantification [21]. To prepare the calibration curve, a mass of fullerenes was carefully weighed using a microbalance, dissolved in toluene, and a range of concentrations were tested to produce a linear regression fit [27]. Recoveries for fullerene extractions from natural waters were 80-100 %. Extractions of natural waters without fullerenes did not give peaks at 335nm and 407 nm.

Shapes and sizes of fullerenes in the suspensions were characterized by transmission electron microscopy (TEM). Suspensions were diluted to 2 mg/L with artificial freshwater to reduce particle agglomeration during drying; in this report, the term agglomeration is used to refer to groups of C<sub>60</sub> particles which may be broken into smaller particles upon processing (i.e., sonication), while aggregates are strongly bonded together (i.e., fused) (29). For TEM sample preparation, 8 µL of natural water or diluted suspension was dropped on a Formvar coated 150-mesh copper grids (Leica, Wetzlar). Samples were then air-dried and analyzed by a ZEISS 900 TEM (Oberkochen) operating at 80 kV beam energy with magnification ranging from 7 000x to 20 000x. Measurements for particle size distributions ( $n = 400$  measured agglomerates for each suspension) were made by using imaging program MegaVision by AnalySIS. Particle sizes were confirmed by dynamic light scattering (DLS, Zetasizer Nano ZS (Malvern Instruments)).

#### *Characterization of natural waters*

Total organic carbon contents in natural waters were measured by a total organic carbon analyzer Shimadzu TOC-5000A with ASI-5000A auto sampler. Conductivity and pH measurements were measured by SCHOTT's Multiline P4 and pH-meter G822, and water hardness was measured using a Perkin-Elmer AS90 AAnalyst. Aromatic content of DNOMs in the water samples were determined as UV-VIS absorption at wavelength 254 nm, and calculating  $sUV_a = A_{254}/DOC$  (mg carbon/L). Parameter  $sUV_a$  represents the degree of aromaticity with increasing value indicating more aromatic character and also larger molecular size. Characteristics of natural waters and DNOMs are presented in the table 2. Molecular size distributions for DNOMs (Table 3) were determined by high performance size exclusion chromatography (HPSEC)

with a pre-column TSKgel SW (7.5 mm I.D. x 7.5 cm length) and a column TSKgel G3000SW (7.5 mm i:D. x 30 cm length, Tosoh Bioscience).

Nuclear magnetic resonance (NMR) spectroscopy gives information about the number and the types of atoms in molecule.  $^{13}\text{C}$  solid-state CPMAS NMR (Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance) was used to semi-quantitatively determine the chemical characteristics of DNOM in waters from Lake Höytiäinen, Lake Mekrijärvi, and Lake Kontiolampi. DNOM of Lake Kuorinka was excluded because the organic carbon content was too low to yield a sufficient quantity of DNOM to obtain accurate results. To obtain solid-state NMR samples, natural waters were lyophilized and dried matter collected by a Hetosicc cryo drier with a vacuum pump Pfeiffer. Each homogeneous dry matter was packed in a 7 mm rotor of zirconium oxide with Kelf cap.

The CPMAS  $^{13}\text{C}$  NMR spectra were recorded with a Bruker AMX 400 high-resolution NMR instrument operating at a carbon frequency of 110.6 MHz and at a temperature of 295.16 K. The spectrometer was equipped with a multinuclear magic-angle spinning probehead. The contact time was 1 ms, delay time 2 s, number of transients 140 000, and sample spin 4000 Hz [30, 31, 32]. Glycine was an external standard for the calibration of the chemical shift scale.

#### *Settling experiments*

One-year long settling experiments were performed. Prepared  $\text{nC}_{60}$  suspensions in natural waters at two nominal initial concentration 100 mg/L was stored in glass bottles protected from the light at room temperature ( $20\pm 2^\circ\text{C}$ ). At time intervals between two days and one year for water suspensions, three 1.5 ml samples from 2 cm below water surfaces were collected, and fullerene concentrations quantified as described above. Suspension pH values were measured before and after the one year period (Table 2). Samples of each  $\text{nC}_{60}$  suspensions were also filtered through 1  $\mu\text{m}$ , 0.45  $\mu\text{m}$ , and 0.2  $\mu\text{m}$  filters after mixing for four weeks, and fullerene concentrations in each size fraction were quantified. During the one-year settling experiment, fullerenes exhibited profoundly different water stabilities in waters from Lake Kontiolampi and Lake Höytiäinen. These

two waters were thus chosen to assess the influence of DNOM concentration on fullerene settling. Initial DNOM concentrations of these waters differed twofold (see Table 2). Thus, Lake Kontiolampi water was diluted using AFW to decrease the DNOM concentration to that of Lake Höytiäinen. This sample was labeled “diluted Lake Kontiolampi” and fullerene settling in this sample was followed one month to pseudo-steady state and compared against those of unmodified Lake Kontiolampi and Lake Höytiäinen waters.

#### *Toxicity studies*

Toxicity of nC<sub>60</sub> suspended in natural waters was determined by the OECD acute immobilization test for *Daphnia magna* (33). The immobilization test was performed with six fullerene concentrations ((0.05, 0.1, 0.2, 2, 5, 10) mg/L) and natural water controls without fullerenes. Four or five replicates with five daphnia per container were tested for each concentration. This concentration range was based on the results from the settling experiment, where it was determined that the fullerene concentration stable in solution was typically less than 5 mg/L; two additional higher concentrations of 5 mg/L and 10 mg/L were tested in an attempt to identify an EC50 value. Because fullerene settling would likely occur during the immobilization experiment, concentrations were measured during the test. For this purpose, 0.25 mL samples were collected at the beginning and after 24 h and 48 h from 1 cm below the water surface from each container and their fullerene concentrations were measured. It was also expected that organisms could cause additional settling by removing fullerenes from the water phase in their feces [21, 24]. To assess effects of organisms themselves on fullerene concentrations in the aqueous phase, duplicate replicates were prepared using similar exposure conditions but without organisms and concentrations at the time points described above determined. Settling was taken into account by using average concentrations between 0 h and 24 h, or 0 h and 48 h respectively, in toxicity calculations.

