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The Determination of the Potential Risks Associated with Engineered Nanoparticles in Aquatic Environments

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Abstract

The presented body of work is submitted for examination to the degree of Masters of Philosophy, (MPhil). The objective of the overall project is to assess the potential risks associated with engineered nanoparticles in the aquatic environment. The current study assesses the potential toxic effects of C_{60} and CB with a range of ecotoxicological end points. The tests employed here include cytotoxicological assessment using two end-points; the Alamar Blue Assay and the Neutral Red Assay on two fish cell lines; PLHC-1 and RTG2. Growth Inhibition of algae *Pseudokirchneriella subcapitata*, OECD test 201, acute toxicity test of the crustacean *Thamnocephalus platyurus,* acute immobilization test with the invertebrate *Daphnia magna*, as well as the toxicity assessment of the luminescent bacteria *vibrio fischeri* using the Microtox test. A series of methods were also employed to initially characterise the test particles, necessary to obtain as much information as possible before exposing the particle to the test systems, in order to ensure results obtained are true values of toxicity and not dependant on external parameters. No significant toxicity was recorded in the cytotoxicological studies, in the algal assessment, *Daphnia* study and *Thamnocephalus* test; this suggests that C_{60} does not pose as an ecotoxicological threat to these species. Toxicity was observed for CB in the *Daphnia* and *Thamnocephalus* studies. A concentration range of 0-833ppm of each particle was assessed with the Microtox test, an EC_{50} after 30 minutes exposure to the bacteria of 467.33ppm and 119.03ppm for C_{60} and CB respectively were obtained. For the *Daphnia magna* test concentration ranges of 0-1000ppm and 0-120ppm were assessed for C_{60} and CB respectivbely, results were inconclusive for C_{60}, an average EC_{50} of 15.11ppm was determined for CB after 48 hours exposure. All results are discussed and future work recommendations are made.

Declaration

I certify that this thesis which I now submit for examination for the degree of Masters of Philosophy is entirely my own work and has not been taken from the work of others, and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any Institute.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

The Institute has permission to keep, lend or copy this thesis in whole or in part, on condition that any such use of the material of the thesis be duly acknowledged.

Signature _____ Date _____

Candidate

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List of Abbreviations

AB……..... Alamar Blue™

AM……..... Algal medium

AF4 Asymmetric Flow FFF

AFM……..... Atomic Force Microscopy

CCAP..... Culture Collection of Algae and Protozoa

C_{60}..... Carbon 60

CB…….. Carbon Black

CHSE-sp…… Chinook Salmon Oncorhynchus tshawytscha embryo cell line

CNT……. Carbon Nano Tubes

CYP…… Cytochrome P450

D.magna……. *Daphnia magna*

DLS..... Dynamic Light Scattering

DM……..... Daphnia medium

DMBA..... 7,12-dimethylbenz(a)anthracene

DMEM-F12 ……..……..…..... Dulbecco's modified essential medium-F12 nutrient mix

DMSO……..... Dimethylsulfoxide

EC_{50}...……..……..... Effective concentration leading to a 50% response

ENP…… Engineered Nano Particle

EPA……. Environmental Protection Agency

FBS..... Foetal Bovine Serum

FFF…….. Flow Field-Flow Fractionation

FU……..…….. Fluorescent units

FWS……..... Fullerene Water Suspension

HeNe…… Helium-Neon

HOC…… Hydrophobic Organic Compounds

IPAC……... International Union of Pure and Applied Chemistry

HRTEM……. High-Resolution TEM

$K_2Cr_2O_7$..... Potassium dichromate

LC_{50} ..... Concentration leading to 50% Lethality

LOEC..... Lowest Observed Effect Concentration

MIC……. Minimal Inhibitory Concentration

MTT..... 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Assay

MWCN…….. Multi Walled Carbon Nanotubes

NOEC..... No Observed Effect Concentration

NM…….. Nano Material

NOM……..... Natural Organic Matter

NP……... Nano Particle

NR..... Neutral Red

PBS..... Phosphate Buffered Saline

PLHC-1…….. *Poeciliopsis lucida* hepatocellular carcinoma cell line

PEC……... Predicted Environmental Concentration

QSAR Quantitative Structure Activity Relationship

RT…… Room Temperature

RTG-2…….. Rainbow Trout Gonad tissue cell line

SATL…….. Shannon Aquatic Toxicity Laboratory

SD…… Standard Deviation

SDS……..... Sodium dodecyl sulphate

SEM…….. Scanning Electron Microscopy

SOP……..... Standard Operational Procedure

SWCNT…….. Single Walled Carbon Nanotubes

TEM Transmission Electron Microscopy

THF……... Tetrahydrofuran

TRP……..... Traffic-Related Particles

ZP..... Zeta Potentiall

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Chapter 1: Defining Nanotechnology and Engineered Nanomaterials, (ENM)

1.1: Introduction

The term "nanotechnology" was first coined by Japanese professor Nario Taganuichi at a conference on precision engineering in 1974 (Rem et al., 2012). At the time, the term was used to describe novel ultra-fine materials used in engineering methods. Today, nanotechnology has evolved into a highly competitive multidisciplinary research area encompassing an unprecedented range of sectors from medical applications to fuel cells, (Danilov et al., 2007) and food technology (Chaudhry et al., 2008). The terminology has also grown to reflect this diversity with numerous broad definitions of nanotechnology emerging from a variety of bodies. The working definition of nanotechnology which will be used throughout this report will be that of The European Cosmetic Products Regulation, defined at a meeting between the chemicals committee and the working party on chemicals, pesticides and biotechnology in 2011 (ENV/JM/MONO(2011)52), OCED, 2011) which defines a nanomaterial, (NM) as: "An insoluble or biopersistent and intentionally manufactured material with external dimensions, or an internal structure, on the scale from 1 to 100nm." In this context, a nanoparticle, (NP), is considered to have all three dimensions on the scale 1 to 100nm.

1.2 Classification of ENMs

The range and diversity of NMs and NPs is quite significant and methods employed to define or classify these materials are often difficult. Nevertheless, the subdivision of NMs based on their innate chemical nature can be done as follows; Inorganic, Organic, Polymeric, and Nanoclays.

Inorganic

These NPs are based on inorganic materials such as metals, and derivatives of silicon. Examples include metallic NPs and oxides. They find many uses in high tech areas right down to food storage devices.

Organic

In this instance, organic will refer to NMs which have a biological source or application. Organic NPs have found their way into many areas of food science and pharmaceuticals, from drug delivery systems to active ingredients (Augustine et al., 2009) These materials are typically protein or lipid based but also include carbon based NMs, the focus of this work.

Polymeric

Polymeric NPs are any number of the large amount of plastics available in the nanoscale. Despite the fact that they fall under the chemical definition of organic, the number of polymeric NPs in existence warrants their own classification. Nano scale polymers are utilized in nanocomposite formation. Typically, they find applications as a dispersion matrix for other NMs (Costa et al., 2008) or as encapsulants in drug delivery (Alexis et al., 2007).

Nanoclays

Nanoclays are inorganic NPs; typically they maintain a uniform shape. Nanoclays have found use as strengtheners in polymers and as fillers for example as constituents in beverage containers in which they are said to curb the slow release of carbon dioxide through the beverage packaging. (Chaudhry et al., 2008). These four primary classifications describe the inherent chemical nature of NMs. However, some overlap does exist in these materials, for example in nano-composites where polymeric and inorganic nano-species are mixed to exploit the properties of both materials (Costa et al., 2008), and in the nutraceutical field, where nano-composites based on polymeric organic particles are in widespread use as delivery systems for active ingredients, (Weiss et al., 2008). The materials upon which this report is based are Buckminsterfullerene (C_{60}) and Carbon Black, (CB), the structure and applications of which are described in the following sections.

1.3 Release of ENP's into the environment

The routes of release of NMs into the environment and the consequences of this is currently being assessed by the research community, including the interaction of NPs with sediments and colloid particulates in the environment, the binding of lipophilic organic compounds and metals, synergistic effects of NPs and other chemical pollutants that may affect toxicity. (Moore et al., 2006). As treated water from industrial waste and urban water sewage is discharged into waterways after treatment, in rivers, lakes, and coastal waters, it's highly plausible that industrial nanoscale products and by-products will enter the aquatic environment and contribute to air pollution (Daughton et al., 2004). Indirect release through use of by the public of NP containing products is also a plausible route of release, for example in food containers and medicines. Another source of release that has been identified is the NPs used in environmental remediation may directly enter water systems (He et al., 2014).

Previous reports have highlighted that suspended sediment particles in aquatic environments affect the transport of chemical pollutants and the hydrodynamic and morphological characteristics of rivers and coastal zones determine the distribution of bound NPs, (Smedes et al., 1994). The makeup of the receiving waters can greatly influence the behaviour and so toxicity of the NPs, for example in marine environments, the sea-surface microlayer of water, containing sugars, proteins and lipids, play an important role in the behaviour of NPs (Wurl et al., 2004).

The lipid moiety provides a medium to partition, which influences the NPs bioavailability in the receiving water. In the case of fresh water systems the presence of natural organic matter influences bioavailability in this way. The fate and bioavailability of ENPs in natural aquatic systems are strongly influenced by their ability to remain dispersed in water. Consequently, understanding the colloidal properties of ENPs through rigorous characterisation of physicochemical properties and measurements of particle stability will allow for a more accurate prediction of their environmental effects in aquatic systems, which is reflected in the use of physiochemical measurements when assessing the toxicity of ENP's in the environment. This study also included techniques to assess the morphology of the NPs being studied and their stability in a suspension of water.

A study conducted by Köhler et al., in 2008 assessed the release of CNT's during the product life cycle of lithium-ion batteries and synthetic textiles, the findings suggested release of NPs were observed during the production, usage and disposal of the products and the likelihood and form of release is determined by the way CNT's are incorporated into the material. Other methods used currently to assess the risk of release of ENPs into the environment include predictive modelling, one such study carried out by Nowack et al., 2007. Factors considered during the study included estimated worldwide production volume, particle release from products, and flow coefficients in environmental compartments, predicted environmental concentrations (PEC) were then compared to the predicted no effect concentrations (PNEC) published to date to estimate the potential risk of release. For CNT's a PEC of 10% release at the disposal stage was recorded.

Knauer et al., 2007 reported the dissolved organic carbon quantity of CB in estuarine and coastal waters to be 8.9% with higher levels recorded in urban areas, which correlates with the suggestion of emissions of CB and urban runoff (Hwang et al., 2006) posing a risk in the environment. Knauer's study also suggested that the presence of CB in aquatic environments acts as a supersorbant of some chemical compounds in the environment, such as polycyclic aromatic hydrocarbons (PAHs), dibenzo-p-dioxins (PCDDs), non-ortho-polychlorinated biphenyls (PCBs) and polybrominated diphenylethers. This would suggest that CB may strongly reduce the risk posed by organic contaminants in the aquatic environment (Knauer et al., 2007). Another source of NPs in the environment that has been suggested is storm runoff, and studies are now assessing toxicity of NPs in storm water to various species, such as the assessment of *Pseudokirchneriella subcapitata* post storm by Knauer et al., 2007.

Moving forward in assessing the sources and risk of release of ENPs into the environment requires the development of reliable and standard tests to clarify the real-time states of targeted ENMs in natural aquatic environments and nanotoxicological study with a holistic approach (He et al., 2014). Taking in to account that routes of NPs exposure to organisms is also significant in relation to the NP stability in vivo and their toxicokinetics, absorption, distribution, conversion to more toxic metabolites, and interaction with macromolecules. This highlights the importance of including physiochemical characterisation assessment in conjunction with the toxicological assessment (Mytych et al., 2012.).

1.4. Nanomaterials of study: C_{60}, Carbon Black

1.4.1. C60-Structure

Prior to the discovery of fullerenes by Kroto and Smalleyin 1985, the most common forms of pure carbon were graphite and diamond, both of which are crystalline materials (Kroto et al., 1991). By contrast, the materials formed from fullerenes are molecular solids, and their structure and properties are of considerable interest both in the solid form (fullerite) and isolated molecular form. The sixty carbon atoms in the C_{60} molecule are arranged at the vertices of twenty hexagonal and twelve pentagonal rings. Each carbon molecule is in a sp^2 hybridised bonding arrangement which gives rise to sixty delocalized π-electrons around the closed carbon cage. These electrons are of particular interest when considering the electronic and optical properties of $C_{60.}$ Although each carbon atom is equivalent, the three bonds emanating from each atom are not equivalent, two being electron deficient single bonds, and one being an electron rich double bond, (Metanawin et al., 2011). This affects the length of the bonds, making single bonds around each pentagon longer than the double bonds at the edge of each pair of adjacent hexagons. The bond lengths are 1.40 Å for a hexagon edge and 1.46 Å for a pentagon edge.

The bond angles on the hexagonal and pentagonal faces are 120.0^{0} and 108.0^{0} respectively. Figure 1.1 (d) shows the structures of diamond, grapheme, C_{60}, as well as other forms of carbon and NP arrangements. As previously mentioned, the electrons of C_{60} are of particular interest when considering the electronic and optical properties of C_{60} but it is important to note that they may also significantly affect the molecular toxicity (The Nanoage, 2012).

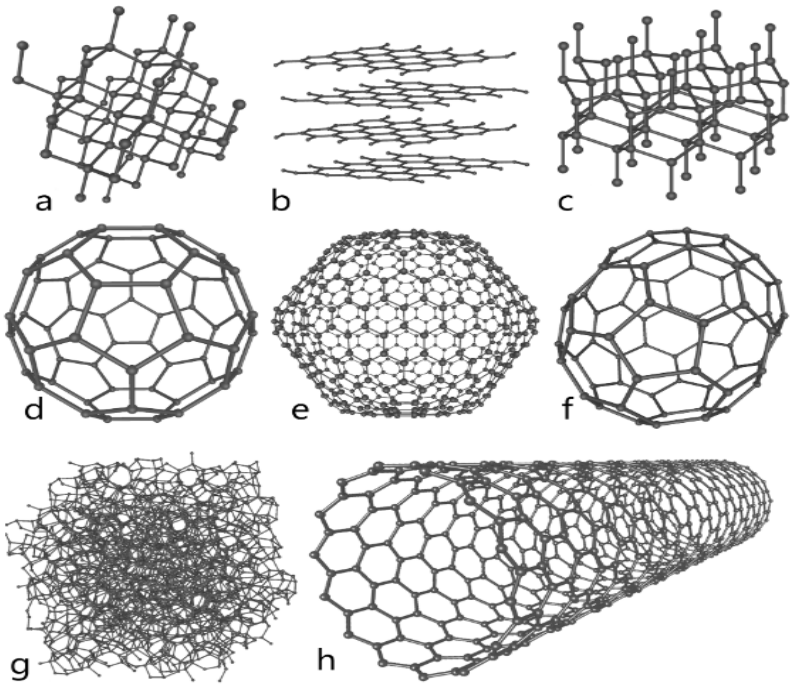


Figure 1.1: NP Structure: a) Diamond, b) Graphite, c) Lonsdaleite, d) C_{60} (Buckminsterfullerene), e) C_{540} (Fullerene), f) C_{70} Fullerene, g) Amorphous carbon, h) single-walled carbon nanotube. (Michael Ströck under the GNU Free Documentation License: The structures of eight allotropes of carbon).

1.4.2 C_{60} Solubility

C_{60} is a hydrophobic material and exhibits a reverse solubility in most organic solvents, that is, the sample usually requires refrigeration, (0-5^{o}C) for a minimum of 12 hours to achieve a high degree of solubility in contract to heat being used to aid solubility. This was demonstrated by (Ruoff et al., 1993) who reported the highest C_{60} solubility in toluene and hexane at 280K, (6^{o}C). The resultant solutions have a characteristic deep purple colour. The hydrophobic character means that molecular C_{60} is expected to have a low solubility in polar solvents such as water (approximately <10^{-9}mg/L) (Torres et al., 2011). However, upon contact with water, C_{60} forms a water stable colloidal aggregate often referred to as nano-C_{60}. These aggregates are composed of C_{60} clusters with diameters of 5-500nm (Sayes et al., 2005). In this study, no distinction will be made between C_{60} and these aggregates, since this is the state of C_{60} in water. The smallest aggregates are typically circular in cross section, intermediate and large particles are mostly rectangular, and the very largest particles often appear to be triangular (Fortner et al., 2005). This aggregation allows for concentrations of up to 100mg/L which is ~11 orders of magnitude more than the estimated molecular solubility (Fortner et al., 2005). Several studies have reported shape evolution of NPs in varying solutes and temperatures, one such study was conducted by Tang et al., 2008 who observed the shape evolution of silver NPs on Silicon Dioxide substrates over varying temperatures.

At room temperature the silver NPs were semi spherical in shape, with an average size of 9nm, when the temperature was increased to 700 °C, where surface diffusion and surface premelting took place, spherical particles were observed. When the heating temperature was increased up to 750 °C, the spherical Ag NPs were found to desquamate from the substrates due to the decreases of the contact area and the binding force between Ag NPs and SiO2 substrates. Indeed, the solution colour, (yellow) and the hydrophobicity and reactivity of individual C_{60} molecules at the aggregates surface are substantially altered with some researchers suggesting a degree of oxidation of the surface sites (Cataldo et al., 2010). The discovery of fullerene solubility in lipids has had enormous implications on their potential use in biological applications, including medicinal chemistry (Cataldo et al., 2010). Fullerenes are also well known as free radical scavengers and have been defined as "free radical sponges", and therefore their antioxidant and unique properties can be used in many different fields of medicinal chemistry, ranging from the treatment of cancer to the treatment of certain neurological diseases (Cataldo et al., 2010). Fullerenes are also employed in the areas of antimicrobial therapy, cytoprotection, enzyme inhibition, controlled drug delivery and contrast or radioactivity based diagnostic imaging (Markovic et al., 2008). However, the potential use of fullerenes in the field of medicinal chemistry and also in the field of cosmetics has been hindered by their insolubility in water and in aqueous environments. To circumvent this water solubility problem, fullerenes have been functionalized with hydrophilic chemical groups or encapsulated in supramolecular complexes such as cyclodextrin or polyvinylpyrrolidone (Cataldo et al., 2010).