### *Fullerene accumulation by D. magna*

Accumulation of fullerenes by *D. magna* in each test water was determined after 48 h at two low exposure concentrations which caused either no or low toxic responses. Non-immobilized daphnia from each replicate exposed at concentrations of 0.05 mg/L and 0.2 mg/L were collected after the toxicity tests, and fullerene uptake was measured using the method previously described by Tervonen et al. [21]. Extraction of control organisms did not yield a peak at 335 nm, and spiking known masses of daphnia and then following the extraction procedure yielded recoveries ranging from 85 to 98% [21].

### *Calculations and statistics*

SigmaPlot for Windows 11.0 (Systat Software, Inc., Germany) was used for fitting settling data in equations 1 and 2 (see below) and for statistical analyses. Estimating toxicity parameters such as the concentration needed to cause 50 % and 20 % immobilization (EC<sub>50</sub>, EC<sub>20</sub>) was performed by probit analysis using SPSS 17.0 (IBM Corp.).

For comparison of treatment averages of fullerene water concentrations between water sources in time range, filtered size fractions, and accumulated masses of fullerenes one way ANOVA (with Dunnett's test or with Tukey's test for post hoc comparison) or ANOVA for repeated measurements (RM ANOVA) were used when data were normally distributed. RM ANOVA was required since the same replicate beakers were sampled for nC<sub>60</sub> water concentrations over time and were thus not independent observations. One way ANOVA with Student-Newman-Keuls comparison (SNK) was used for all-pairwise comparisons of the mean responses among the different treatment groups. Results were deemed significant if p<0.05. When data were not normally distributed, Kruskal-Wallis one way ANOVA was applied. The effect of the presence of organisms on decrease in fullerene concentration in toxicity tests was assessed using the Mann-Whitney Rank Sum test.

Equations 1 and 2 were used to fit settling data and are used to describe exponential decays

$$C_0/2 = e^{-kt} \quad (1)$$

$$y = ae^{-bx} + ce^{-dx} + ge^{-hx} \quad (2)$$

Equation 1 describes first-order decay and was used to calculate half-lives for fullerenes' remaining in water phase. In equation 1,  $C_0$  is the initial concentration of fullerenes in water (mg/L),  $k$  the rate coefficient, and  $t$  represents the half-life (h). Equation 2 describes third-order exponential decay and was fitted to settling data, because equation 1 would give too gentle slope to describe settling process (Fig. 1). In equation 2,  $a$ ,  $c$ , and  $g$  represent three stability fractions of fullerenes remaining in the water phase in a time range,  $b$ ,  $d$ , and  $h$  are rate constants,  $x$  represents time (h), and  $y$  is the concentration at time  $x$  (mg/L).

## RESULTS

### *Settling experiments*

Rapid settling was observed during the first few days in all tested waters (Fig. 1), but differences in water stable fullerene concentrations varied substantially among samples (Table 3).

Rapid settling was followed by slow settling after one week in waters from lakes Höytiäinen and Kuorinka, and after three weeks for Mekrijärvi and Kontiolampi.

Water characteristics, such as pH, conductivity, and hardness are known to impact the agglomeration of fullerenes [13, 34]. Nevertheless, these parameters were similar in all tested waters (Table 2). Additionally, ionic strength (measured as conductivity) and hardness were at relatively low levels compared to those which have been reported to affect agglomeration efficiency of fullerenes [35], thus they should not strongly affect agglomeration. Water hardness of the lake waters ranged from 0.10 to 0.33 mmol/L and did not correlate with nC<sub>60</sub> water stability. The highest water-stability was observed with AFW which had a hardness (0.5 mmol/L) greater than those of all of the lakes, indicating that these hardness levels were not high enough to cause enhanced settling in these conditions. The pH values of test waters should also not have significantly impacted the agglomeration, because the fullerene agglomerate size was nearly constant when formed using waters with pH values between pH 5 and 9 [34].

Generally, particles smaller than 450 nm in diameter are assumed to remain in water while larger particles settle more readily [36]. Measured average agglomerate sizes for nC<sub>60</sub>+DNOM by DLS were about 235 nm in waters from Lakes Kontiolampi, Mekrijärvi, and Höytiäinen but more than 1000 nm in Lake Kuorinka (Supplemental data, Table S1). Formed nC<sub>60</sub>+DNOM agglomerate sizes could not have been predicted from particle sizes of DNOM; in addition, measurements for Lake Kuorinka were not reliable due to high PDI value (Supplemental data, Table S1). Size distributions by TEM for colloids in unmodified lake waters and agglomerates in lake waters with fullerenes added are shown in figure 2. Mainly small particles (< 450 nm) were formed, excepting Lake Kuorinka where the particles were much larger, a finding that helps explain why the most rapid settling occurred in the water from Lake Kuorinka. Measured fullerene concentrations for filtered size fractions after mixing for four weeks are presented in table 3. All pairwise comparisons after ANOVA revealed statistically significant differences among waters for all size-fractionated concentrations, except for the fraction < 0.45 among lakes Kontiolampi and Höytiäinen. Very small nC<sub>60</sub>+DNOM particles (< 50 nm) occurred mainly in Lake Kontiolampi, where almost 15 % of particles belonged to this fraction according to TEM analysis (an arrow in Fig. 2).

Settling rate constants calculated by fitting equation 1 increased with larger initial concentrations except for Lake Kontiolampi (Supplemental Data, Table S2). Half-lives for water stabilities (Supplemental Data, Table S2) indicate that settling in all waters was very rapid after stirring was stopped. Nevertheless, it was followed by slower settling continuing for months, as seen in figure 1. Thus, a three phase equation (Eqn. 2) with three different settling fractions was fitted to describe settling behavior more accurately, and fractions for rapidly and moderately settled fraction and a fraction with extended water stability were determined (Table 4).

The impact of DNOM on fullerene settling was also investigated. The significance of aromatic content and proportional amounts of some functional groups in DNOM on the agglomerate size and fullerene settling was assessed by <sup>13</sup>C-NMR and UV-absorption data. The

DNOM in the test waters represented a variety of selected functional groups (Supplemental Data, Table S3). Increasing aromatic content of DNOM seemed to cause decreasing fullerene particle size, whereas carboxylic and oxygen-substituted aliphatic groups might have the opposite effect. However, it is important to note that DNOM from only three lakes were tested, thus the relationships observed here would need to be confirmed across a broader set of DNOM to yield definitive conclusions among these parameters.

Both UV-absorption and NMR data revealed that Lake Kontiolampi had the highest aromatic content. The parameter  $sUV_a$  depends both on aromatic content and molecular size; thus, UV-absorption measurements were consistent with  $^{13}C$ -NMR, because higher  $sUV_a$  in Lake Mekrijärvi than in Lake Höytiäinen can be explained by the larger DNOM's molecular sizes in Lake Mekrijärvi. Molecular size distributions by HPSEC (Fig. 3) revealed that molecular sizes were mainly large molecules in lakes Kontiolampi and Mekrijärvi, which is consistent with the  $sUV_a$  results. The proportion of fullerenes in the most stable fraction (g) decreased with increasing agglomerate size (Fig. 4a). Formed  $nC_{60}$ +DNOM particle sizes appeared to correlate with the molecular sizes of DNOM (Fig. 4b). There appeared to be a trend of increasing  $sUV_a$  and larger extended stable fractions (Fig. 4c). Increasing aromaticity also seemed to lead to larger water stable fractions, but the correlation was not as clear as for  $sUV_a$ . Instead, increasing un-substituted aliphatic content seemed to lead to higher water stability, and increasing carboxylic content decreased water stability.

Two waters with opposite settling behavior and different DNOM concentrations, namely Lake Kontiolampi and Höytiäinen, were compared to investigate if DNOM concentration would predict settling rates. Diluting the DNOM concentration for Lake Kontiolampi to that of Lake Höytiäinen did not change the settling rate of fullerenes in Lake Kontiolampi water (Fig. S1) suggesting that under these conditions the DNOM concentration only had a minor impact on the settling rate.

### *Toxicity and accumulation tests*

Concentrations causing 50 % *D. magna* immobilization (EC<sub>50</sub> values) were not achieved in any of the test waters (Supplemental Data, Fig. S2 parts a and b). Instead, SPSS software was used to attempt to determine values for immobility of 20 % (EC<sub>20</sub>) of the organisms after exposure for 24 and 48 h, but upper and lower limits for EC<sub>20</sub> confidence intervals could not be estimated using the software and thus EC<sub>20</sub> values were not reliable. Slight increases in *Daphnia* immobility from fullerene treatments were observed in waters of lakes Kontiolampi, Mekrijärvi, and Höytiäinen at the lowest concentrations after 24 h and in all waters after 48 h. There was some immobilization also in the controls after 48 h: for lakes Kontiolampi, Kuorinka, Mekrijärvi, and Höytiäinen, immobilization was 15±10 %, 5±10 %, 5±10 %, and 4±9 %, respectively.

During the toxicity tests, the fullerene concentrations decreased substantially even though the starting concentrations were at least five times lower than those in the settling experiments. Although concentrations were decreased by organisms (data not shown), these differences were not significant (Mann-Whitney Rank Sum test,  $p > 0.05$  for each cases).

Accumulated masses of fullerenes per daphnia wet mass at 0.05 mg/L were (1.3±0.9, 1.2±0.4, and 1.4±0.3) µg/mg for waters of Höytiäinen, Kuorinka, and Kontiolampi, respectively, but were 5±3 µg/mg in Lake Mekrijärvi (see Fig. 5). For the higher concentration of 0.2 mg/L, accumulated masses were (2.4±0.5) µg/mg and (3.1±0.8) µg/mg for lakes Höytiäinen and Kontiolampi, and (7.2±0.6) µg/mg for Lake Kuorinka. The standard deviation for the *Daphnia* exposed to Lake Mekrijärvi water was so high that the result for the 0.2 mg/L treatment was excluded from statistical analysis. Interestingly, the differences among lakes were clearer at higher concentration. Accumulated fullerene masses were increased by higher fullerene concentrations in each lake water, although this difference was significant only in Lake Kuorinka ( $p < 0.001$ , t-test). There were no differences among lakes (one way ANOVA) in treatments at 0.05 mg/L, but at 0.2 mg/L, the accumulated mass of fullerenes per daphnia wet mass in Lake Kuorinka differed significantly both from Lake Höytiäinen ( $P < 0.001$ ) and Kontiolampi ( $P < 0.001$ ).