1.4.3 Second Nanomaterial of study: CB

CB is a generic term for a family of products used widely to modify the mechanical, electrical, and other physical properties of the medium in which it is dispersed (Wang et al., 2003). It is thought to be the most widely used nanomaterial, with particle dimensions ranging from tens to a few hundred nanometers, (Wang et al., 2003). Figure 1.2 shows the structure of CB. CB plays a major role in

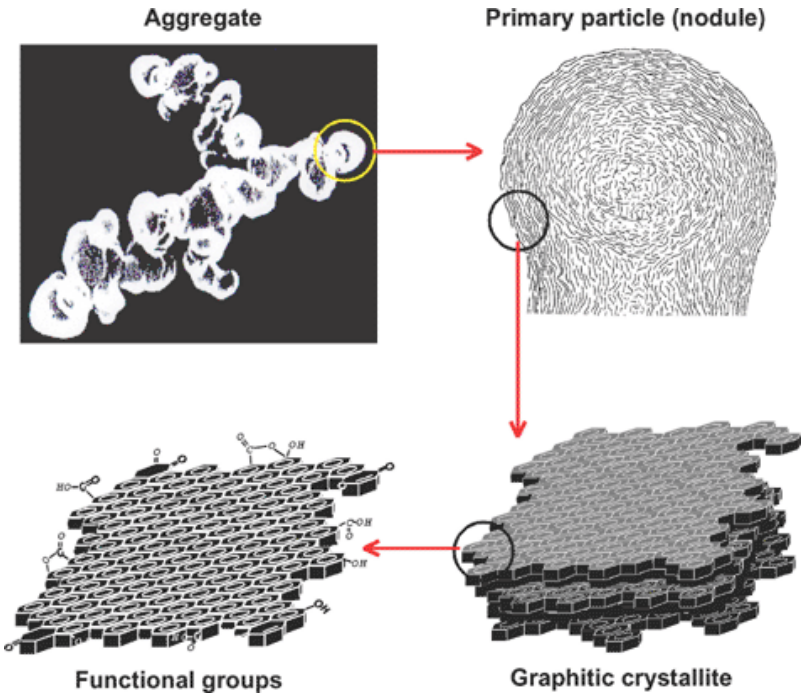


Figure 1.2: CB Structure (Wang et al., 2003).

The properties of the various types of CB are a function of the fuel source used during manufacture and the type of combustion process applied. CB is graded by the following properties; its ability to colour (known as tint), surface area, primary particle size, structure (the number of primary particles that fuse to form each secondary particle), and conductivity in the case of electrically conductive blacks. When listing uses of CB's, there are two main categories; rubber grades and special grades. The rubber grades are primarily used as reinforcing agents in tires and other rubber products to improve their mechanical properties. Special grades are manufactured to meet specific product specifications for imparting certain properties required by the end use products, such as dispersion, pigmentation and electrical conductivity. Today, over 95% of the estimated 10 million tons of CB produced annually worldwide is produced by the oil furnace process (Voll et al., 2002). These substances exhibit a high sorption capacity for organic contaminants in the environment. Therefore, CBs can be regarded as adsorbents ubiquitous in soil and aquatic environments, consequently influencing the chemical fate in the environment (Shih et al., 2012). This highlights the importance of studying the fate of CB in the environment, and in this context, not only do direct effects need examination but also indirect effects in the way that, in the presence of CB, the toxic effect of organic contaminants may be seen to increase (Nowack et al., 2007).

1.5 Toxicology and Nanotoxicology

Mammalian toxicology is the assessment of the adverse effects of test substances on mammalian species. In general, mammalian toxicology dominates much of the published literature, due to the fact that it directly impacts human health. ecotoxicology, on the other hand, encompasses the study of both animals and plants within their specific environment. In other words, entire food webs are analyzed to determine if there is a toxic response to all or some of the trophic levels in that environment. Also of concern is the potential for toxins to accumulate in the environment. This is called bioaccumulation, where concentrations are observed to accumulate in species due to repeated exposure. Bioaccumulation leads to the possibility of affects being carried along the food chain and exacerbated, as well as adverse effects on reproduction. Bioavailability is another phenomenon studied widely in this area, which is the description of whether the make-up of a species allows for exposure to a toxicant, for example a filter-feeder by the means by which it feeds is exposed to toxicants in the water when feeding. The suggestion that ecotoxicology be separated into its own area of research came from Rene Truhaut in 1969 (Truhaut et al., 1969). Aquatic toxicology is a branch of ecotoxicology that assesses the effects of substances and physico-chemical conditions on plants and animals that live in water environments. This includes the study of mechanisms by which changes in the quality of water or food supply affect the growth, reproduction, behaviour or survival of aquatic organisms.

The evaluation of the effects of NPs on living organisms, which is an extensive area of research worldwide at present, is termed Nanotoxicology; examples of the work being carried out include the "Nanosafety Cluster (http://www.nanosafetycluster.eu/)", a consortium of European projects on the Health and Environmental Impact of Nanomaterials, funded by the European Union through FP7. In order to understand what Nanotoxicology involves, we must first define the term toxicology; "Toxicology is the study of the adverse effects of chemical, physical or biological agents on living organisms and the ecosystem, including the prevention and amelioration of such adverse effects" (American Society of Toxicology, 2014). Given such a broad definition, toxicology is often sub-divided into specific research areas; most commonly mammalian and eco-toxicology. Nanotoxicology combines physiochemical characterisation and the determination of bio- molecular interactions with traditional toxicity screening in order to formulate a valid risk assessment. Toxicity is chemical and organism specific i.e. different chemicals act in different ways and different organisms respond in different ways. And in some cases different organs of the test species can display a stronger reaction to the test substance than other organs in the system as reported by (Keene et al., 2012) where gold NPs demonstrated specific distribution sites and blood serum data difference in a range of tissues. This is called organ specificity and must be considered when performing *in vivo* testing. In the case of aquatic toxicity testing, no one species is always the most sensitive for all chemicals or effluents being tested (Aquatic Toxicity Testing in Ireland, 2011). Therefore it is imperative to test a battery of organisms from different trophic levels, generally a fish, a crustacean and an algal species.

Recently, many commentators have expressed concerns about the use of nanotechnology in consumer products and markets, with fears and uncertainties about the safety of free engineered nanoparticles, (ENPs), being paramount. A number of international bodies have begun to recognise the potential toxicity of these particles. For example, the Canadian government in 2009 introduced a series of regulations concerning the reporting and tracking of NMs (Stone et al., 2010). The US EPA has also reclassified a number of NPs most notably nano-silver, as pesticides (Weiss et al., 2006). This lack of publication and awareness of the environmental and health impacts of NPs is in part due to difficulties in establishing efficient and reliable toxicological assays for the assessment of NPs with respect to cell lines and also the potential that once in an open environment NPs will coalesce into large aggregates, thereby exhibiting behaviour more a kin to the bulk material. Indeed the high surface area of NPs maximizes any chemical interactions with the environment, and could make such particles less mobile in environmental systems e.g. groundwater systems, due to increased interaction with porous media, therefore slowing transport (Handy et al., 2008). On the other hand, like naturally occurring colloids, the high surface area may facilitate the adsorption of molecular contaminants leading to concentration of contaminant molecules and an avenue for long-range contaminant migration. All of these questions are the focus of on-going NP toxicological studies worldwide. It has recently been demonstrated that NPs in aquatic environments can in fact remain as isolated free NPs depending upon the organic or biological content of the water in which it is suspended (Handy et al., 2008).

It is thus postulated here that, in a manner similar to the protein-NP interactions in the body, the key to understanding the fate and eco-toxicity of NPs is to determine the degree of NP interaction with its own local environment i.e. whether the NP binds to organic matter which may subsequently be consumed by a filter feeder, thus entering the food chain or will it sediment and aggregate. The evidence suggests that, in aquatic systems with suitable organic matter, NPs can be dispersed to form stable colloidal suspensions. However, the degree and extent of the NP reactivity, mobility, eco-toxicity and persistency in the environment has to date not been determined, leading to speculation regarding potential adverse effects. Environmental fate models have been established and used to assess the fate and transport of organic chemicals for 30 years. Such models explore the relationship between the physicochemical properties of a chemical and its behaviour in the environment, coupled with a detailed description of environmental processes. The use of these models has enabled scientists to make accurate predictions of the fate of many different chemicals in various environmental systems (Praetorius et al. 2012). This method is now being applied to the study of NPs in the environment, an area from which publications are now emerging. For example (Mueller et al., 2008) derived predicted environmental concentration, (PEC), values for silver, and titanium dioxide NPs as well as carbon nanotubes in air, water, and soil. Literature reports have shown that filter feeders like *Daphnia magna* can in fact ingest carbon nanotubes, but that the organism is incapable of excreting the NMs, thus leading to speculation and significant concern about bioaccumulation of carbon nanotubes in aquatic environments.

It was further shown that the rate of uptake can be influenced by functionalising the carbon nanotube surface with lipids. These models are being developed further by using a probabilistic material flow analysis to account for the large uncertainties and variables of the model input parameters, such as production volumes and NP behaviour within and between the model compartments (Gottschalk et al., 2009).

1.5.1 Eco-Toxicity of C_{60} Findings published to date.

There are a wide variety of routes by which NPs may reach humans and other organisms. For example, organisms may ingest materials that have entered a water system or that have been deposited on vegetation. Once materials have been inhaled or ingested, they may enter the food chain, leading to the possibility of bioaccumulation and ingestion by organisms further up the food chain. The bioaccumulation of NPs will depend on their surface properties, which will determine whether they are likely to be taken up by the fatty tissue, bone or proteins in the body. Low aqueous solubility generally favours the persistence of a chemical in the environment and its uptake by biological systems, where it can persist for long periods of time and bioaccumulate, as has been shown for DDT and dioxins (Hoet et al., 2004). (Fortner, et al., 2005) reported some of the first studies on how C_{60} affected bacteria and simple organisms like worms.

They also explored the potential of the fullerene to move up the food chain. The initial results showed that the NP can accumulate in living cells over time, with ever-increasing concentrations in microbes, in the worms that eat those microbes, and in animals higher up the food chain. It was this data that showed significant potential for these NPs to reach humans (Fortner et al., 2005).There are several reported studies evaluating fullerene toxicity in aqueous systems. However, they have been the subject of significant debate with conflicting results being published. C_{60} has been shown to inhibit the motility and phagocytosis of macrophages in the lungs, and the authors postulated that similar effects might be expected in simple soil organisms (Lam et al., 2004). Lovern et al., 2007 found that exposure of *Daphnia magna* to filtered C_{60} caused an increase in mortality with increasing concentrations. It should be noted, however, that the upper dose rates were well in excess of realistic exposure scenarios, >100ppm. The first known published work on C_{60} toxicity to organisms concluded that C_{60} produced oxidative damage in the brains of exposed largemouth bass (Oberdorster et al., 2004). The tendency of C_{60} to aggregate and deposit was proposed to play a key role in determining its longevity in aquatic systems and, therefore, provide key information on the exposure risk presented by these colloids. In one case, it was shown that hydrophobic contaminants can irreversibly interact with fullerene aggregates in water and that these species showed a high capacity for concentrating a model aromatic hydrocarbon (Cheng et al., 2004). Lecoanet et al., 2004 evaluated the mobility of eight particulate fullerenes in a well-defined porous medium, to assess their potential for migration in porous media such as groundwater aquifers and water treatment plant filters.

They found that the particles exhibited widely differing transport behaviours. Their results suggest that the potential for exposure to C_{60} through groundwater transport may be less than that of other fullerenes (Lecoanet et al., 2004). Their results were supported by observations made by (Brant et al., 2005) who suggested that, under some conditions present in natural aquatic systems, these materials have limited mobility as they form large aggregates that may settle out of suspension or deposit on surfaces. These phenomena may, at least partially, offset any risk presented by C_{60} toxicity due to a reduced potential for exposure (Brant et al., 2005). Such investigations will increase understanding of the potential uses of such NPs to clean-up groundwater pollution, as well as aid in the assessment of any environmental risks the materials may present. More recent data, however, has shown that fullerenes, including C_{60}, might interact with natural organic matter in real water systems (Hyung et al., 2007) and be stabilized as individual entities with their potential for dispersal increased dramatically. In fact, natural organic matter stabilizes the carbon nanotubes, (CNT), in the aqueous phase more efficiently than any surfactant (Hyung et al., 2007). Another concern regarding this interaction in aquatic systems is that if NPs are released into polluted waters the affects may be exacerbated. For example (Baun et al., 2008) showed that phenanthrene uptake by *Daphnia magna* was more rapid in the presence of C_{60}, as the C_{60} interacted via a hydrophobic interaction with the phenanthrene and enabled its delivery to the *Daphnia*. Similarly (Navarro et al., 2008) showed that C_{60} NPs may adhere to algae which may then be ingested by filter-feeders such as *Daphnia magna*, possibly leading to a bioconcentration of NPs and transfer to higher trophic levels.

(Zhu et al., 2006), carried out acute toxicity tests with *Daphnia magna* and showed a median lethal concentration, LC_{50} value for tetrahydrofuran, (THF), solubilised C_{60} to be 0.8mg/L. For water-stirred C_{60} the LC_{50} was observed to be 35mg/L, meaning a concentration of 0.8mg/L. THF-solubilised C_{60} was sufficient to cause death to 50% of the *D. magna* tested, whereas in the populations treated with C_{60} alone, 50% mortality was observed at a concentration of 35mg/L, thus showing that C_{60} toxicity increased in the presence of THF. This result also brings into question the use of solvents in ecotoxicity testing in that the solvents can influence the results obtained and such results may therefore not be reflective of the C_{60} toxicity exclusively. As well as solvent use, the presence of different functional groups on NPs have been shown to affect toxicity. Lovern et al., 2007 examined behavioural affects of *Daphnia magna* when exposed to C_{60} and a fullerene derivative $C_{60}H_xC_{70}$. C_{60} elicited an increase in heart rate, although $C_{60}H_xC_{70}$ did not produce this affect, indicating the presence of the functional group affected toxicity, in this case decreasing the effect. However, this study shows the importance of considering that the response may differ and should be taken into account when employing solvents. (Kim et al., 2010) carried out an investigation into the toxicity of fullerenes and developmental changes using embryos of the fish, Japanese medaka, (*Oryzias latipes*), in which they report that the physicochemical properties of fullerene water suspensions and their subsequent toxicity were influenced by different preparation methods. Suspensions were produced by three methods: toluene exchange, DMSO dissolving, and stirring over time.

This study also reported particle size, zeta potential, and C_{60} structure, which have been found to be strongly dependent on both the type of aggregates formed and the test medium; Tol/C_{60} exhibited small and spherical closed aggregates, whereas DMSO/C_{60} and Aqu/C_{60} presented mesoscale aggregates of smaller spherical aggregates. The highest mortality and glutathione induction rates were observed by the Tol/C_{60}, attributed to its closely packed fullerene structure, followed by the DMSO/C_{60} and Aqu/C_{60} (Kim et al., 2010). A report by Henry et al., 2011 has, however, brought much of the known literature on aquatic C_{60} toxicity into question. They claim that C_{60} can indeed affect environmental fate, transport, and bioavailability of co-contaminants in aquatic environments. In addition, they claim that the techniques for evaluation of reactive oxygen species, (ROS), production and toxicity of aqueous preparations of C_{60} have evolved over time, and led to unintentional erroneous reports of C_{60} ROS generation and toxicity. Their results showed minimal ROS production by aqueous C_{60} when appropriate experimental controls were employed to eliminate artefacts (i.e., solvent effects and controlling light). Their results have thus reignited interest in the toxicity of this NP and in particular the methods used in previous testing. One such method endeavoured to overcome this issue by employing extended stirring in water without the aid of organic solvents to create colloidal suspensions of C_{60} and C_{70} with the intention of creating fewer preparation artefacts which the author claimed is more representative of natural environmental systems (Xin et al., 2011).

1.5.2 Findings of Eco-Toxicity of CB published to date.

There is an alarming lack of literature on the toxic effects of CB in the environment to date, particularly when considering its wide spread usage, (ENRHES Review 2011). The current study aspires to contribute towards the knowledge gap in understanding the behaviour of CB in the environment. The majority of toxicological studies published to date have focused on mammalian toxicology. Where CB has been used as a model particle in particle toxicology investigations, with extensive study in both *in vivo* and *in vitro* mammalian laboratory experiments. For example the study carried out by Barlow, et al,. 2005, confirmed that CB particle exposure to type II epithelial cells increased the release of macrophage chemoattractant compared to the control, which mediate macrophage migration such as macrophage chemoattractant protein 1, (MCP-1), which is a potent mediator of macrophage migration and thus plays an essential role in early events of inflammation, (Nam et al., 2002). In order to aid in the recruitment of inflammatory cells to sites of particle deposition and the subsequent removal of the particles by phagocytosis. Other examples where CB was used as a reference toxicant was in the study of single walled carbon nanotubes, (SWCNTs), toxicity carried out by Lam, et al. 2004 Printex-90, (CB) was injected into $B6C3F_1$ mice for 90 days, after which time the lungs of the mice were excised, fixed and stained for histopathological assessment.

The particle size characteristics of manufactured CBs have been extensively studied and it has been reported that inhalable airborne CB particles are significantly larger in aerodynamic diameter than particles that occur in the early stages of CB formation, (McCunney et al., 2001) Other studies involving CB include assessment of traffic-related particles (TRPs), as one of the main uses of CB is in car tires. (Fang et al., 2012) reported associated adverse cardiovascular events in humans. Navarro, 2008 et al., proposes that the possible ecotoxicological impacts of NPs remain unknown. It is also imperative to note that the majority of cell culture work assessing CB ecotoxicity has been carried out on mammalian cells. The structure and processes compared to plant cells differ greatly, namely the presence of cell walls in plant, algal and fungi cells. The cell wall alters the entrance of NPs into these cells as well as providing a primary site for interaction with NPs. In this respect, there are several questions remaining regarding the bioavailability of ENPs, their uptake by algae, plants, and fungi and the toxicity mechanisms involved (Navarro et al., 2008). A study conducted by (Cheng et al., 2007) investigated the ecotoxicity of a number of NPs, one of which was CB, to zebrafish, (*Danio rerio)* hatching rates; CB did not show an effect on hatching rates. Mwangi, et al., 2012 correlated the findings presented by Cheng et al., 2007, suggesting that CB did not cause embryo hatching delays, in comparison with Carbon Nano tubes, (CNTs) where a hatching delay was recorded, the suggested reason for this being the surface coatings/functionalizations that are added to the CNT's for the various applications.

Fernandes et al., 2007 showed that, by exposure of aquatic crustaceans *D. magna*, *Artemia salina* and Gammarids to TiO_2, fluorescent polystyrene particles, and CB, particles are readily ingested and accumulated in the gastrointestinal tract and distributed into body lipid droplets. (Canesi et al., 2010) reported that suspensions of CB induce oxyradical production and lysosomal enzyme release in the hemocytes of the marine mussel, *Mytilus galloprovincialis in vitro*. Mussels were exposed to different concentrations; 0.05, 0.2, 1, & 5 mg/L of NP suspensions for 24h and different biomarkers were evaluated. Results showed that CB induced significant lysosomal membrane destabilisation in both hemocytes and in the digestive gland where lysosomal lipofuscin accumulation was induced. An increased activity of the antioxidant enzyme catalase was also reported. In a study assessing the partitioning and toxicity of carbon NPs in the aquatic environment, Kennedy et al., 2008 reported CB produced a higher mortality of the test amphipods, *Leptocheirus plumulosus* and *Hyalella azteca*, compared to multi-walled carbon nanotubes, (MWNTs), recording median lethal concentrations, (LC_{50}) of 50-264 g/kg and 18-40 g/kg respectively. This study also reported that a higher NP toxicity was observed with the smaller particle size samples tested. (Smith et al., 1969) reported that CB inhibited growth of tobacco plant cells; a 20% concentration of CB in the growth culture media gave a 50-90% inhibition compared with untreated cultures. Free proline in the cells was also increased by CB, the function of which is to protect the cells by inhibiting lipid peroxidation.

One of the current uses of carbonaceous materials is as a method for sediment remediation, in order to reduce porewater concentrations and risks by binding hydrophobic organic compounds, (HOCs) present in aquatic sediments (Rakowska et al., 2012). The authors also review the extent to which carbonaceous materials may reduce bioaccumulation and toxicity of HOCs and whether carbonaceous materials have negative effects on benthic species and communities, which they reported as a low negative effect on benthic species present.

1.6 Thesis Outline

The emergence of nanotechnology out of the research lab and into the consumer market has exposed a number of concerning gaps in the assembled knowledge to date and raised a number of concerns regarding the environmental and health impacts of NMs (Handy et al., 2008). The knowledge gaps predominantly concern the exposure risks associated with NMs and approaches to characterise these novel systems. Research has a key role to play in this context. It develops new technologies for application in industry while simultaneously investigating the potential risks. The questions or aspects of nano-research which this report and purposed future work will attempt to address include the impact of nanotechnology on the environment, specifically the aquatic environment and assessment of current methods of *in vitro* toxicity testing to investigate the potential toxicity of NPs and the underlying mechanisms of NP ecotoxicity.

In addition, there are numerous regulatory knowledge gaps. These gaps are due, in part, to the lack of a standardised terminology and invalid risk assessments leading to unregulated nanotechnologies finding their way into consumer markets and subsequently into ecosystems. A key objective of this work therefore is to constructively contribute to the growing body of knowledge on the impact of NPs in the environment, by assessing the ecotoxicity of C_{60} and CB. The study involves the determination of particle size, surface area, and testing of three environmental test species, selected due to their relevance to the test system being assessed. The species selected were an algal strain, an invertebrate, and a crustacean, chosen to represent a broad spectrum of the fresh water food web. The species are recommended by the global scientific community for analysing fresh water aquatic life; the crustacean *Daphnia magna*, the algae *Pseudokirchneriella subcapitata*, the bacterium, *Vibrio fischeri*, and a fish cell line from the rainbow trout gonad tissue, *Oncorhynchus mykiss*, and a cancerous liver from the topminnow, *Poeciliopsis lucida*. Table 1.1 shows the species employed in both fresh water and marine water ecotoxicological tests. As well as the three freshwater species outlined in table 1.1, a forth invertebrate species was included in this body of work; *Thamnocephalus platyurus* (Aquatic Toxicity Testing in Ireland, 2011, Enterprise Ireland annual report). The Environmental Protection Agency, (EPA), came into being in Ireland in 1992 and introduced toxicity testing requirements into its IPPC licences. Prior to 1992 the Local Authorities issued licences and only a few of these, mainly Cork County Council, required toxicity testing of effluents. The aforementioned tests are those employed currently to assess aquatic toxicity nationally, (Aquatic Toxicity Testing in Ireland, 2011, Enterprise Ireland annual report).

Table 1.1: Receiving Waters and Appropriate Test Species, (SATL, 2011)

Trophic Level	Marine Species	Freshwater Species
Fish	*Psetta maxima*	*Oncorhynchus mykiss*
Crustacean	*Tisbe battagliai*	*Daphnia magna*
Plant/Alga	*Skeletonema costatum*	*Pseudokirchneriella subcapitata*
Bacterium	*Vibrio fischeri*	*Vibrio fischeri*

Standardised ecotoxicological test methods were employed, two of which followed OECD test guidelines. This battery of bioassays, that represent several trophic levels of the aquatic environment with varied habitats, followed the approach for ecotoxicological testing of all new test substances. This affords a greater insight into how the particles affect the aquatic environment as a whole. The principle of the majority of ecotoxicity tests and the tests carried out here is as follows; test organisms are exposed to a concentration series of the test substance for a designated time, generally short term (acute, 24-48 hours), but can also be long term (chronic, several days). At the end of the exposure time, the effects, e.g. lethality, immobilisation, growth inhibition are assessed by comparison to untreated controls. This allows for the construction of a concentration-effect graph and calculation of an LC_{50}, (Median Lethal Concentration) or $EC_{50,}$ (Median Effective Concentration), the concentration that kills/immobilises/inhibits 50% of the population tested. The following chapters present the information of the material outlined; Chapter 2; Experimental Techniques and Procedures, Chapter 3; Physical Characterisation, Chapter 4 and 5; Results, Chapter 6; detailed discussion and future work recommendations.

Chapter 2 Experimental Techniques and Procedures

2.1 Introduction

The previous chapter discussed the background theory, findings to date in this area of research and objectives of this study. An underlying theme of the chapter was the need to ensure tractability and standardisation in all aspects of nanotoxicology research, including both the materials and methods. This chapter further develops the latter point and describes the general experimental techniques used in characterising and assessing the toxic effects of the NPs studied. A brief introduction to the underlying theory for each technique is given, after which the general experimental arrangement and sample preparation is detailed.

2.2 Nanoparticle Characterization

The NPs employed in this study were purchased commercially, and then characterized in-house to compare with the manufacturer's specifications and the stability of the particles, an issue which has been one of the most challenging to the scientific community in its quest to quantitatively and accurately determine the level of toxicity of NPs that are now being employed in hundreds of products and by-products worldwide (Project on Emerging Nanotechnologies, 2014).

The importance of including physico-chemical characterisation in toxoicolological assessment of NPs was highlighted in a publication by (Bouwmeester et al., 2011) following a workshop held by members of NanoImpactNet. The NanoImpactNet is a European Network on the Health and Environmental Impact of NMs, who agreed that data published in this field, should be based on well characterized dose-response relations derived from the systematic study of the bio-kinetics and bio-interactions of NMs at both organism and sub-cellular levels. Bio-kinetics being the evaluation of the deposition pattern of inhaled particles and their subsequent dissemination in the respiratory tract and the entire organism being studied (Geiser et al., 2010). Bio-interaction of NMs involves the assessment of the interactions of NMs with biological systems and the following adverse effects (Pelaz et al., 2013). In addition to developing and validating analytical methods to determine the metrics stated. The testing programme of OECD Working Party on Manufactured Nanomaterials, 2008, specifies a non-prioritized list of physico-chemical properties and material characterization end-points. The current study employed a range of these techniques which are designed to characterize particle size, aggregation state and particle stability in solution. The following sections describe the techniques and methods employed during this study.

2.2.2 Particle Sizing: Dynamic Light Scattering

Dynamic light scattering, (DLS) is a technique used for measuring the size of particles, typically in the submicron region. DLS (also known as PCS, Photon Correlation Spectroscopy), measures frequency fluctuations in the interference pattern caused as a beam of light is scattered by the Brownian motion of the solvated particles. Brownian motion is the random movement of particles due to the bombardment by the solvent molecules that surround them. The larger the particle, the slower the Brownian motion. In order for the Brownian motion to be considered truly random, the temperature must be kept stable to prevent convection currents from influencing the movement. Similarly, the solvent viscosity will also play a vital role and needs to be factored into the final calculation of particle diameter. In doing so, DLS does not measure physical particle size, but rather the effective particle diameter in its local environment. This effective diameter, known as the hydrodynamic diameter, can be calculated from the Stokes-Einstein equation, (Equation 2.1.) From the Stokes-Einstein equation, the hydrodynamic diameter's dependency on the particle's translational diffusion, (velocity), as well as the solvent viscosity and temperature, is clear. In essence, the hydrodynamic diameter refers to how a perfectly spherical particle diffuses within a fluid and in cases where the particle is non-spherical, an equivalent sphere of the same diffusion coefficient is assumed (Hackley et al., 2011).

$$d(H) = \frac{kT}{3\pi\eta D}$$

Where: *d(H)* = hydrodynamic diameter
D = translational diffusion coefficient
k = Boltzmann's constant *T* = absolute temperature η = viscosity

Equation 2.1: Stokes-Einstein equation, (Hackley and Clogston 2011)

In this study, a Malvern Zetasizer Nano ZS was used for determining the hydrodynamic diameter/particle size of the NPs in distilled water. The Zetasizer Nano ZS has a size measurement range of 0.6nm to 6μm hydrodynamic diameter. The laser used to illuminate the sample is a He-Ne gas laser with a wavelength of 633nm. The detector used to measure the intensity of the scattered light is an avalanche photodiode, Q.E. >50% at 633nm and measures at 173° to the incident beam, (backscatter detection). The instrument contains automatic laser attenuation transmission, ranging from 100% to 0.0003%, to reduce or increase the intensity of scattering to be detected. Measurements were carried out with a concentration of 100ug/mL of NP; the samples were gently agitated for approximately 10 minutes before being tested. Measurements were carried out at 25^{0}C, three measurements were taken, after which the average particle size was then calculated and the standard deviation determined. DLS measurements are ideally suited to determine the diameter of spherical particles, which raises questions when using DLS to measure the particle size of non-spherical particles, with this in mind several studies had suggested it can be useful in providing the hydrodynamic diameter for CNTs and this data can be used to evaluate variation of length distributio, (Cheng et al., 2011).

2.2.3 BET Measurement

The BET theory put forward by Brunauer in 1938 (Sing et al., 1985), which was devised to explain how gases adsorb on solids in multilayers when the pressure is gradually increased at the boiling point of the gas being used. It contains a derivation of an adsorption equation, (the BET Equation) based on an extension to multilayers of the Langmuir treatment of monomolecular adsorption, which serves as the basis for an important analysis technique, for the measurement of the specific surface area of a material. The concept describes monolayer molecular adsorption, to multilayer adsorption, with the following hypotheses: (a) gas molecules physically adsorb on a solid in layers infinitely; (b) there is no interaction between each adsorption layer; and (c) the Langmuir theory can be applied to each layer, (NANOGENOTOX Nanomaterials, 2012).

The resulting BET equation is expressed by Equation 2.2:

$$\frac{1}{v\left[(P_0/P)-1\right]} = \frac{c-1}{v_m c}\left(\frac{P}{P_0}\right) + \frac{1}{v_m c}$$

Where:
P/P_0 =relative pressure of adsorbates at the temperature of adsorption
v = adsorbed gas quantity, (for example, in volume units)
v_m= monolayer adsorbed gas quantity
c = BET constant.

Equation 2.2: The BET equation (Sing et al., 1985).

In this study, 0.0751g of C_{60} and 1.000g of CB was weighed out and this sample was then dehydrated for two hours at a temperature above $100^{O}C$. After the dehydration period, the sample was allowed to cool for approximately an hour. Once the sample was prepared, a P_0 measurement was taken, that is, the atmospheric pressure at the time of the measurement. The sample was then inserted, and, using the P_0 reading, the instrument adjusts for the sample and calculates a BET reading of the surface area and generates a BET plot.

2.2.4 Particle sizing: Atomic Force Microscopy

The Atomic Force Microscope, (AFM) is an instrument that can analyze and characterize samples at the microscopic level with a resolution from 100μm to less than 1nm. Figure 2.1(a) shows the general set up of an AFM. The AFM operates by allowing an extremely fine sharp tip to come in contact with, or be in very close proximity to, the sample that is being imaged. This tip is usually on the order of 100μm long and less than 10nm in diameter at its point. This makes AFM a powerful method to measure structures and topographies of NMs. When combined with chemical force microscopy, single molecules can be identified on surfaces (Noy et al., 1997). This gives information about a single particle, its chemistry, charge, and magnetic properties. The tip is located at the free end of a cantilever under which the sample is scanned.

Between the tip and the sample, a variety of forces exist depending on the situation. For example, there can be mechanical contact forces, capillary forces, chemical bonding, or electrostatic forces acting between the sample surface and the tip (Wang et al., 2011). The most common force, however, is the van der Waals force. The force will either attract or repel the tip. An AFM can be operated in a number of different modes, namely contact, non-contact and tapping. Figure 2.1(c) displays the different modes used. In the contact mode, the cantilever is held less than a few tenths of nanometers from the sample surface, and the interatomic force between the cantilever and the sample is repulsive. In the non-contact mode, the cantilever is held on the order of nanometers from the sample surface, and the interatomic force between the cantilever and sample is attractive. In tapping mode, the cantilever is driven to oscillate near its resonance frequency by a small piezoelectric element mounted in the AFM tip holder, similar to non-contact mode. This method of 'tapping' lessens the damage done to the surface and the tip compared to the amount done in contact mode. Regardless of which mode of operation, tip deflections are recorded via a laser focused on the back of the cantilever and any variations in cantilever height are deciphered by measuring the interference it causes to the light beam. The interpretation of the interference patterns results in a topographical representation of the sample surface. In this study, an Asylum MFP-3D BIO Atomic Force Microscope was used. It uses a helium-neon, (He-Ne) laser light source to detect any deflections. In order to measure the particles using the instrument, the NPs were drop cast on to a clean silicon wafer and the solvent allowed to evaporate.

All measurements were done in tapping mode. There are several types of images that can be recorded when using AFM, some examples include; amplitude images, which show the z-scale deflections that of the tip as it comes in contact with the sample's topography. These images are useful for showing the slope of the sample and sample shape but do not allow for sample structure investigation. Phase images are obtained in tapping mode, and are a map of how the phase of cantilever oscillation is affected by its interaction with the surface. In addition to topographic information, the phase can be affected by the relative softness/hardness of the sample, or the chemical nature of the sample (Cleveland et al., 1998).

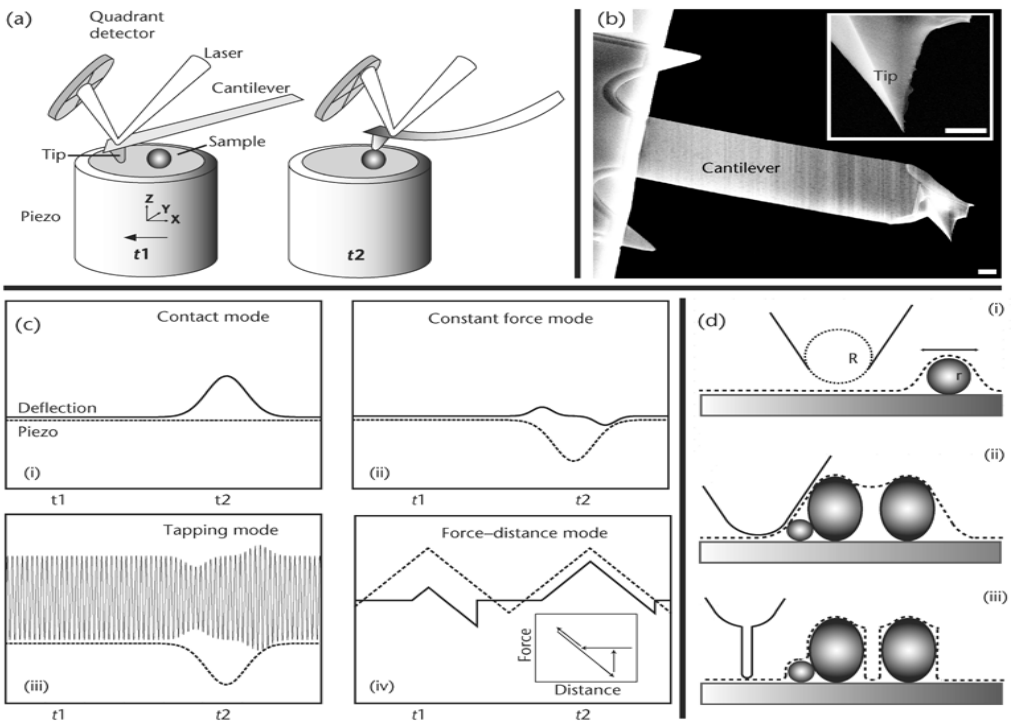


Figure 2.1 (a)-(d): Modes of AFM. (a) General layout of AFM. (b) Scanning electron micrograph of an AFM cantilever. (c) Different modes of AFM operation. The curves represent the response of the microscope as a function of time in (i) contact mode, (ii) constant force mode, (iii) tapping mode and (iv) force–distance mode. (d) Tip convolution effects. (i) Large objects will obscure adjacent features and deep cavities are not accessible (ii). Tips that combine a small end radius with a high aspect ratio can significantly relieve tip convolution effects (iii) (Jager et al., 2007).