## DISCUSSION

Settling experiments revealed that there was substantial variability in the impact of natural waters on fullerene settling with fullerenes having higher stability in one lake (Lake Kontiolampi) and low stability in others (Lakes Höytiäinen, Kuorinka, and Mekrijärvi). Smaller fullerene agglomerate sizes in the various natural waters correlated with increased water stability (Fig. 3 part a), a result that mirrors an earlier study [37]. Thus, the impact of various factors on fullerene particle sizes among the different lake waters was investigated. Colloidal sizes of DNOM measured by TEM in lake waters without added fullerenes (Fig. 2) did not explain observed  $nC_{60}$ +DNOM particle sizes. Instead, sizes of  $nC_{60}$ +DNOM agglomerates correlated with the molecular sizes of DNOM measured by HPSEC. Large DNOM molecules may contain functional groups (e.g. aromatic as NMR measurements here revealed) which are likely to adsorb fullerenes enclosing fullerenes inside of formed DNOM+ $nC_{60}$  particles. Thus, fullerenes could be trapped and prevented from further agglomeration (see Supplemental Data, Fig. S3 A) whereas small DNOM particles could leave fullerenes to interact together (Fig. S3 B). In addition, Mashayekhi and coworkers [38] indicated that the steric stability by NOM dominates agglomeration of fullerenes rather than electrostatic one, and this might be seen also in the current study. Higher  $sUVa$  values, which generally indicate more hydrophobic DNOM, correlated with larger fractions of  $nC_{60}$  that were water-stable for extended periods (Fig. 4c). High aromatic content of DNOM offers fullerenes the possibility of  $\pi$ - $\pi$  interactions, interactions that have been reported to be important for adsorption between carbon nanomaterials and humic material [39, 40]. Hydrophobic interactions between humic material and  $nC_{60}$  suggested by Wu et al. [41] can also be responsible for observed high water-stability of  $nC_{60}$  in water indicating that  $sUVa$  could be used as a parameter in assessing fullerenes' settling behavior. The immobilization assay did not show any significant toxicity. In lakes Höytiäinen and Mekrijärvi after 24 h, less than 10 % were immobilized at the highest concentrations while none were immobilized in Lake Kuorinka waters. Immobilization remained at 20 % or less in these waters after 48 h indicating low acute toxic responses. These results are

similar to those that reported only minimal toxic effects for organisms exposed to fullerenes suspended in AFW without solvents [20, 22, 42].

Despite fullerenes' general tendency to settle, there was also a fraction that was stable in water for one year. For Lake Kontiolampi, the water stable fullerene concentration after one year was still  $4.3 \pm 1$  mg/L. This concentration was observed to immobilize about 12% of *D. magna* in toxicity test in 24 h, and 32% after 48 h, respectively. Thus, high water stability can maintain sufficiently high concentrations to pose potential ecotoxicological risks. Also, the toxicity was higher in Kontiolampi than in other waters. This may be due to a notable portion of very small particles (less than 50 nm, see Fig. 2), which could be responsible for higher toxicity in this water than the others. Similar size-dependent toxicity of  $nC_{60}$  has recently been reported by Song et al. [43]. This indicates that acute toxicity is possible and should not be ignored in nanoparticle risk assessment at these aqueous concentrations although it may not be the main effect. A dose-response curve in toxicity was clearly observed only for Lake Kuorinka after 48 h. In Lake Mekrijärvi, the immobilization percentage after 48 h rose with increasing concentration for the two lowest concentrations, but no immobilization was observed at higher concentrations. Immobilization did not change between 24 h and 48 h in lakes Höytiäinen and Kontiolampi, a result probably due at least in part to decreased concentrations by fullerene settling. It is possible that effective filter feeder organisms, such as *D. magna*, could decrease aqueous phase fullerene concentrations by collecting masses of fullerenes in their bodies without real absorption into tissues and then excrete larger fullerene agglomerates to the bottom of the container as feces [21, 24]. In these experiments, there appeared to be a trend of decreased aqueous phase fullerene concentrations in containers with organisms, but the results were not statistically significant.

Fullerene accumulation in the different waters was also investigated (Fig. 5). Gut volume has been reported to define the mass of accumulated fullerenes [21]. Organisms in this study were younger and thus smaller than ones in the previous study, which may have affected organisms' capacity to retain fullerenes without affecting the water concentration significantly (see Table 5 for

a summary of fullerene uptake results with *D. magna*). Two potential explanations for this result that could be investigated in future studies are effective particle packing in the organism gut tract and a decreased ability to depurate fullerenes. Differences among the results obtained in *D. magna* accumulation studies may partly stem from differences in the ages of *Daphnia* tested, exposure durations, and from fullerene suspension and extraction methods. A preliminary assessment of the effects of DNOM on accumulation was described by Waissi [44] and maximum accumulation was reported to be 2.5 µg/mg daphnia for fullerene particles suspended in Lake Kontiolampi water, a result close to the amount measured here.

## CONCLUSIONS

This study investigated the impact of different lake waters on the settling of fullerenes and their ecotoxicological effects. Although settling occurred rapidly during the first days, it became slower within time and detectable fullerene concentrations remained in suspension over a one year period. These highly stable fullerenes are significant with regards to fullerenes' mobility in natural aquatic systems, their effects on aquatic species, and being susceptibility to modifications by interactions with sunlight. This study indicated that the quality and molecular size distribution of DNOM were important factors with regards to fullerene stability. According to previous modeling studies, sediments have been predicted to be sinks for fullerenes in aquatic systems [45]. This study confirms that modeling and revealed that a large proportion of fullerenes will rapidly settle to the sediments regardless of freshwater characteristics of lake waters. The environmental significance of effective settling behavior is that fullerenes will accumulate in sediments and thus cause higher exposure risk to species living on the sediment surface or in sediment. The effects observed in the *Daphnia magna* studies suggest that the acute toxicity results were generally low and highly variable under the testing conditions tested but that the organisms can uptake a substantial quantity of fullerenes in natural waters, which may have long term implications.

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Table 1: Aims of the study.