2.2.5 Suspension Stability and Zeta Potential

Zeta potential, (ZP), is often employed as an assessment of the stability of a colloidal system (de Morais et al., 2006). When particles are suspended in a medium of a different phase, (e.g. solids suspended in liquid), their interaction with the medium surrounding them determines whether the particles will coagulate or stay dispersed. The interactions with the surrounding medium occur in the area directly surrounding the particle. This region may be considered to consist of two separate layers, the Stern layer and the diffuse layer, figure 2.2 (Salopek et al., 1992). The Stern, (innermost) layer, is the region where ions suspended in the medium adhere to the particle quite strongly. The outer layer, (diffuse), contains ions which are not as strongly bound and are said to be diffuse. The boundary of this diffuse layer has a specific potential which is known as zeta potential, (ZP).

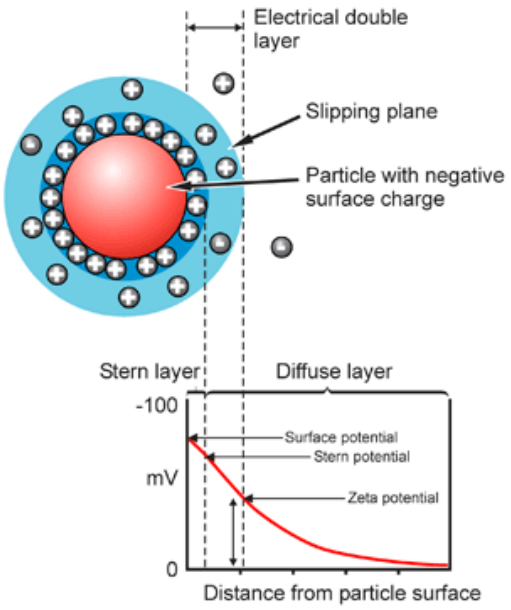


Figure 2.2: Schematic representation of Zeta Potential. (Malvern Instruments Ltd © Copyright 2012)

In general, if all particles in suspension have a large negative or positive ZP, they will tend to repel each other and there is no tendency to flocculate. However, if particles have a low ZP value, then there is no force to prevent the particles coming together and flocculating, (process of reversible aggregation of particles). The dividing line between stable and unstable suspensions is generally taken at zeta potentials of either +30mV or -30mV. Particles with ZP more positive than +30mV or more negative than -30mV are normally considered stable. A Malvern Zetasizer Nano ZS was used for ZP measurements throughout this project, a schematic of which is shown in figure 2.3. The laser used to illuminate the sample is a He-Ne red gas laser with a beam wavelength of 633nm. The detector used to measure the intensity of the scattered light is an avalanche photodiode, Q.E. >50% at 633nm at 17°, backscatter detection. The instrument contains automatic laser attenuation transmission, ranging from 100% to 0.0003%, to reduce or increase the intensity of scattering to be detected. The light scattered at an angle of 17° is combined with the reference beam. This produces a fluctuating intensity signal where the rate of fluctuation is proportional to the speed of the particles. A digital signal processor is used to extract the characteristic frequencies in the scattered light. Samples were prepared in the same method described in section 2.2.2 above.

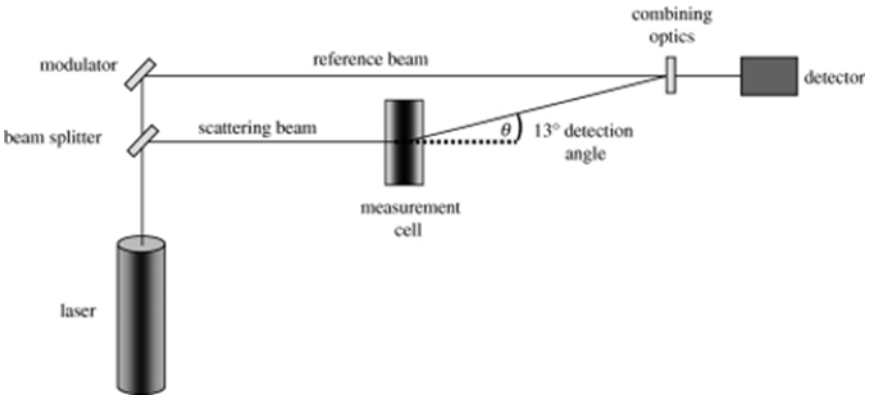


Figure 2.3: Optical configurations of the Zetasizer Nano series. (Kaszuba, Corbett et al. 2010).

2.3 Toxicological techniques

The toxicological tests employed in this study were selected as they are among the species recommended for analysing fresh water aquatic life by the OCED, which are; the crustacean *Daphnia magna*, the algal species *Pseudokirchneriella subcapitata,* the bacterium, *Vibrio fischeri, the* freshwater crustacean *Thamnocephalus Platyurus,* a fish cell line from the rainbow trout, *Oncorhynchus mykiss*, and the topminnow, *Poeciliopsis lucida,* (OECD Guidelines for the Testing of Chemicals, 2011).

2.3.1 Microtox Acute Toxicity Test

The test exposes luminescent organisms, (*vibrio fischeri*) in Microtox Acute Reagent to water borne samples, and measures the increase or decrease in light output by the test organisms. The reagent contains living luminescent bacteria that have been grown under optimal conditions, harvested, and then lyophilized, (freeze-dried). The lyophilized bacteria are rehydrated with reconstitution solution to provide a ready-to-use suspension of organisms. The test system measures the light output of the luminescent bacteria after they have been challenged by a sample toxicant and compares it to the light output of a control, (reagent blank), that contains no sample. A difference in light output, (between the sample and the control), is attributed to the effect of the sample on the organisms. The first step carried out is sample preparation. The NPs were diluted in Microtox Diluent to the desired higher concentration of the test, (normally 1000 ppm or 100 ppm, in 30mL). The next step is to run the accompanying software; 'MicrotoxOmni' which is supported by Microsoft Windows. The procedure is to, click on the Start button and select MicrotoxOmni from the programs menu, insert username and password. The window that opens up allows the user to select a test template and click on the list selection arrow which will display all of the test templates available. The '90% Basic Test for Aqueous Extract' was selected for this study. Once selected, the test parameters can be modified. Test times of 5, 15, and 30 minute time points were selected and an initial concentration of 833ppm is entered. Once the parameters are selected, the Model 500 well display and the Data/Graph window will open on the screen and update as the test is being carried out, (Figure 2.5).

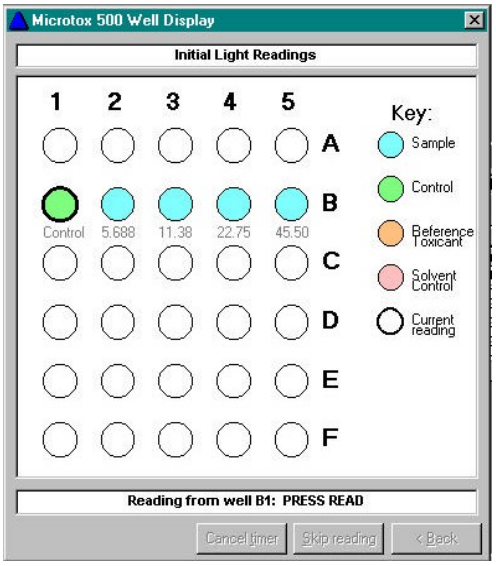


Figure 2.4: Well Display from MicrotoxOmni™ Software prior test to initiation.
(MicrotoxOmni™ Software for Windows® 95/98/NT user manual)

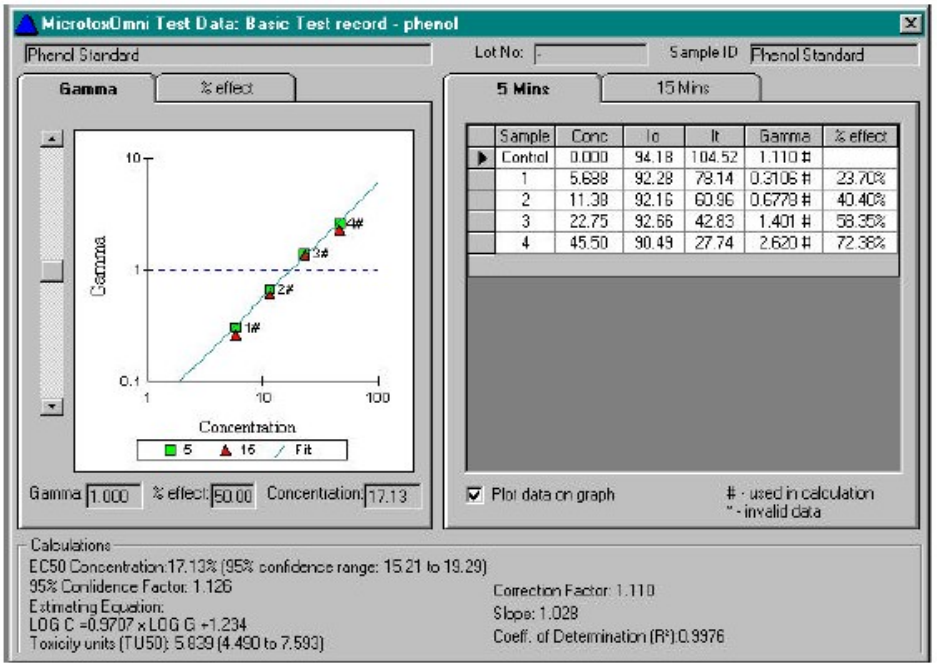


Figure 2.5: The Data/Graph window displaying analysis captured by the analyzer 500.
(MicrotoxOmni™ Software for Windows® 95/98/NT user manual).

2.3.1.1 Methodology for the "90% Basic Test for Aqueous Extract"

Disposable plastic cuvettes are placed in incubator wells: A1 through A5, B1 through B5, C1 through C5, D1 through D5, F3 and Reagent Well. 1000µl of Reconstitution Solution is pipetted into the Reagent Well. 1500 µl of Diluent are pipetted into F3.1000 µl of diluent are pipetted into A1 through A5 and C1 through C4, (leave C5 empty). 1000µl of sample are pipetted into C4 and C5. 1:2 serial dilutions are prepared by transferring 1000µl and mixing after each transfer, (changing pipette tips every time), starting from C4 to C3, C3 to C2, C2 to C1, C1 to A5, A5 to A4, A4 to A3, A3 to A2 and finally 1000µl are discarded from A2. 5 minutes incubation time is required. Once the test is set up, the Microtox Acute Toxicity reagent is reconstituted; a single vial of reagent is removed from the freezer and care is taken to open it with the minimum of handling, thereby reducing warming of the vial. The vial is shaken gently to ensure the pellet of bacteria is seated on the bottom of the vial. The precooled cuvette of reconstitution solution is taken from the reagent well, then the solution is quickly poured into the bacteria vial. The vial is swirled 3 or 4 times, then the mixture is quickly poured back into the cuvette and returned to the reagent well. The bacteria is mixed thoroughly using the pipette by aspirating and dispensing 0.5mL of solution at least 10 times. Reconstituted bacteria should be used within 3 hours of reconstitution. Further tests after this period require the preparation of freshly reconstituted bacteria. At this point, the bacteria are added to the necessary wells. 150µL of reagent are added to F3 and mixed. 100µL of diluted reagent (from F3), are added to B1 through B5 and D1 through D5 and a 15 minute incubation time is allowed.

The B1 cuvette is placed in the READ well and the SET button is pressed, the computer 'space bar' key is then pressed, the instructions the software displays are followed. The I_0 light levels are then read, that is the light levels at time zero. The cuvettes are read in the following order, prompted by the software; B1, B2, B3, B4, B5, D1, D2, D3, D4 and D5. Then immediately the following 500µL transfers are made: A1 to B1, A2 to B2, A3 to B3, A4 to B4, A5 to B5, C1 to D1, C2 to D2, C3 to D3, C4 to D4 and C5 to D5. When completed, the computer 'space bar' key is pressed to start the timer for the first time point, 5 minutes. When the timer sounds, (after 5 minutes, 15 minutes and 30 minutes), light levels are read as prompted by the software B1, B2, B3, B4, B5, D1, D2, D3, D4 and D5. The data is plotted in the data window as the test proceeds. The database function will prompt the user to save the test data to the database once the test has been completed. The data can be sent to another database, if required.

2.3.1.2 Purpose of a standard

Testing a standard, (reference toxicant), whose test results are well characterized, confirms the validity of a test protocol and checks the performance of the complete test system, (e.g. analyzer, reagent, diluent and reconstitution solution). The "3 basic test protocol" is the preferred protocol for testing standards as it provides the highest confidence level. When testing "Standards" an EC_{50} derived from extrapolated data must not be accepted. In this case retesting is performed on diluted standards.

The reference toxicant commonly used for the Microtox Acute Toxicity Assay is phenol. This was the toxicant used in this study also; the reference test was carried out prior to each sample being tested. The reported phenol EC_{50} after 5 minutes exposure is 13-26 mg/L. Therefore, to test the test system being employed, an EC_{50} of 13-26 mg/L must be obtained. This is carried out by initially preparing the phenol standard; 50mg, (0.050g) of crystalline phenol is weighted out and added to a 500mL amber volumetric flask, or 10mg in 100mL. If an amber volumetric flask is not available, the entire flask is covered with aluminium foil to protect the phenol standard from light. Diluent is added to the 500mL mark on the volumetric flask, the flask is sealed, and the solution is mixed well by inverting the volumetric flask. The flask is labelled and stored at normal refrigerator temperature, (2-8°C). The phenol standard can last for 3-4 months when stored in this manner. The software is initiated as described above in section 2.5.1, with the exception of selecting the "3 Basic Test" template instead of the "90% Basic test for Aqueous Solutions".

2.3.1.3 Methodology for the "3 Basic Test"

Cuvettes are placed in incubator wells: A1 through A5, B1 through B5 and reagent well. 1000µL of reconstitution solution are pipetted into the reagent well. 500 µl of diluent are pipetted into B1 through B5. 1000µL of diluent are pipetted into A1 through A4, (leave, A5 empty). 2500µL of sample, (phenol 100 ppm), are added to A5. 250 µL of osmotic adjusting solution are added to A5 and mixed.

750µL are then discarded from A5. 1:2 serial dilutions are prepared by transferring 1000µL and mixing after each transfer, (changing pipette tips every time), starting from A5 to A4, A4 to A3, A3 to A2 and finally 1000µL are discarded from A2, a 5 minute incubation time is allowed. As described in section 2.5.1.1 above, the reagent, (bacteria) is reconstituted at this point. Cuvette B1 is placed in the 'READ' well and then the 'SET' button is pressed. The 'space bar' key is pressed and the instructions of the software are followed. The zero time light levels, I_0 light levels, are read as prompted by the computer monitor: B1, B2, B3, B4 and B5. Immediately, the following transfers are made: 500µl from A1 to B1, A2 to B2, A3 to B3, A4 to B4 and A5 to B5. The computer 'space bar' key is pressed again. After the 5 minute exposure time, the light levels are read again as prompted by the computer monitor: B1, B2, B3, B4 and B5. Once the test is completed the data can be saved in Excel files and saved for the statistical calculation of an EC_{50}. As outlined in section 1.6 of chapter 1, an EC_{50} value is the measurement of the toxicological affects of the NPs in the ecotoxicological tests employed in this study, therefore section 2.5.6 describes the statistical analysis of the tests described here.

2.3.2 Freshwater Alga and Cyanobacteria, Growth Inhibition Test, (OCED Test No. 201)

Pseudokirchneriella subcapitata was originally obtained from the Culture Collection of Algae and Protozoa, (CCAP) Argyll, Scotland. Cultures are incubated at 25^{0}C in a specific algal incubator and continuously shaken under constant illumination of 4000 lux. *Pseudokirchneriella subcapitata* is the test alga used for the 72 hour algal growth inhibition test. *Pseudokirchneriella subcapitata* are single celled green algae that survive in water by means of photosynthesis, that is, they need only carbon dioxide, water and sunlight to survive. They are found in most fresh water systems.

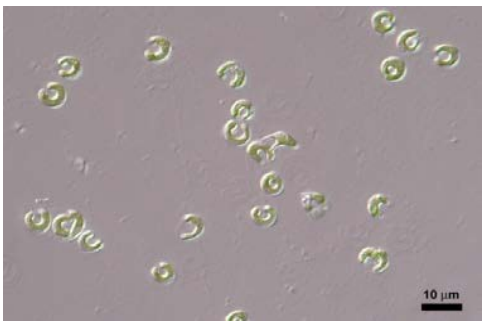


Figure 2.6 & 2.7: Light Microscopic observations of *Pseudokirchneriella subcapitata*. (Held et al., 2011).

The purpose of this test is to determine the effects of a test substance on the growth of freshwater microalgae. Exponentially growing test organisms are exposed to the test substance in batch cultures over a period of 72 hours. The system response is the reduction of growth in a series of algal cultures, (test units) exposed to various concentrations of a test substance.