QUESTION	IMPLEMENTATION	DURATION
What is the long term stability of fullerenes in natural lake waters?	Measure aqueous phase fullerene concentration in natural waters from four lakes. Investigate effects of water quality characteristics (pH, hardness, and DNOM molecular size distribution) on agglomeration potential.	12 months
Does the quality of DNOM determine the fullerene settling rates?	Characterization of lake DNOM by UV-VIS, HPLC, and <sup>13</sup> C-CPMAS-NMR. Agglomerate size determinations by TEM and DLS.	-
How does the initial C <sub>60</sub> concentration affect settling rates?	Concentration monitoring for two different initial concentrations in natural waters from four lakes.	6 months
Does toxicity and accumulation potential vary between natural lake waters?	OECD acute immobilization test and accumulation test on <i>D. magna</i> in four freshwaters.	48 hours

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Table 2: Characterization of natural waters and their DNOMs, and pH values for nC<sub>60</sub> suspensions. Errors for pH, DOC, conductivity, and hardness values represent standard deviations, respectively. Values represent the mean of triplicate measurements.

Lake	pH	DOC mg/L	Conductivity μS/cm	Hardness <sup>1</sup> mmol/L	sUV <sub>a</sub> <sup>2</sup>	pH after nC <sub>60</sub> addition	
						initial	1 year
Höytiäinen	6.5±0.01	6.2±0.4	52±0.1	0.197	2.6±0.4	6.5±0.01	7.0±0.01
Kuorinka	6.5±0.01	2.2±0.1	47±0.1	0.15	0.8±0.1	6.8±0.01	7.0±0.01
Mekrijärvi	6.5±0.01	13.1±0.2	31±0.1	0.104	4.0±0.2	6.8±0.01	7.2±0.01
Kontiolampi	6.0±0.01	14.4±1.2	45±0.1	0.33	4.7±1.2	5.5±0.01	7.2±0.01

<sup>1</sup> [Ca<sup>2+</sup>+Mg<sup>2+</sup>]; previous research on the water hardness of these lake waters indicated coefficients of variation of 0.2 to 0.3 for triplicate measurements, and thus provide estimates of the uncertainty for these measurements.

<sup>2</sup> sUV<sub>a</sub>=A<sub>254</sub>/DOC (mg C/L) = the degree of aromaticity

Table 3: Fullerene concentrations (mg/L) in selected size fractions after mixing for four weeks.

These data represent the mean of triplicate samples and uncertainties represent standard deviations.

ND indicates that the values could not be detected.

<b>Sample</b>	<b>&lt; 1 <math>\mu\text{m}</math></b>	<b>&lt; 0.45 <math>\mu\text{m}</math></b>	<b>&lt; 0.2 <math>\mu\text{m}</math></b>
Lake Kontiolampi+ C <sub>60</sub>	7.2 $\pm$ 0.01	3.00 $\pm$ 0.4	0.90 $\pm$ 0.03
Lake Mekrijärvi + C <sub>60</sub>	ND	ND	ND
Lake Höytiäinen+ C <sub>60</sub>	6.3 $\pm$ 1.65	4.7 $\pm$ 0.48	0.30 $\pm$ 0.079
Lake Kuorinka + C <sub>60</sub>	1.9 $\pm$ 0.2	0.4 $\pm$ 0.02	0.07 $\pm$ 0.01

Table 4: Fractions for rapid (a) and moderately fast settling (c), and the fraction with an extended water stability (g) using equation 2. Uncertainties represent standard deviations from modeling.

Lake	a	c	g
Höytiäinen	0.44±(<0.001)	0.41±(<0.001)	0.15±0.002
Kuorinka	0.75±(<0.001)	0.10±(<0.001)	0.16±0.001
Mekrijärvi	0.38±0.09	0.32±0.06	0.30±0.01
Kontiolampi	0.34±(<0.001)	0.26±(<0.001)	0.39±0.05

Table 5: A summary of fullerene accumulation studies by *D.magna*.

Reference	Daphnia age (days)	Fullerene concentration (mg/L)	Exposure duration (days)	Body burden $\mu\text{g}/\text{mg ww}$
Current study	<1	0.05 and 0.2	2	3.1 **
Tervonen et al. 2010	3-5	0.5 and 2*	1	4 to 5 *
Oberdörster et al. 2006	n.a.	30*	5	2.3
Tao et al. 2009	adult	0.2*	1	0.24
Waissi 2010	3-5	2.5**	1	2.5

\* in artificial freshwater, \*\* in Lake Kontiolampi water, n.a. = not available