The response is evaluated as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures. Growth and growth inhibition are quantified from measurements of the algal biomass as a function of time. Algal biomass is defined as the dry weight per volume, e.g. mg algae/litre test solution. However, dry weight is difficult to measure and therefore surrogate parameters are used. Of these surrogates, cell counts are most often used, which is the method used in this study. The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure period. From the average specific growth rates recorded in a series of test solutions, the concentration bringing about a specified x% inhibition of growth rate (e.g. 50%) is determined and expressed as the percentage effective concentration, EC_X (e.g. EC_{50}). In addition, the lowest observed effect concentration, (LOEC) and the no observed effect concentration, (NOEC) may be statistically determined. The following procedure that was followed in this study is in accordance with the OCED guidelines for the testing of chemicals; Freshwater Alga and Cyanobacteria, Growth Inhibition Test Guideline number 201.

2.3.2.1 Measurement of Cell Number

The first step of the procedure is to determine the cell density, (No. of cells per mL of culture medium), of the test culture. This is done by using an Improved Neubauer Counting Chamber (Figure 2.8). The counting chambers are flooded with the algal culture, the chamber is placed under a light microscope and manual counts, (counting each visible cell in the squares of the chamber, on a hand held counter), are used to calculate the average of cells/mL. The required concentration of algae for the test is 10,000 cells/mL. The quantity of algae used per test vessel is calculated from the average count of the test algae per mL.

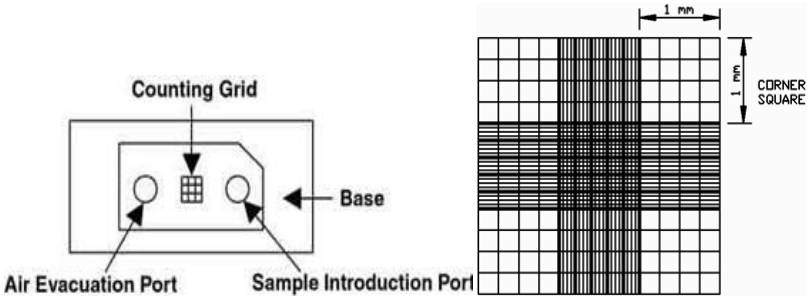


Figure 2.8: Improved Neubauer Counting Chamber, (Nexcelom Bioscience LLC).

2.3.2.2 Preparation of Test Substances

A range finding study is carried out initially, whereby a wide range of concentrations are used to find the range in which up to 75% of the algal growth is inhibited. In the case where NOEC can be found, concentrations are not increased to more than 100ppm, (100mg/L), as it is thought to be not environmentally relevant in this test system. For this series of tests initial concentrations of 100ppm C_{60} and 10ppm CB were used to carry out the range finding experiments. Prior to assessing the test substance, a series of experiments must be carried out to validate the test procedure. This is done by using a positive control. For this, potassium dichromate is used; serial dilutions are prepared and exposed to the algae. The following steps describe the remaining procedures for preparing the dilutions for both the test substance and positive control. All tests require negative controls, (blanks), which are test vessels with algae and growth media only. Each concentration has six replicates. In the case of the range finding study, three vessels per concentration are employed. The volume prepared allows for the number of replicates required. The volume of each vessel is 100mL. An initial stock concentration of the test substance is prepared firstly; in the case of C_{60} 100ppm and for CB 10ppm. These stock solutions are sonicated for 30 minutes. Following sonication, the remaining test dilutions are prepared.

2.3.2.3 Exposure to Test Substances

The volume of algae added to each vessel is determined by calculating the amount required to give 10,000 algal cells/mL of test substance. This is calculated using the initial count of cells/mL of the test algae, as described in section 2.5.2.1. Once the algae are added, the vessels are placed into specific algal incubators, (New Brunswick^{TM} Innova Shaker 43) wherein they are exposed to constant light, constant temperature of 20^{O}C and constant shaking at 100rpm for 72 hours.

2.3.2.4 Scoring of Results

After incubation, the vessels are removed from the incubator and a manual count, (as described in section 2.5.2.1) is carried out for each replicate of each concentration, i.e. each vessel used for test (30 test vessels plus 6 control vessels). The average specific growth rate, (μ) was calculated using equation 2.3.

$$\mu = \text{Ln}\,(X_n - X_o)/\,(t_n - t_o)$$

Where: X_n = cell number at time n
X_o = cell number at time zero (10,000)
t_n = test time duration, (72hrs)
t_o = time zero.

Equation 2.3: The average specific growth rate of algae. (OECD Test procedure 201, 2002).

The cell concentration in the control vessels should be increased by a factor of at least 16, after the three days (72hours), in order for the test to be valid. The % Growth Inhibition of the algae due to the test substance is then calculated by the following formula;

$$\% \text{ Growth Inhibition} = \mu_r/\mu_c * 100$$

Where: μ_r = average specific growth rate of replicates of test substance
μ_c = average specific growth rate of controls

Equation 2.4: The Percentage Growth Rate of Algae. (OECD Test procedure 201, 2002).

The % Growth Inhibition is plotted against the concentration of the test substance and the EC_{50} is calculated statistically, (section 2.5.6). To validate the results, the test must be carried out under the exact set of procedures outlined above a minimum of three times.

2.3.3 Thamnocephalus Platyurus Acute Toxicity Test

The *Thamnocephalus platyurus* acute toxicity test is a simple and cost-effective bioassay for screening toxicity in freshwater. Using Instar II-III larvae of the fairy shrimp *Thamnocephalus platyurus* hatched from cysts, an acute toxicity test is executed within 24 hours. Each 'THAMNOTOXKIT F' provides for 6 complete tests, (range-finding or definitive 24h-LC_{50}), or 5 bioassays and 1 quality control test with a reference toxicant.

2.3.3.1 Preparing Standard Freshwater

The *Thamnocephalus platyurus* cysts are stored at 4^{o}C, and are hatched in standard freshwater, which is prepared by dissolving 96mg sodium hydroxide, 60mg calcium chloride, 60mg magnesium sulphate and 4mg potassium chloride up to 1L with deionized water. Before hatching the cysts, the media is pre-aerated for at least 15 mins by connecting it to an aeration pump. As 1 litre of standard freshwater suffices for the 6 bioassays of each toxkit, and if all 6 tests are not carried out within a few days after preparation of the medium, the standard freshwater is stored in the refrigerator in darkness, wrapped in aluminium foil. However, prior to the next use, the cooled medium must be allowed to come back up to room temperature gradually and aerated.

2.3.3.2 Hatching the *Thamnocephalus Platyurus* Cysts

The hatching medium is prepared by transferring 2.5 mL standard freshwater into a vial and adding 17.5mL deionized water, (i.e. dilution 1:8). Hatching of the *Thamnocephalus* cysts should be initiated 24 hours before the start of the toxicity test. The cysts must be prehydrated; a tube of cysts is removed from the refrigerator and filled with hatching medium, (approx. 1mL). The tube is closed and must be shaken at regular intervals for approximately 30 minutes. 10mL hatching medium is pipetted into a small Petri dish and the contents of the vial emptied into it. The majority of cysts are transferred by rinsing the tube with hatching medium. The Petri dish is swirled gently to distribute the cysts evenly. The hatching Petri dish is wrapped in aluminium foil and incubated at 25°C for 20-22 hours, under continuous illumination, (light source of 3000-4000 lux). The New Brunswick^{TM} Innova Shaker 43 was used in this case.

2.3.3.3 Preparing Toxicant Dilution Series

The recommended test substance dilution series given by the kit manufacturer is as follows: 100%, 50%, 25%, 12.5%, and 6.25%, diluting the previous concentration by half with standard freshwater. If the approximate toxicity of the chemical is not known, a range finding test must be carried out initially, which is advised, using the following concentration range; 1000mg/L, 10mg/L, 1mg/L and 0.1mg/L.

2.3.3.4 Filling the Test Plate

The test design of the THAMNOTOXKIT employs one control and five toxicant concentrations, each with 3 replicates of 10 larvae. Each bioassay is performed in a new multi-well plate with a new micropipette. Glass pipettes were used here as it was found they were more effective than plastic pipettes, as the static of the plastic caused the NPs to stick to the inside of the pipette and the larvae were easier to see through the glass pipette. As stated above, the bioassay is conducted in a disposable plastic multi-well test plate with 24 (6 x 4) test wells. The wells are labelled as columns 1 to 6 across, and rows A to D down. The distribution of the test solutions should always be carried out starting from the control, (column 1, left) towards the highest concentration (column 6, right). To fill the control column, 1mL Standard Freshwater is added to the four wells of column 1. This procedure is repeated, (1mL) for the other columns with the respective toxicant concentrations, progressing from low to high concentrations in columns 2 to 6.

2.3.3.5 Adding the Larvae

For this step, the use of a dissection microscope at magnification 10-12x can be beneficial, however it was not used in this case. Approximately 50 Instar II-III larvae were transferred with a micropipette from the hatching Petri dish to each well in row D, (rinsing wells), of the multiwell plate. Subsequently, 10 larvae are transferred from the rinsing well of column 1 to the three wells of this column, care must be taken during this operation.

To minimize the transfer of medium along with the larvae to avoid dilution of the test substance in the receiving well. This operation is repeated for columns 2 to 6. The intermediate passage of the fairy shrimp larvae from the Petri dish to the definitive test wells via rinsing wells "washes" the larvae in the appropriate test solution before they enter the actual test well, thus minimizing dilution of the test solution during transfer.

2.3.3.6. Incubation of Test Plate and Scoring of Results

A strip of parafilm is put on the test plate, covered with aluminium foil and incubated at 25°C in darkness. After 24 hours, the number of dead larvae in each test well is determined, counted and recorded, Larvae are considered dead if they do not exhibit any internal or external movement within 10 seconds of observation. The percentage mortality is calculated and, for the definitive tests, the 24h-LC_{50} value is calculated, (section 2.5.6). For a test to be deemed valid, control mortality should not exceed 10%.

2.3.3.7 Reference Test

It is recommended that every 5 to 10 assays, a quality control test be carried out to check proper adherence to the test protocol, as well as the test sensitivity. Such a quality control test can be performed with the reference chemical potassium dichromate, ($K_2Cr_2O_7$). When performing this quality control test, the 24h LC_{50} should be within the 95% confidence limits stipulated in the specification sheet.

The following concentrations were made up to carry this out; 100mg of potassium dichromate were added to 100mL of deionized water to make a 1000ppm stock solution. A dilution series of the following concentrations was then prepared; 0.32, 0.18, 0.10, 0.056, and 0.032 mg/L and the test procedure was followed as outlined in sections 2.5.3.4-2.5.3.6.

2.3.4 Daphnia Magna Acute Immobilization Test, (OECD Test Number 202)

Freshwater crustaceans of the genus *Daphnia magna*, *(D. magna)* are distributed throughout the temperate regions of the northern hemisphere in ponds and lakes rich in small algae. *D. magna* play an important role in freshwater food chains. They are often dominant consumers of phytoplankton and are an important food for invertebrate and vertebrate predators. *D. magna* are used extensively in ecotoxicological assessment studies due to their relative ease of culture, low costs and high yields, as well as the fact that they are a vital connection in the food chain between the algae that they consume and the ecologically and economically important fish that consume them. Therefore it is imperative to understand the toxic response of NPs to *D. magna*. *D. magna* are filter-feeders that have combs of setae on their 'limbs' in the trunk formed by the bivalved carapace. These combs serve as a mesh by which water is filtered and particles caught. Approximately 16.6mL of water is filtered per hour by each *daphnid*, which makes it an ideal fresh-water test species. *D. magna* exhibits two forms of reproduction, asexual and sexual.

During most of the year, populations of *D. magna* consist almost exclusively of females, the males being abundant only in spring or autumn. Under favourable conditions, females reproduce by diploid parthenogenesis to produce genetically identical female offspring. Production of males appears to be induced principally by declining food quantity or quality and possibly short day lengths. These conditions also induce the females to produce two eggs that require fertilization. When fertilized, these are enclosed, as a pair, by a dense brown/black case, (the ephippium) which is released by the female at the next moult. These "ephippial eggs" are resistant to adverse conditions such as drought and cold, and may remain dormant for a period of years. When hatched, the young from the ephippia develop as females. Laboratory cultures of *D. Magna* are maintained in the asexual phase of reproduction. Cultures are checked daily for the presence of males or ephippia and if either are present the culture is immediately discarded. This is documented in the relevant culture maintenance records. Under favourable conditions, the first brood is released after approximately 8 days at a temperature of 20°C. Subsequent broods are produced every 2 to 3 days during the female lifecycle.

2.3.4.1 *Daphnia Magna* Acute Immobilization Test Method

The daphnia neonates, (offspring) required for this test must be less than 24 hours old, and are cultured continuously in-house. In order to ensure this, the neonates are separated daily from the adults and discarded if not required for testing or used to set up a new culture of 20 neonates per flask.

Ninety neonates are required per test carried out. Once separated, the test substance is prepared. In this case a 1000ppm solution of C_{60} and 100ppm CB were prepared in daphnia medium and sonicated for thirty minutes. Once sonicated, the remaining range of test concentrations are prepared from the stock 1000ppm solution. In both cases, several range finding studies were carried out to assess if a range could be found that causes 10% mortality in the lowest concentration and 90% mortality in the highest concentration tested. Once prepared, 10mL of each test concentration are pipetted into a 15mL plastic test tube; there are three replicates for each concentration set up and three control tubes. Starting with the control tubes and progressing from the lowest to the highest concentrations, five neonates are placed in each tube. A glass, narrow bore pipette is used for this, a number of daphnia are taken up at one time, the pipette is then held below the line of the liquid in the tube and the daphnia swim into the tube. No additional liquid can be added to the tube, as this would alter the concentration. To ensure this, as much liquid and air as possible is removed from the pipette before being put into the tube. Once all neonates are transferred, the test is left in conditions identical to that of the culture, which is 21^{0}C in a controlled temperature room, and 16 hours light and 8 hours darkness where the light is controlled by a timer switch. The test is left static for 48 hours, that is, the media is not changed or the daphnia are not fed.

2.3.4.2 Scoring Test

After 24 hours, the number of immobilised/dead daphnia are recorded per test tube; any other abnormalities are recorded also, including signs of stress such as swimming in a circle or at the top of the tube. After a further 24 hours, the count is repeated and recorded. A test is deemed invalid if >10% of the controls show stressed behaviour or mortality. The results obtained are used to calculate the EC_{50}, that is the effective concentration at which 50% of the exposed neonates die, details of which are described in section 2.5.6 below.

2.3.5 Cytotoxic Evaluation

Two fish cell lines were employed to assess the cytotoxicological effects of C_{60} and CB, these fish cell lines are RTG-2 and PLHC-1. The PLHC-1, (*Poeciliopsis lucida* hepatocellular carcinoma) cell line was derived from an adult female *Poeciliopsis lucida*, a topminnow from the Sonoran Desert. A transplantable tumour was induced by multiple doses of 7,12-dimethylbenz(a)anthracene, (DMBA), treatment of the fish in 1982 by Mary E. Schultz. (ATCC product sheet). The tumour was adapted to cell culture in 1985 by Lawrence E. Hightower. These cells maintain a number of differentiated cell functions of hepatocytes. The cells possess inducible and stable cytochrome P450, (CYP) activity. PLHC-1 cells can be used in an *in vitro* system to screen environmentally relevant stressors such as heavy metals using a combined stress protein and cytotoxicity assay.

Retention of hepatocyte properties has made this cell line ideal for *in vitro* toxicology assays and it has been well characterized by environmental toxicologists, (Raja, Connolley et al. 2007). The RTG-2, Rainbow Trout Gonad tissue cell line originated from pooled male and female gonad tissue of yearling rainbow trout, (*Oncorhynchus mykiss*) in 1962 by K. Wolf and M.C. Quimby (Bols et al., 1991). It was the first cell line to be established from cold-blooded vertebrates and so has been used extensively in the assessment of ecotoxicological contaminants. One of the most important points to note when culturing this cell line is that there is an 8-fold difference in the growth rate between the temperature range 4-24°C. That is, this cell line can be cultured from between 4 and 24^{o}C. A temperature of 20^{o}C gives confluent cultures between 10 and 14 days.

2.3.5.1 Seeding Plates for Cytotoxic Evaluation

Cells are visually assessed to ensure the correct confluence level, (70% optimum) and the health of the cells. Cells are trypsinised and a cell suspension in created with fresh cell culture media. A cell count is performed using a Coulter Counter. This can also be carried out manually using a haemocytometer and a light microscope. The average number of cells per mL of three samples is calculated. The concentration of cell suspension required for testing is calculated according to length of exposure time being assessed; 24, 48, or 72hours, the concentration used was $1x10^5$ cells/ml in 100mL culture media.

This can also be dependent on cell type, as different cell types have different doubling times. Plates are seeded with the desired cell concentration, (e.g. for 96 well plate, 100µl of cell suspension per well is pipetted). All plates are labelled with cell name, passage number, date and tester initials. Plates are covered with Parafilm to avoid risk of spills in the incubator and placed in the incubator, for PLHC-1 cells 30^{0}C and for RTG-2 cells 20^{0}C. Cells are allowed to attach to the plastic well plates for 24 hours prior to exposing to test samples.

2.3.5.2 Exposing Cells for Cytotoxicity Evaluation

After 24 hours incubation, the medium is removed from the plate, (this will also remove any unattached or dead cells), and each well is rinsed with 100µL of sterile PBS. 100µL fresh cell culture medium is placed in the control well column on the plate. If using a solvent to disperse test particles, a second control column with medium and solvent is used to mimic exposure solvent concentrations. Cells are exposed to 100µL of test solution, (test material dispersed in 5% or 10% fresh media, 5% for PLHC-1 and 10% for RTG-2). Plates are resealed with Parafilm and incubated for the exposure time period being assessed; 24, 48, 72, or 96 hours.

2.3.5.3 Cytotoxicity Evaluation Using Alamar Blue & Neutral Red Assays

AlamarBlue®, (AB), is a proven and widely used cell viability indicator that uses the natural reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin (Takhar et al., 2011). The active ingredient of AlamarBlue®, (resazurin) is a nontoxic, cell permeable compound that is blue in colour and virtually nonfluorescent. Upon entering cells, resazurin is reduced to resorufin, which produces very bright red fluorescence. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability and cytotoxicity. The procedure is relatively simple, viable cells are exposed to the test substance, incubated for the desired exposure time, and after this time has lapsed, the fluorescence is read. The amount of fluorescence or absorbance is proportional to the number of living cells and corresponds to the cells metabolic activity. Damaged and non-viable cells have lower innate metabolic activity and thus generate a proportionally lower signal than healthy cells. This activity is compared to the control and statistical analysis is then performed (Al-Nasiry et al., 2007). The neutral red, (NR) uptake assay is another assay that is used extensively to quantitatively estimate the number of viable cells in a culture. It is one of the most widely used cytotoxicity tests with many biomedical and environmental applications. The principle of the test is based on the ability of viable cells to incorporate and bind the supravital dye NR in the lysosomes. The setup is similar to that of AB, in that cells are seeded in 96-well tissue culture plates and are treated for the appropriate period.

The plates are then incubated for 2 hours with a medium containing the NR dye. In addition to these steps in the case of the NR assay, the stock solution must be prepared also, by weighing 0.4g NR dye, adding it to 100mL phosphate buffered saline, (PBS), (with Ca^{2+} and Mg^{2+}). This solution can be stored for up to two months in a dark environment, (covered with aluminium foil), at room temperature. After the required exposure time has lapsed, the cells are observed under an inverted microscope at 10X and any visual differences are noted, including loss of monolayer, or changes in shape. The AB and NR assays can be carried out on the same 96 well plate and the dyes mixed and end points assessed separately. This was carried out during this assessment. The AB/NR solution is prepared by adding 0.5mL of AB dye (AB solution must be kept sterile) and 0.125mL of NR stock solution, (as described above), for each 10mL of un-supplemented medium, without serum or antibiotics. The test medium is removed from the plate and the plate is washed with 100µL of sterile PBS. 100µL of AB/NR solution are added to each 96 well to be tested. The plate is then incubated for 3 hours before fluorescence measurement. The AB fluorescence is measured first by placing the plate in the plate reader and reading at the following wave-lengths; 540nm excitation and 595nm emission. Once AB measurements have been completed, the AB/NR media is discarded from the plate, and the plate is rinsed again with 100µl PBS. 150µl of NR fixative solution, (1mL acetic acid, 50mL ethanol, 49mL distilled water) are then added and the plate is shaken for 10min at 240 RPM using a SciQuip Microplate Shaker. NR fluorescence is then read in the plate reader using 540nm excitation and 650 nm emission wavelengths. Statistical calculations are then used to assess the effect of the particles on the cell lines tested.

2.3.6 Statistical Analysis of Ecotoxicological Tests

The recommended statistical analysis was carried upon data collection of each set of tests described in the above sections. There are several software statistical programs available to carry out these calculations. Two statistical programs were used to analyze the results obtained during this study; the REGTOX software program (Garric et al., 1990) and the MasterPlex 2010 program, (MasterPlex, 2012). The REGTOX software program utilises the Hill model to calculate the EC_{50} values. In this study this was used to determine EC_{50} values obtained in the toxicological assessment of *Vibrio fischeri, Pseudokirchneriella subcapitata, Thamnocephalus Platyurus,* and *Daphnia magna.* REGTOX is a Macro that allows calculation of dose-response parameters using several models, the model parameters are calculated by non-linear regression, their confidence intervals are estimated using a Bootstrap (simulation). On examining the cytotoxicological end points of three independent experiments carried out, single factor ANOVA calculations were carried out, ANOVA models are used to analyse the differences between group means, used in comparing three or more means for statistical significance. In addition where possible EC_{50} values were calculated using the software program MasterPlex 2010 using the equation below, which is curve-fitting software designed for the analysis of data from multiplexing platforms. In this study the Dunnett test was used, which is the mean of each test sample is compared to the control mean (Day et al., 1989). Other studies use the Tukey test which compares every test mean with every other mean in the test (Day et al., 1989).

$EC_F = (F/(100-F))^{(1/H)} * EC_{50}$

Where: F=EC value being calculated, ($0 < F < 100$)
H= Hill Slope for the curve fit

Equation 2.5: MasterPlex 2010 EC_{50} calculation.

2.4 Chapter Summary

This chapter has detailed the experimental processes that were carried out in the physio-chemical assessment of the NPs tested and the ecotoxicological assessment of C_{60} and CB, namely; the Microtox Acute Assay, the *Pseudokirchneriella subcapitata* Growth Inhibition Assay, the *Thamnocephalus Platyurus* acute toxicity test, the *Daphnia magna* acute immobilisation test and cytotoxic assessment in two fish cell lines. The procedures detailed in this chapter are followed by the results of the tests described, sstarting with results of physicochemical analysis of C_{60} and CB. Results of toxicological screening follows, displaying results of five toxicological tests, these results are followed by detailed discussion and recommendations for future testing.

Chapter 3: Physicochemical Characterization of Nanoparticles

3.1 Introduction

In the previous chapter, the various techniques that were employed to build a physical characterization profile and a biological toxicological profile of C_{60} and CB in an aquatic environment were discussed in detail. It was highlighted that nanotoxicological studies require special consideration of the physicochemical properties in conjunction with the biological characterization. Several properties were highlighted in Chapter 1, which are often heralded as the key properties requiring characterization in advance of a toxicity study being performed on a nanomaterial (Warheit et al., 2008). Indeed, physiochemical parameters such as size, shape, and surface characteristics play crucial roles in the elucidation and determination of the biological properties of NPs, often influencing how these particles interact with proteins, lipids and surfactants in the local environment (Warheit et al., 2008). As a result, such information can be used to determine how particles behave in biological media and subsequently within cells during *in vitro* studies, as performed in this study on two fish cell lines. Characteristics such as changes in the environment or ionic strength, or a change in the particle concentration, may result in some aggregation or a shift in the particle size distribution, which will subsequently have an effect on the manner in which the particles are presented to the cell and/or cellular structures. Hence, the nature of the particles in the test media is undefined, making the interpretation of toxicity results with NPs questionable.

This latter point is particularly important when interpreting results of *in vitro* cytotoxicity studies and comparing to particles behaviour in an aquatic environment. The importance of 'in lab' characterization is further highlighted by (Park et al., 2010), who showed differences between label specifications of commercial NPs and independent characterization. Their study highlighted important insights with respect to self-life and stability, again crucial in order to fully interpret the results of toxicity assessments. The present chapter presents the findings of a number of characterization techniques recommended by Zuin et al., 2007 that were performed in advance of the cytotoxicological and eco-toxicological evaluation of carbon nanotubes, fullerenes, metal oxides, silica and quantum dots. The properties assessed include; particle size and particle size distribution measurement, zeta potential and surface area analysis. As well as being recommended by Zuin et al., 2007, these end-points were also highlighted as being important properties in the physico-chemical assessment of NM by the OCED NM working party in 2008. The report included agglomeration, aggregation, water solubility, particle size distribution, specific surface area, zeta potential, and surface chemistry, several of which were carried out during this study, the results of which are presented in the following sections.

3.2 Particle size/distribution

Particle size is one of the main factors that affect the biological applications of NPs. One of the major concerns is the possible toxic affects due to the capacity of these materials to penetrate cells and potentially translocate to other cells, tissues and organs around living systems. The size and shape of mammalian and fish cells are varied, mostly related to their specific functions. A typical mammalian cell is approximately 12μm in diameter. It is also known that the cell membrane, which is approximately 8 nm–180 nm in thickness, and has an overall negative charge, allows certain substances to across, through selective permeability by methods such as diffusion, facilitated diffusion, active transport and endocytosis (Nel et al., 2009). Surface area, particle size, temperature, concentration and surface charge are all factors, which affect particle movement across the membrane, (Nel et al,. 2009). Taking this information into consideration, correct and accurate particle size measurement is an undeniably important factor for evaluating toxicity and uptake of NPs in aquatic species. There are many techniques employed for the measurement of particle size such as AFM, Scanning Electron Microscopy, (SEM), and Transmission Electron Microscopy, (TEM), (Kalantzi et al., 2014). While most of the mentioned techniques are more modern methods of particle size measurement, there are also many older available techniques for the measurement of particle size. Dynamic light scattering, as explained in more detail in Chapter 2, was the technique used for measuring the size of particles in this study and has been used widely used for the assessment of NP size studies, (OCED working party, 2008).

3.2.1 DLS Results

Size measurements were made using DLS on a Malvern Zetasizer Nano ZS, as described in Chapter 2. Measurements were carried out in de-ionized water at three time points, with a C_{60} and CB concentration of 1000µg/l (all measurements were performed in triplicate). The following figures and tables represent the data collected for both particles. Size results obtained for C_{60} were in the main monomodal curves, as can be observed by the size distribution graph shown in figure 3.1. The results from DLS size measurements for C_{60} have been tabulated in table 3.1.

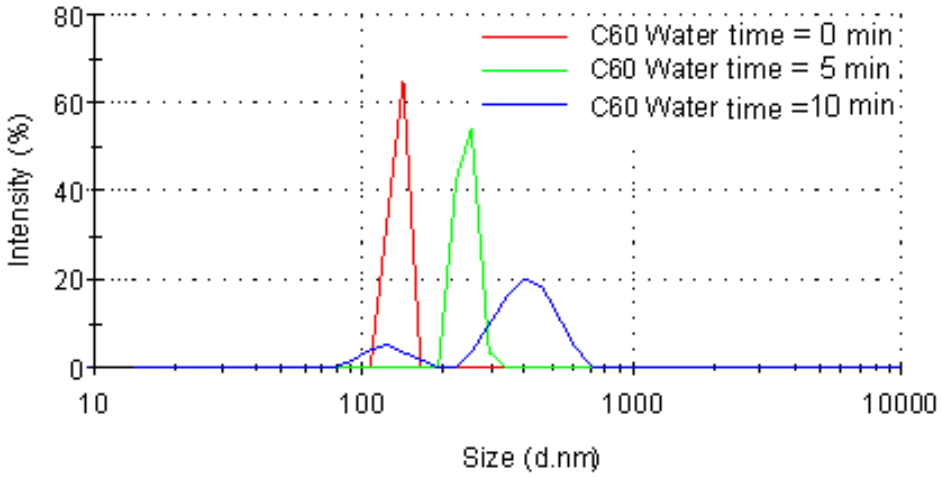


Figure 3.1: Size distribution graph of C60 in water at time points 0 minutes, 5 minutes and 10 minutes. After the time points the curve can be seen to evolve form a monomodial curve to a bi-modial curve.

Table 3.1: Size distribution and mean size of C_{60} for time points 0 minutes, 5 minutes, and 10 minutes.

	Pk 1 Mean Intensity	Peak 2 Mean Intensity	Peak 1 Area Intensity	Peak 2 Area Intensity
	d.nm	d.nm	%	%
0 mins	135		37	
5 mins	241.6		63	
10 mins	110	414	122	254
Mean 1-3	162.2	414	74	254
Std Dev	69.89		43.55	
Minimum	135	414	37	254
Maximum	414	414	122	254

The well-known and well documented diameter of molecular C_{60} is 1nm. However, as can be seen from the DLS measurements, the lowest recorded diameter was 135nm and, in addition, the measured size evolves with time. After ten minutes, no further evolution is recorded. However, at this time point, a bimodal profile is observed. These observations have been attributed to the spontaneous formation of an aggregate form of C_{60}, known as nano-C_{60}, which forms a stable colloid suspension. As mentioned in chapter 1, these aggregates are composed of C_{60} clusters with diameters of 5-500nm and can vary in shape.

The mechanism and kinetics for the formation of these aggregates are the subject of some debate, most believing they are the result of hydrophobic interactions and form immediately upon contact with water. The appearance of the double feature at the 10 minute time point has been attributed to the fact that the reported upper stable size limit for the C_{60} aggregates is 500 nm, an average diameter of 658 nm for C_{60} particles in deionized water was reported by Yuriy et al., 2013. In addition, it has been reported that the hydrophobicity and reactivity of individual C_{60} molecules at the aggregates surface are substantially altered due to local environment interactions. For example, a study carried out by Mashayekhi et al., 2013, to examine the effects of natural organic matter, (NOM), on the aggregation behaviour of a fullerene water suspension, (FWS), reported the absolute value of zeta potential of pure FWS increased after addition of the 4 types of NOM assessed. Lynch *et al*., 2008 have argued that the representation of NPs in a biological media is actually a representation of the proteins, (in terms of media) and the NOM, (in terms of aquatic environment), associated with the NPs surface, rather than the particles themselves and that this causes numerous biological responses. In terms of C_{60}, this means that the altering of the surface chemistry within the aquatic environment will allow the relatively un-reactive molecule to actively interact and bind to other molecules or particles present in its environment. Table 3.2 shows results obtained from measuring particle size by DLS of CB at three time points; 0 minutes, 5 minutes and 10 minutes.

As with C_{60}, monomodal dispersion was initially observed, the profile evolved into a bimodal dispersion over time.The size of particles increased over the three time points, giving a mean size of 5378nm in peak 2 after 10 minutes of measurement. For both particles the formation of large aggregates were observed, particularly in the measurement of CB as shown in table 3.2. The significance of this is discussed in the chapter summary.

Table 3.2: Size distribution and size for CB at three time points 0 minutes, 5 minutes, and 10 minutes.

	T	Z-Ave	Peak 1 Mean Intensity	Peak 2 Mean Intensity	Peak 3 Mean Intensity	Peak 1 Area Intensity	Peak 2 Area Intensity	Peak 3 Area Intensity
	°C	d.nm	d.nm	d.nm	d.nm	%	%	%
0 mins	25	19000	0.6213			100		
5 mins	25	3608	26.57	1.886		64.5	35.5	
10 mins	25	655	294.4	5378	1.405	72.2	15.3	9.3
Mean 1-3	25	7754	107.2	1793	0.4683	78.9	16.9	3.1
Std Dev	0	9850	162.6	3104	0.8112	18.7	17.8	5.4
Minimum	25	655	0.6213	0	0	64.5	0	0
Maximum	25	19000	294.4	5378	1.405	100	35.5	9.3

3.3 BET Measurements

Toxicology is essentially the interaction of the surface states of a material with their local environment. Therefore, a material with a large surface area in general has a higher portion of surface states available for chemical interaction. For a bulk material, the majority of its component atoms/molecules are not at the surface but are rather internalized in the material bulk and so not available for reaction. In contrast, in a NM, the majority of the constituent components are close to the surface and hence are available for interaction. Therefore, with constant mass, a decreased particle size results in increased total surface area in comparison to the bulk material (Nel et al,. 2006).The resultant larger surface area causes surface chemistry to become increasingly important. Hence, smaller particles may exhibit greater biological activity per given mass compared with larger particles. Although a correlation between increasing surface area and biological effects is generally accepted (Warheit et al., 2006) the relationship between size, surface area and toxicity is not straightforward. Therefore, it is not always possible to predict effects on the basis of size or surface area alone. For example, NPs which agglomerate and/or aggregate result in larger particles. The surface area of an aggregate and/or agglomerate generally depends on the local environment such as pH, temperature and pressure. It is therefore plausible that aggregates will have an "outer" and "inner" surface, (inner being interstitial sites or even porous media), which can behave differently with respect to biological reactivity. Nevertheless, an estimate of the total surface area is crucially important for understanding the mechanisms of nanotoxicology.

Indeed, very early studies on the dissolution of porous metal oxide particles intracellularly in alveolar macrophages showed that the entire surface area, including the inner surface area, needs to be considered (Kreyling et al., 1990). The most effective method for surface area analysis with respect to toxicity is to utilize the BET isotherm which essentially examines the absorption of nitrogen gas on to the surface of aggregated or agglomerated powders. The details of the procedure carried out during this study are contained in chapter 2.

3.3.1 BET Results

In this study, for each run, approximately 0.0750g of C_{60} power was weighed out. The sample was then dehydrated for two hours at a temperature above 100^{o}C. Figures 3.2 and 3.3 and table 3.3 show the BET measurements obtained for the C_{60} sample. It can be seen that this isotherm best fits with porous materials with cohesive force between adsorbate molecules and adsorbent being greater than that between adsorbate molecules, this type of isotherm is often referred to as a type VI isotherm. The Type IV isotherm was classified in 1984 by the International Union of Pure and Applied Chemistry, (IUPAC) (Sing et al., 1985). At low pressure, the BET isotherm equation, (Equation 2.2, chapter 2, shown here in equation 3.1) can be used to estimate the surface area. This is done by plotting $\frac{P/P_o}{V(1-P/P_o)}$ against the relative pressure of adsorbates P/P_0.