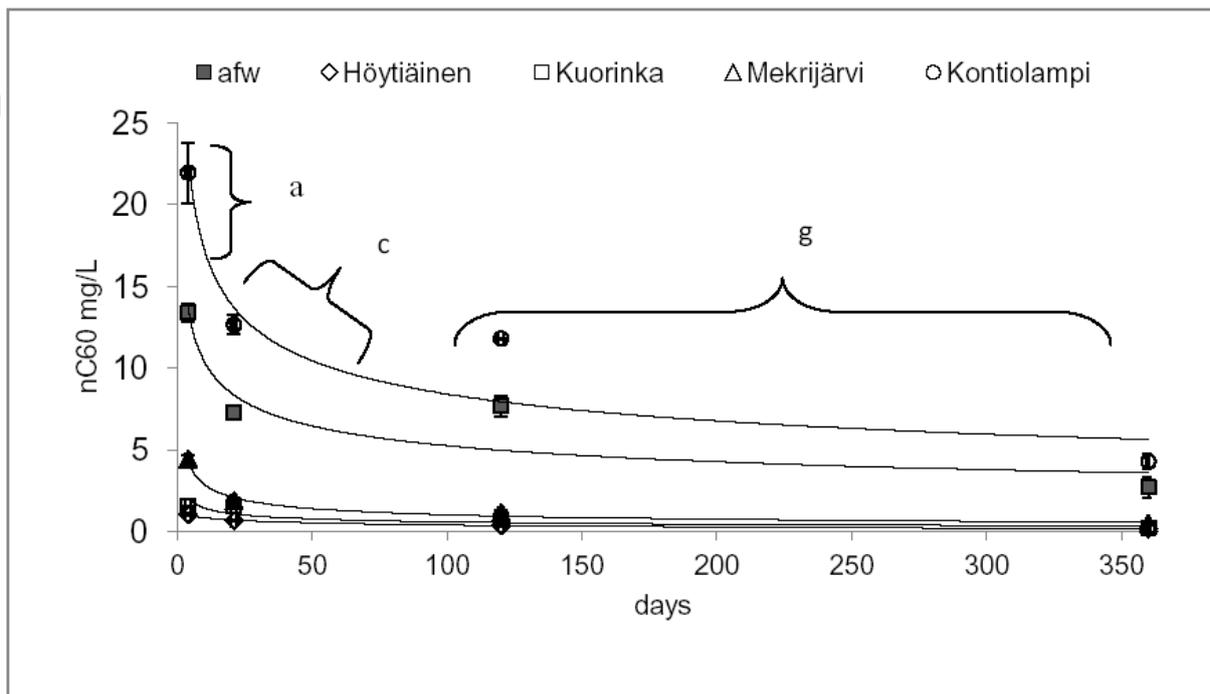


Figure 1: Water stable fullerene concentrations during one year. Initial nC<sub>60</sub> concentration is 100 mg/L. Values represent averages of triplicate data points and error bars represent standard deviations. Fitted curves are from equation 2. Brackets show fractions for rapidly (a) and moderately settled (c) fraction and a fraction with extended water stability (g) using Lake Kontiolampi as an example.

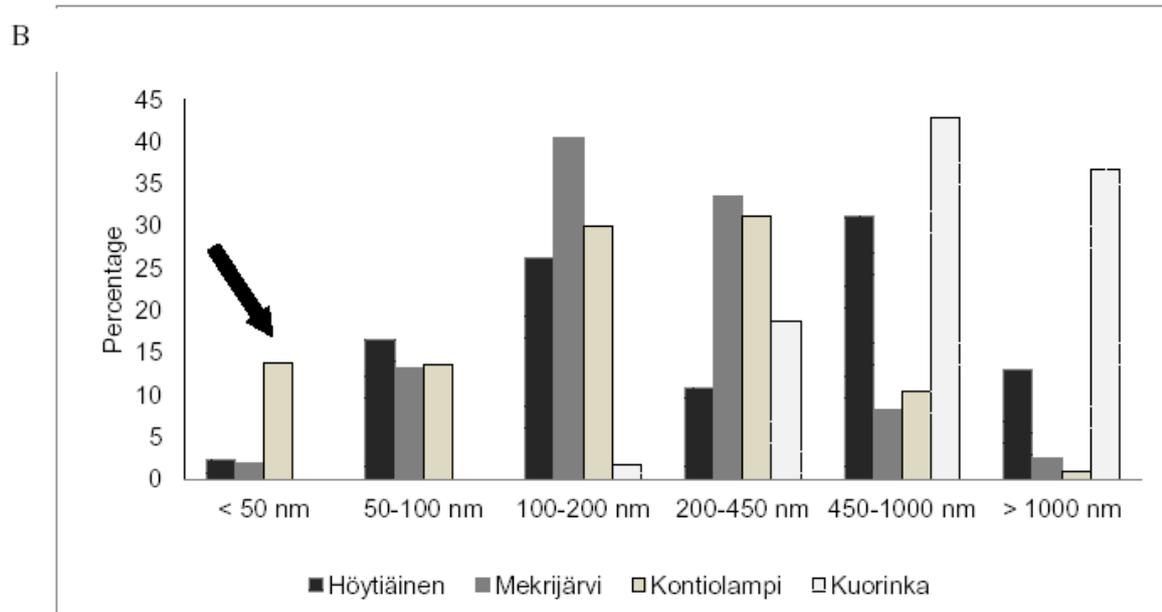
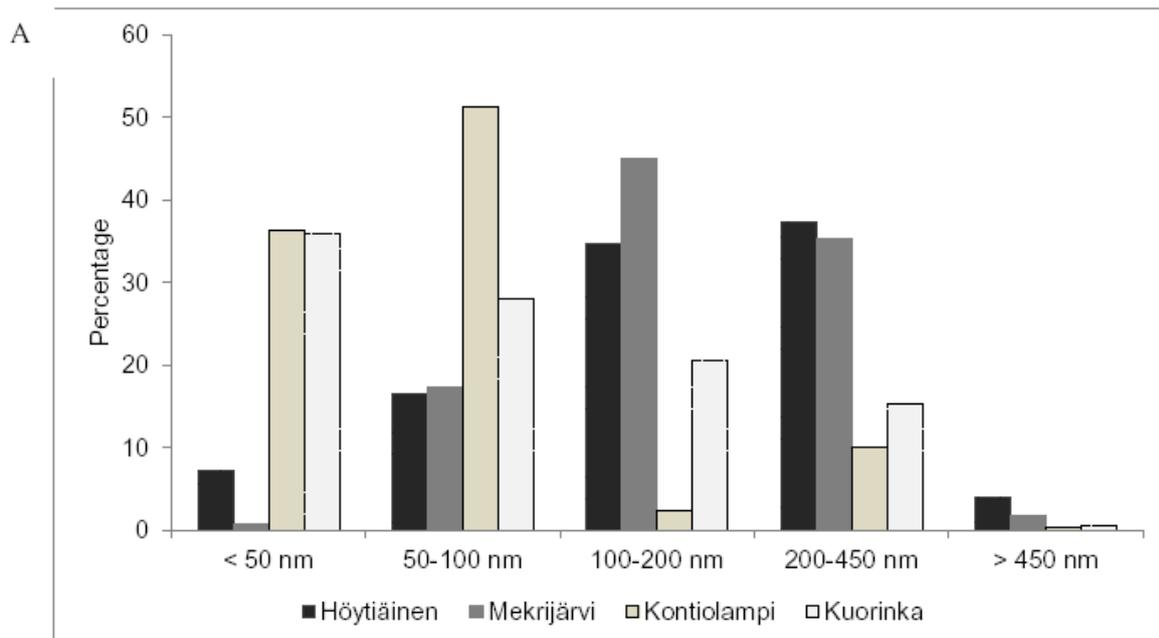


Figure 2: A) Size distributions ( $n = 400$ ) for colloids in clean natural waters and B)  $nC_{60}$  agglomerates in suspensions formed in each of waters immediately after stirring was stopped at an initial concentration of 100 mg/L (lower) measured by TEM. An arrow shows amount of the smallest particle fraction formed in Lake Kontiolampi.

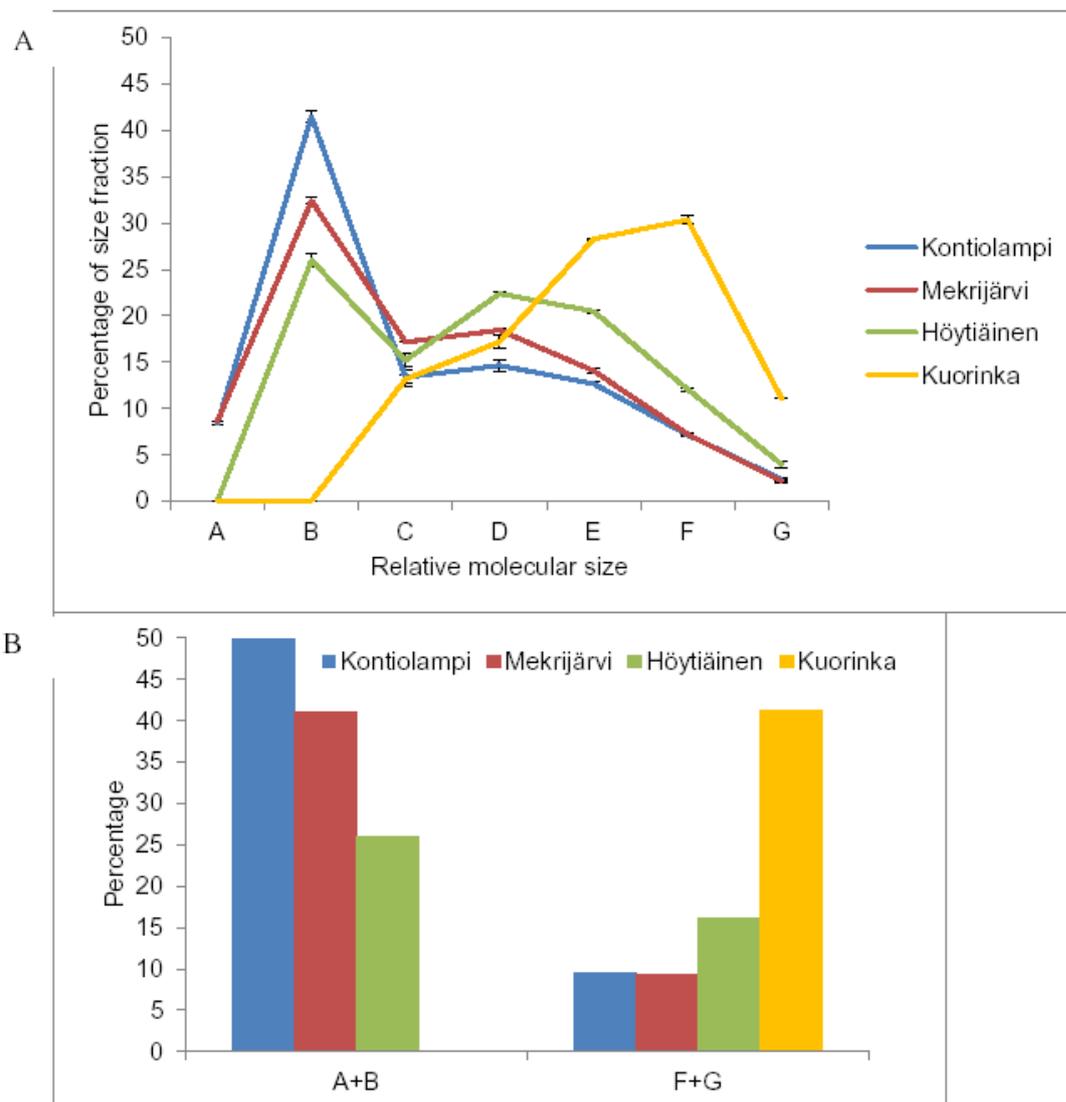


Figure 3: A) Percentages of molecular size fractions in natural waters determined by HPSEC. Codes A-G represent diminishing molecular sizes. Error bars represent standard deviations from triplicate measurements. B) Sum of two largest molecular size fractions (A+B) and two smallest fractions (F+G).