Figure 3.2 shows that this yields a straight line with slope equal to (c-1)/(cV_m) and intersect 1/(cV_m), where V_m is the monolayer adsorbed gas quantity (0.01165mmol/g) and c is the BET constant (8.688109a.u.). Using these figures, the surface area of the fullerene can be determined from equation 3.1 to be 1.1± 0.1m^2/g, with a slope of 75.9g/mmol and an intercept of 9.8g/mmol. The same equation was used to calculate the surface area of a 1.0000g sample of CB. The surface area calculated was 4.9m^2/g with a slope of 19.7g/mmol and an intercept of 0.05068 mmol/g. Figures 3.4 and 3.5 and table 3.4 show the Isotherms obtained for the surface area of the CB sample. The surface area obtained using BET is not a direct reflection of the geometric surface area, as could be calculated from the DLS and AFM measurements as BET considers absorption at specific surface sites only. Previous studies have reported that several NPs have a small number of micro-pores, such as CB reported by Vijayshankar et al., 2013, which is indicative of tightly packed molecules which would not be easily accessible by the N_2 gas when measuring BET, which results in measuring small specific surface areas of the particles. This highlights the importance of incorporating several techniques when assessing the physicochemical characterisation of NPs.

$$A_s = A_m N_m = A_m \frac{V_m}{V_{T,P}} \times 6.022 \times 10^{23}$$

Where: Vm = volume of monolayer adsorbed gas molecules calculated from the plot

VT,P = standard molar volume at Standard temperature and pressure 22.4L/mol

Am = Cross sectional area of N2, 16.2 x 10-20 m2 Avogadro's number 6.022X 1023

Equation 3.1: BET Isotherm Equation (Sing et al., 1985).

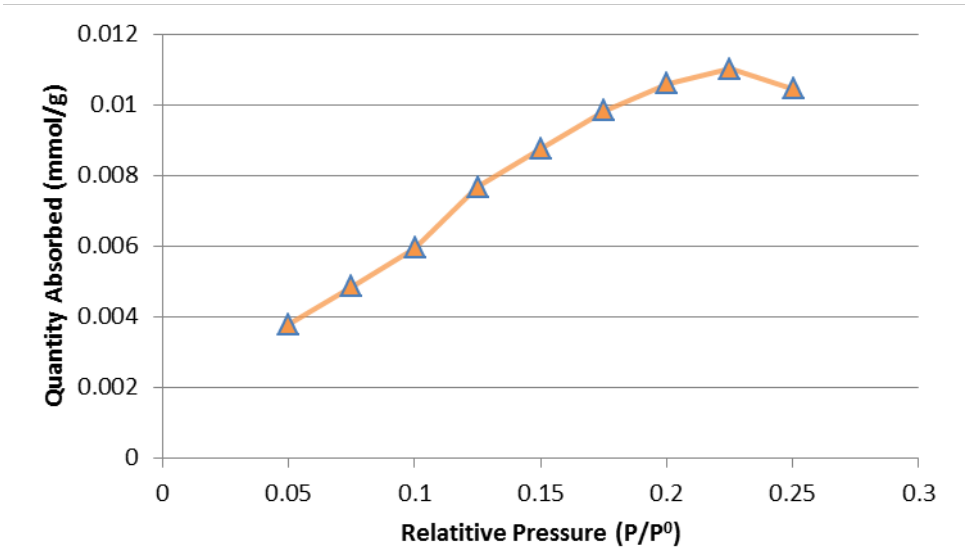


Figure 3.2 BET Isotherm Linear plot of C60.

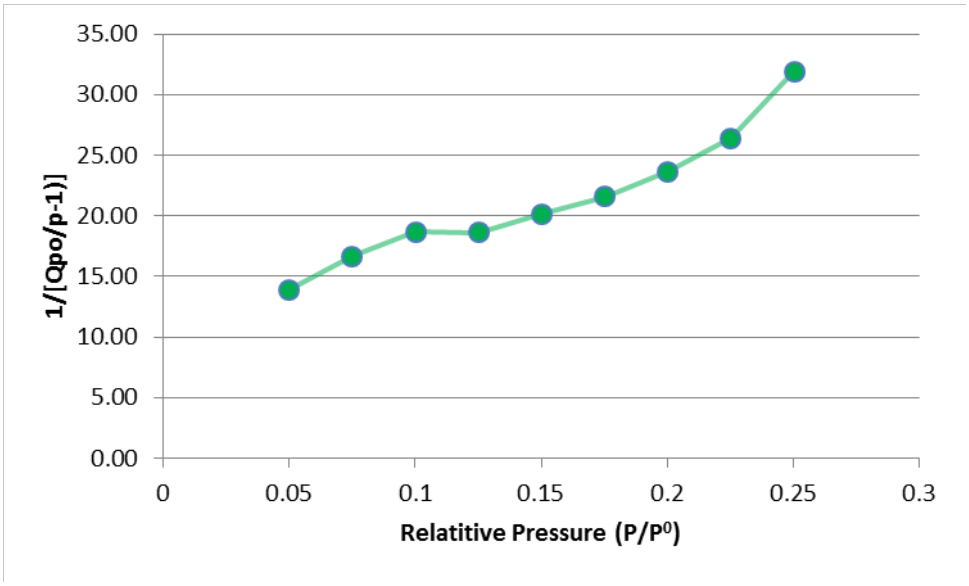


Figure 3.3 BET Surface Area Plot of C_{60}

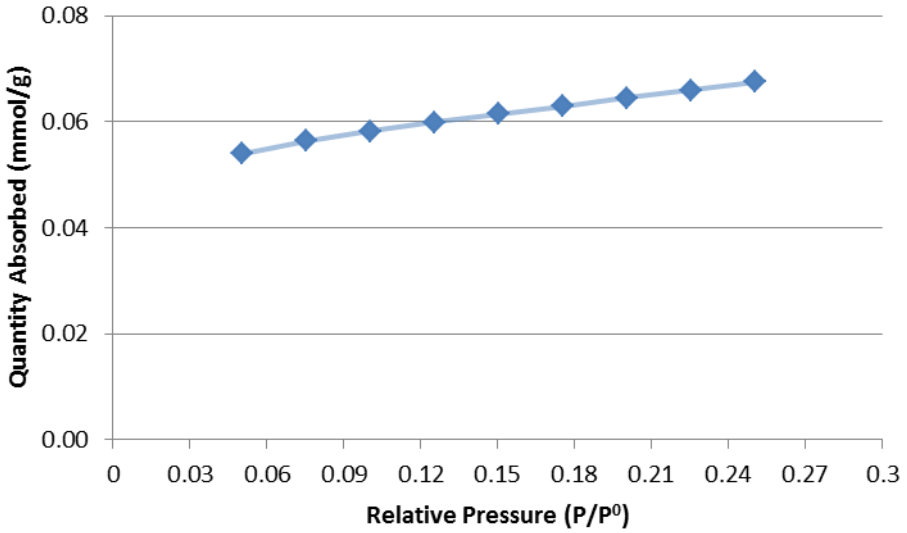


Figure 3.4 BET Isotherm Linear plot of CB.

Table 3.3: BET Surface Area report of C_{60}

BET Surface Area	**1.1364 ± 0.1130 m²/g**
Slope	75.981449 ± 8.424563 g/mmol
BET C constant	8.688109
Correlation Coefficient	0.9595642
Y-Intercept	9.882983 ± 1.376918 g/mmol
Monolayer adsorption capacity per unit mass of sorbent (Qm)	0.01165 mmol/g
Molecular Cross-Sectional Area	0.1620 nm²

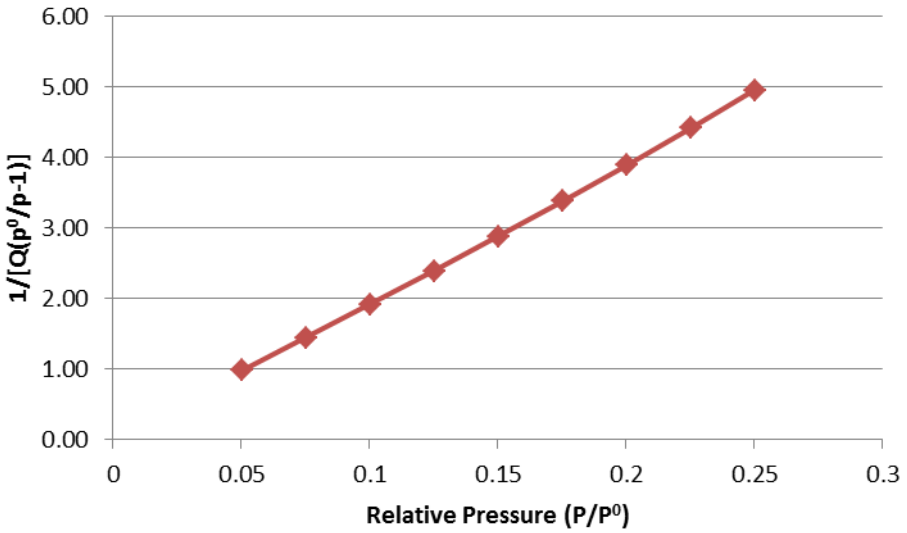


Figure 3.5: BET Surface Area Plot of CB.

Table 3.4: BET Surface Area report of CB

BET Surface Area	**4.9451 ± 0.0473 m²/g**
Slope	19.791532 ± 0.186234 g/mmol
BET C constant	-0.060320 ± 0.030428 g/mmol
Correlation Coefficient	-327.110322
Y-Intercept	0.05068 mmol/g
Monolayer adsorption capacity per unit mass of sorbent (Qm)	0.9996902 mmol/g
Molecular Cross-Sectional Area	0.1620 nm²

3.4 Atomic Force Microscopy

While DLS is the recommended method of particle size measurement, there are also other complementary techniques used to verify particle sizes. AFM is ideally suited for characterizing NPs; it is a well-documented technique for the characterization of NPs (Rao et al., 2007), (Pletikapi et al., 2012). The technique has the capability of 3D visualization and provides both qualitative and quantitative information of many physical properties including size, morphology, surface texture and roughness. A wide range of particle sizes can be characterized in the same scan, from 1nm to 8µm. The technique used for this study is described in Chapter 2.

3.4.1 AFM Results

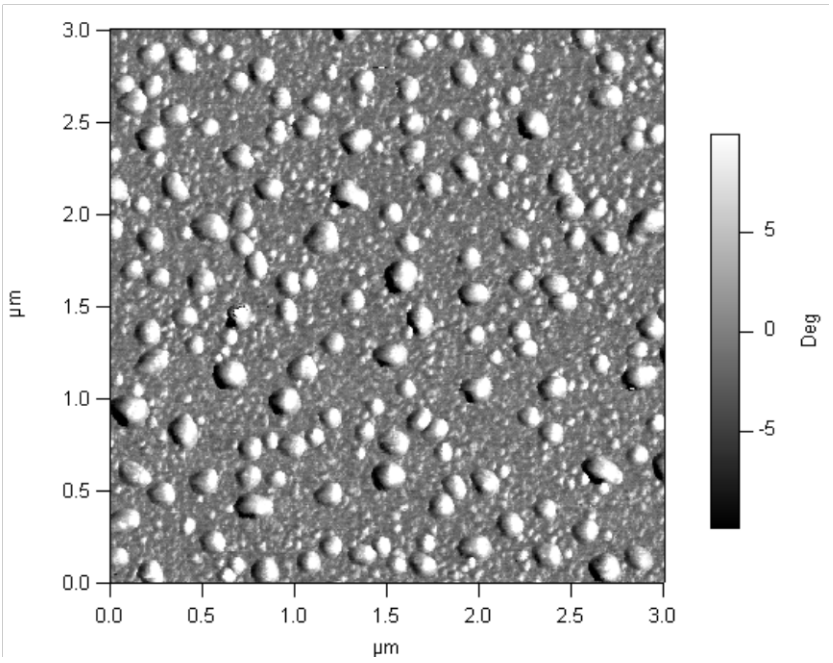


Figure 3.6 (a): AFM Phase image of C_{60} sample performed in tapping mode, (Section 2.2.4)

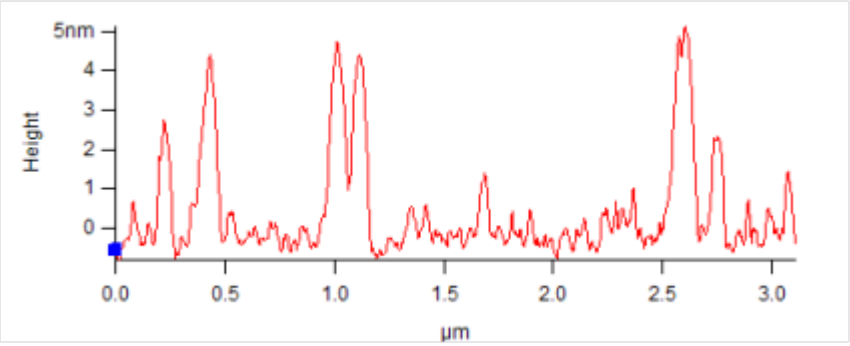
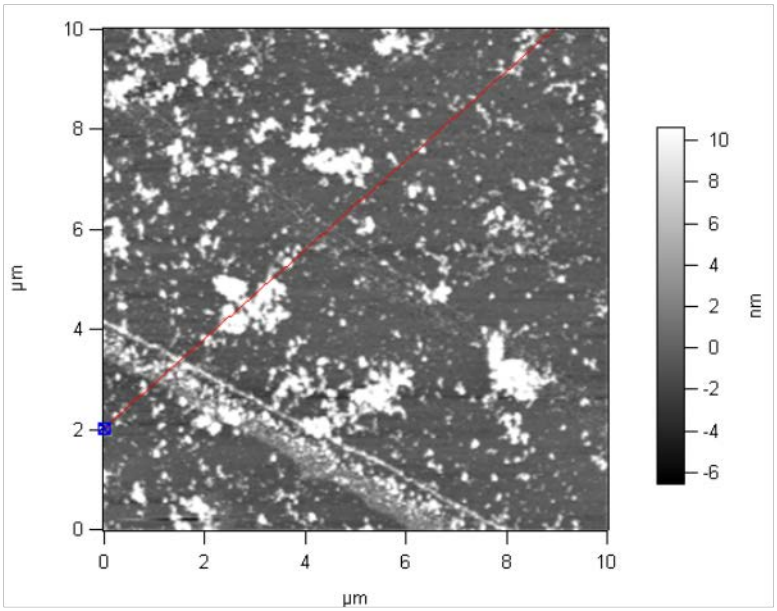


Figure 3.6 (b): AFM Height Profile of C_{60} sample performed in tapping mode.



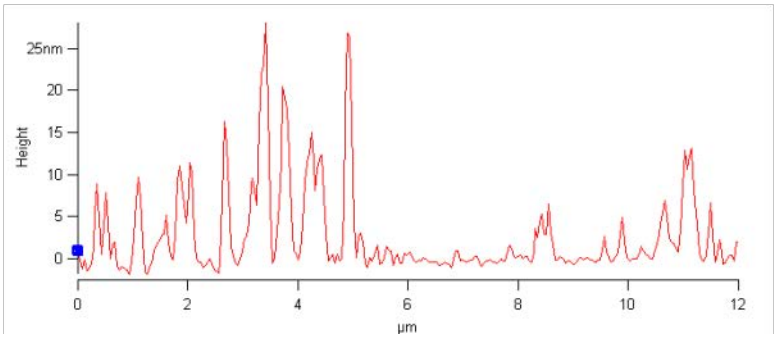


Figure: 3.7 (a) :AFM Phase image of CB sample performed in tapping mode, (b) AFM Height Profile of CB.

Figure 3.6 and 3.7 are examples of AFM images obtained for C_{60} and CB particles respectively drop cast from aqueous solution. Figures 3.8 and 3.9 show more detailed AFM images of C_{60} and CB respectively drop cast from water and the height profiles. It is important to note here that the AFM measurements were not taken over a time course when comparing to the DLS measurements which were taken at three time intervals. DLS indicated an evolution of the particle size in suspension overtime, whereas AFM measured the particles precipitated from suspension. The line drawn through the spots, (particles) is an indication of the particles of which the measurements were taken. On close inspection in Figure 3.6, the particle size is more evident with an average width recorded of 150nm. The height profile however shows a discrepancy with a max height of 6nm recorded for each particle. At this point, no explanation for this has been developed other than the suggestion that the drop casting process dehydrates the aggregates causing them to collapse. It is suggested that liquid AFM may be an alternative approach for characterizing the suspensions. Nevertheless, AFM measurements are in agreement with the DLS study. On examining the CB images obtained, it is evident in the first image, figure 3.7, that large aggregates have formed, which is confirmed when examining the second image, (figure 3.9), in more detail, giving an average particle size of 250nm, with a height of up to 10nm. These results also confirm the DLS measurements which showed the formation of large CB aggregates. However, as stated at the beginning of the section, it is important to note the formation of such aggregates when viewed during the DLS assessment was seen to evolve over time, whereas the AFM measurements were not taken at time intervals, however we suggest that measurements recorded are representative of the majority of the sample being assessed.