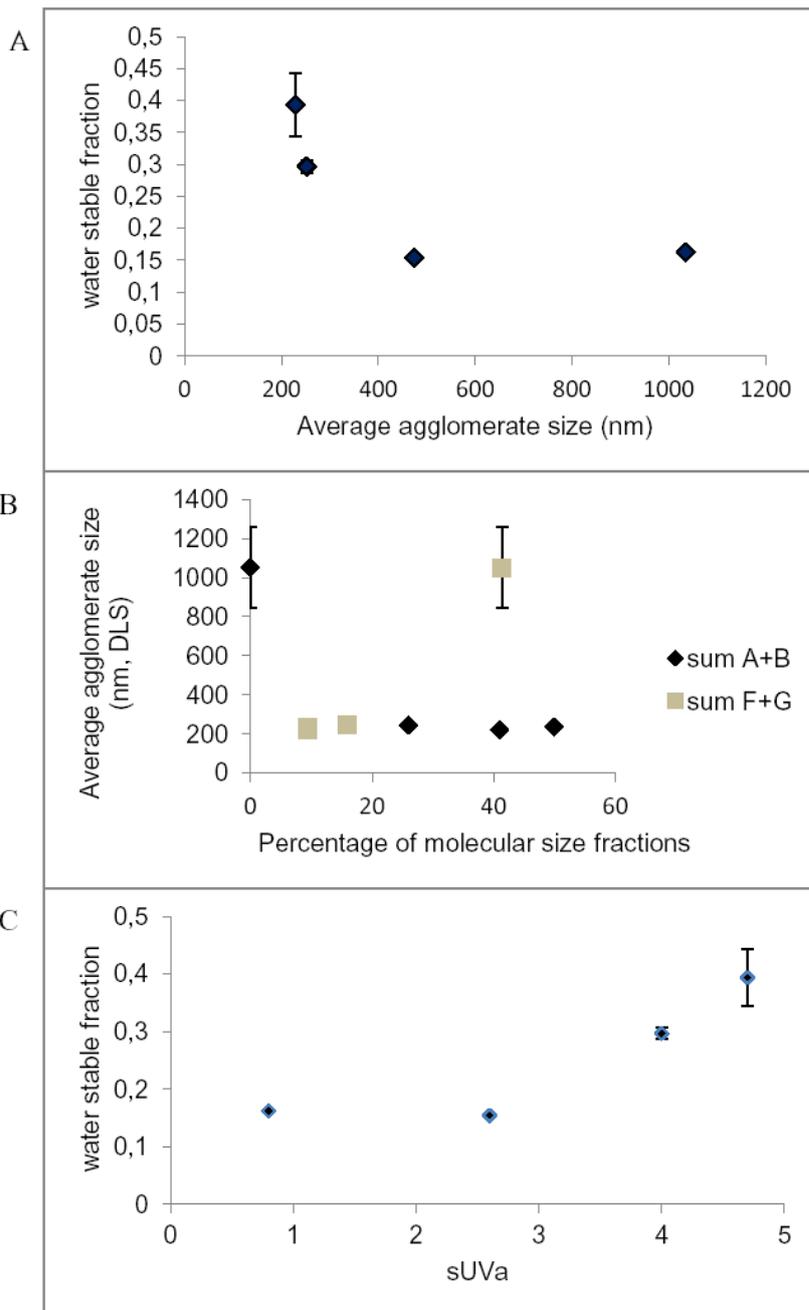


Figure 4: A) Modeled water stable fraction  $g$  (the fraction remaining suspended during one year) for an initial fullerene concentration of 100 mg/L plotted against agglomerate size. B) Formed fullerene agglomerate size against molecular size fractions measured by HPSEC (figure 3). C) Modeled water stable fraction ( $g$ ) for an initial fullerene concentration of 100 mg/L plotted against sUVa. Error bars represent standard deviations for the water stable fraction or DLS measurements.

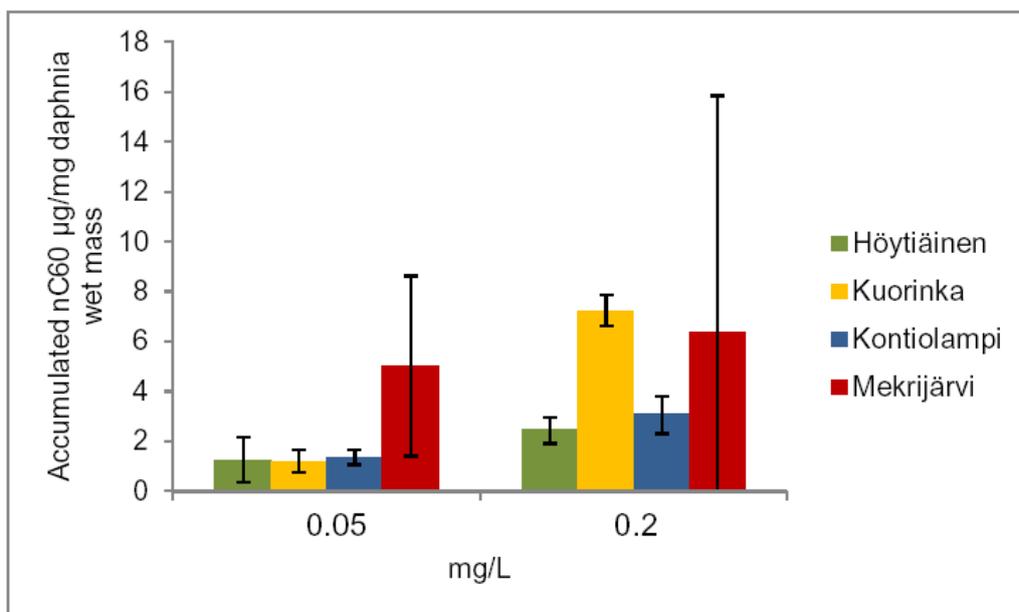


Figure 5: Accumulated  $nC_{60}$  in *D. magna* after 48 h. Data represent the means of 5 (lakes Höytiäinen and Mekrijärvi) or 4 (lakes Kuorinka and Kontiolampi) samples, and the error bars represent standard deviations.