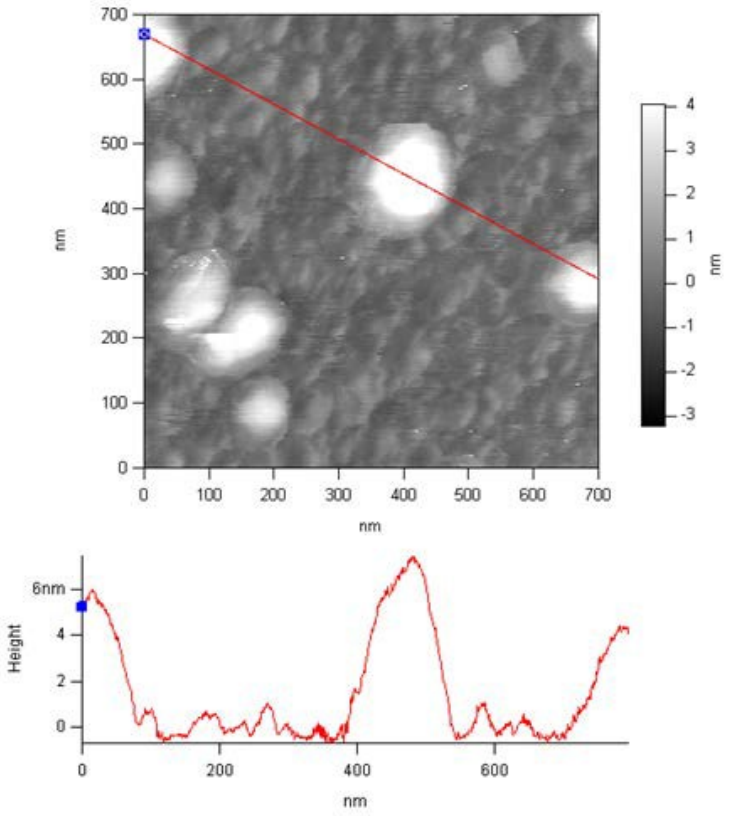


Figure 3.8: AFM of C_{60} drop cast from water

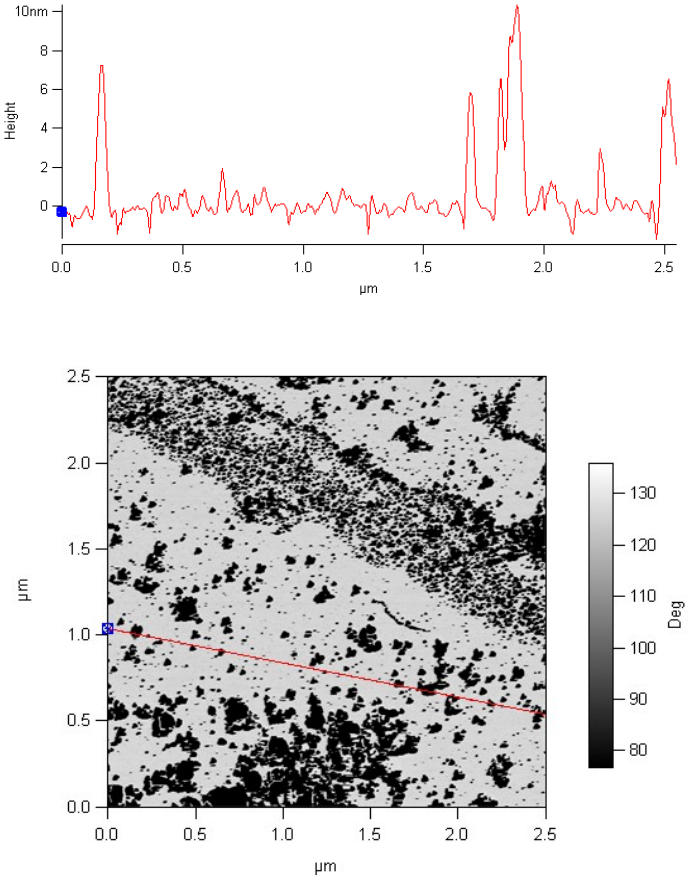


Figure 3.9: AFM Phase image of CB sample performed in tapping mode, (Section 2.2.4)

3.5 Zeta Potential

When examining particle characteristics in advance of any nanotoxicological study, it is imperative to know the state of the NPs being used and in particular any changes in the appropriate test media. This was clearly highlighted by the variation in particle size observed for the DLS with respect to particle height once the particles were removed from the aqueous environment. In addition to size, effective surface charge is particularly important when considering NPs are dispersed in biological test media in ecotoxicological and cytotoxicological studies. Zeta potential and overall surface charge is of particular interest when looking at how particles enter cells and transport contaminants due to the potential for interactions with proteins and ions in its local environment. For example, the permeability of a membrane is the ease with which molecules pass through it, but permeability depends mainly on the electric charge of the molecule and to a lesser extent the molar mass of the molecule. Electrically neutral and small molecules pass through the membrane more easily than charged, large molecules (Pogodin et al., 2012). Within cells, the intermediate extracellular surface of the plasma membrane is negatively charged due to the negative charge of the phospholipid heads. T herefore, the surface of the membrane could influence the way in which charged particles interact with the cell membrane. The cell uses this negative electrical membrane potential to drive the transport of the substances, often against a concentration gradient, into or out of the cell. Pogodin et al., 2012 demonstrated that NPs enhanced lipid bilayer permeability using a bond fluctuation method.

Zeta potential can potentially provide information on the particle's absorbed species, such as proteins or salts. Therefore, from this information it can be concluded that it is imperative that characterization measurements, particularly surface charge and zeta potential measurements, should always be included in the range of physiochemical assessments carried out in the study of NPs. The procedure that was followed to obtain the zeta potential measurements is outlined in chapter 2.

3.5.1 Zeta Potential Results

As with particle size measurements, zeta potential measurements were carried out in de-ionized water at three time points with a C_{60} and CB concentration of 1000µg/l (all measurements were performed in triplicate). NP behaviour is influenced by particle size, shape, surface charge, and the presence of other materials in the environment. NPs tend to aggregate in hard water and seawater, and are greatly influenced by the specific type of organic matter or other natural particles, (colloids) present in freshwater (Velzeboer et al., 2008). In this study, the average zeta potential for the deionized water was -22.10 mV (the negative sign indicating the electrode ordination), figure 3.9. In comparison, for the C_{60} suspension, (figure 3.10), the average zeta potential was found to be -79.2 mV, this is considered to be a stable suspension as the zeta potential falls between the range recommended by the instrument manufacturers, Malvern, 2012, as being indicative of a stable suspension, (< -30mV or > +30mV).

This figure was recorded for the 10 min time point and was found not to change with time. This is a clear indication that the C_{60} suspensions are extremely stable. As indicated by the DLS, the aggregate size stabilizes after the initial ten minutes time point.

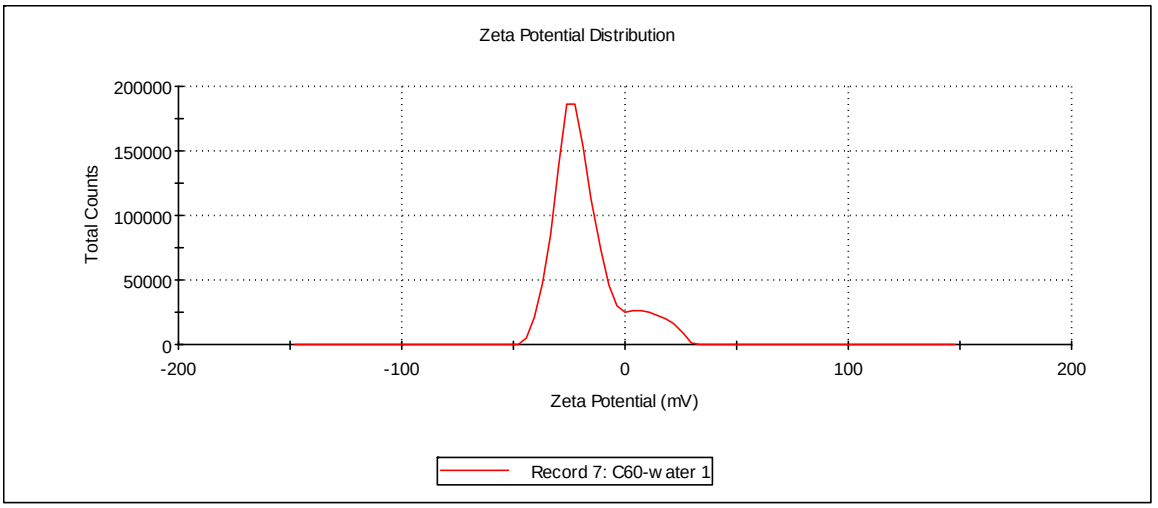


Figure 3.9: Zeta potential of water -22.1 mV.

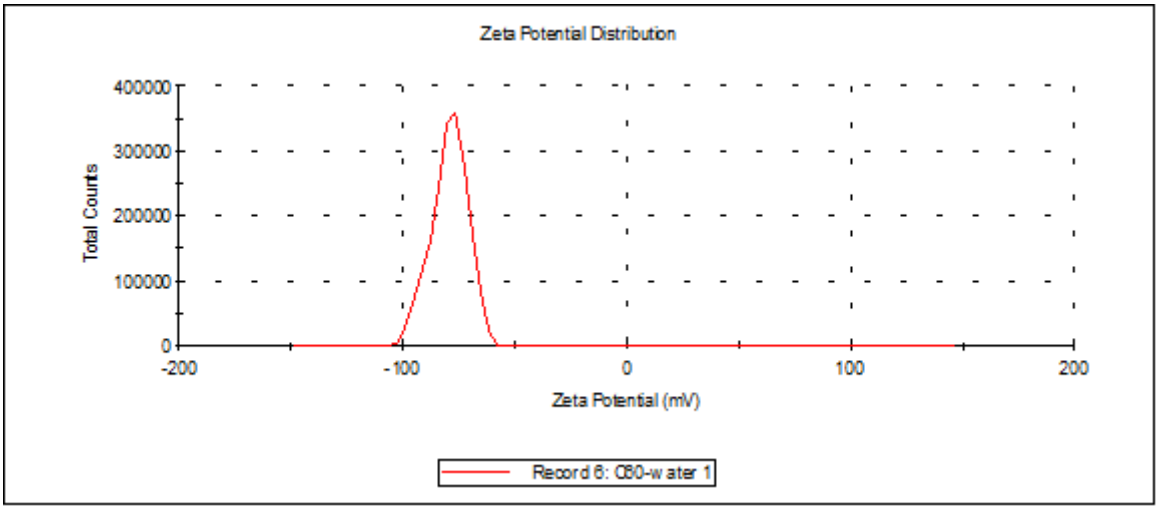


Figure 3.10: C60 in Water average zeta potential -79.2 mV. (Zeta potential didn't change with time).

Table 3.3 shows the results of an experiment to obtain the zeta potential of CB over three time points. On inspection of the data, it is clear that the zeta potential of CB does not change over time, with an average of 27.6mV after the 10 minute measurement time but also that it is an unstable suspension being outside the range of a stable suspension, (< -30mV or > +30mV).

Table 3.3: CB in DI water average zeta potential of 9 readings, three per each time point.

Time Point Mins	**Temp** 0^C	**Zeta Potential** mV	**Mobility** µmcm/Vs	**Conductivity** mS/cm
0 (Rep1)	25	28.3	2.215	0.00158
0 (Rep2)	25	28.8	2.256	0.00138
0 (Rep3)	25	28.5	2.237	0.00161
5 (Rep1)	25	27.1	2.125	0.00242
5 (Rep2)	25	25.9	2.032	0.00196
5 (Rep3)	25	27.2	2.133	0.00288
10 (Rep1)	25	27.3	2.143	0.00215
10 (Rep2)	25	27.4	2.151	0.00207
10 (Rep3)	25	27.8	2.18	0.00235
Mean	25	27.6	2.164	0.00204
Std Dev	0	0.881	0.06816	4.74E-04
Minimum	25	25.9	2.032	0.00138
Maximum	25	28.8	2.256	0.00288

3.6 Chapter Summary

The current chapter presented the results of assessing physiochemical parameters of C_{60} and CB, to assess size, shape, and surface characteristics of the NPs. The assessments employed to determine the physiochemical parameters included; particle size/distribution, (DLS), surface area measurements, (BET & AFM), and zeta potential measurements. The results obtained for the physiochemical assessment of the two NPs are summarized in table 3.4. From the results obtained, it is evident both particles formed large aggregates over time, particularly CB. As can be seen from the DLS results, aggregation or a shift in the particle size distribution was seen over time in both particles. Monomodal dispersion was initially observed, the profile then evolved into a bimodal dispersion over the three time points, giving 2 peaks in the C_{60} measurement, (110nm, 414nm) and 3 peaks in the CB measurements, (294.4nm, 5378nm, 1.405nm). As one of the desired functions of manufactured NPs is that a decreased particle size results in an increased total surface area in comparison to the bulk material, which results in a larger surface area. BET measurements are commonly employed in the physiochemical assessments of NPs. The reason for this is that the changes in surface chemistry of NPs due to the change in surface area in comparison to the bulk material may show different/greater biological activity, which may lead to increased toxicity. As with the DLS measurements, BET results for both particles showed an increase in the absorption of nitrogen gas on to the surface of both C_{60} and CB over time, with CB measuring the larger surface area, ($4.94 m^2/g$) of the two particles, which correlates with the DLS results showing CB to form larger aggregates.

Table 3.4: Summary of Physiochemical assessment results of C_{60} and CB.

DLS (d.nm) after 10 mins		BET (m^2/g)		AFM (nm)		Zeta Potential (mV)	
C_{60}	CB	C_{60}	CB	C_{60}	CB	C_{60}	CB
Pk 1:110 Pk2: 414	Pk1: 294.4 , Pk2: 5378 Pk3: 1.405	1.13	4.94	150	250	-79.2	27.6

The 2nd technique employed to measure particle size was AFM, which measures the particles that are precipitated from suspension after being drop-cast onto the silicon wafer and evaporated. When comparing the results obtained from the DLS & AFM, it is important to note that the DLS measurements were taken over 3 time points in suspension, while the AFM measurements are taken at one time point from a dehydrated sample. Bearing this in mind, the results obtained here are comparable. A size of 150nm was recorded with the AFM for C_{60}, which is comparable to the diameter of the first peak recorded by the DLS; 110nm. This is also the case with the CB measurements, with an AFM size measurement of 250nm and a peak diameter of 294.4nm in the first peak recorded in the DLS. The last technique that was employed during the physiochemical assessment of the NPs was measurement of zeta potential, as described in section 3.5.

As a large number of the traditional ecotoxicological and cytotoxicological test methods employ living cells/organisms the measurement of the stability of a substance in a suspension is extremely relevant, and may aid in predicting NP behaviour in various environments due to the potential for interactions with proteins and ions in the local environment, and transport across membranes. The zeta potential readings recorded for both NPs did not change over time and were found to indicate that the C_{60} was a stable suspension unlike CB, for which the zeta potential indicated it is an unstable suspension. The techniques employed in this study were assessed by using deionized water as a solute for both NPs. From the results obtained here and recommendations from other studies, the author would highlight the importance of considering the range of different media employed in ecotoxicological tests and the nature by which NPs behave differently in varying solutes to include the assessment of the physiochemical characteristics of the NPs suspended in the test medias as well as deionized water. DLS measures the hydrodynamic diameter of a sphere with the same translational diffusion coefficient as the particle being measured within a fluid (Lim et al., 2013). This will depend on the size of the particle core, its surface structure and the concentration and type of ions in the medium, which in the case of ecotoxicological and cytotoxicological test medias can vary greatly in terms of ion constitution and quantity from one test to another. Taking this into account and employing several techniques is important, as depending on the media that the NP is suspended in, the measured size could be larger than that measured by other techniques, such as electron microscopy.

In the case of measuring zeta potential of NPs in cell culture media, a change in the zeta potential when compared to deionized water has been reported to indicate an interaction with the medium, for example formation of a protein corona (Monopoli et al., 2011) or agglomeration (Fubini et al., 2010) in the cell culture media, and in the case of ecotoxicological test media charge neutralisation could occur. This is very plausible as the interactions with the surrounding media occur in the area directly surrounding the particle; the Stern layer and the diffuse layer. When particles move with Brownian motion, this layer moves along with the particle and the zeta potential is the point in the diffuse layer where it moves past the bulk solution, the slipping plane. Therefore it is reasonable to assume that the effective charge at this plane will be very sensitive to the concentration and type of ions in solution. In light of this, a study which includes the assessment of zeta potential of the NPs being studied in the ecotoxicity test solutions as well as the cytotoxicological media would be very valuable in providing information on the toxicity of the NPs to the species being tested, and, as mentioned previously, this would be recommended for all of the assessment techniques to compare the responses recorded when employing the specific test media. The following chapter details the results of the ecotoxicological studies carried out, followed by the cytotoxicological assessments.

Chapter 4: Ecotoxicological Assessment of C_{60} and CB

4.1Introduction

The following two chapters detail the toxicological experimental results obtained in assessing the aquatic toxicity of C_{60} and CB. Ecotoxicological tests usually involve short-term exposures, (acute). However, if further investigation is required, long-term, (chronic) tests are carried out. Substances are deemed harmful to the aquatic environment if they persist, bioaccumulate and are toxic to aquatic life, (SATL Annual Report, 2011). As toxicity is chemical and organism specific, a battery of organisms from different trophic levels is tested. As discussed in chapter one, the salinity of the receiving waters dictates the appropriate battery of aquatic test species selected, the broad distinction being marine and freshwater. All of the species recommended for freshwater toxicity testing, which are carried out on a national level by the Shannon Aquatic Toxicity Laboratory, (SATL), which provide an independent service to industry and local authorities for investigating the hazards posed by substances discharged into the aquatic environment, were applied during this project. As well as salinity, there are a number of additional criteria that influence test species selection, including; practicality of test, documented sensitivity to a wide range of compounds, standardisation of the test, and reproducibility.

The tests carried out during the course of this study include, bacterial assessment using *Vibrio fischeri*, an OCED algal growth inhibition test using the green alga *Pseudokirchneriella subcapitata,* followed by toxicity screening of two fresh water invertebrate species, namely, *Thamnocephalus platyurus* and *Daphnia magna*. The latter test was also carried out following OECD guidelines. In addition to the ecotoxicological tests carried out, results from a series of *in vitro* cytotoxicity studies on two fish cell lines will be presented and discussed (Chapter 5). The principle of the tests carried out are as follows, test organisms are exposed to a series of concentrations of the NP for a designated time, specific to each test. At the end of the exposure time, the effects (e.g. lethality, growth inhibition), are measured. This allows plotting of a concentration effect graph and calculation of an $LC_{50,}$ (Median Lethal Concentration) or $EC_{50,}$ (Median Effective Concentration), that is, calculation of the concentration of NP that is required to kill/inhibit 50% of the population tested. Toxic units, (TU) can also be reported, which are calculated by dividing 100 by the LC_{50} or EC_{50}. National Environmental Protection Agencies set Toxic Unit limits for industry and local authorities with respect to waste-water and waste products, issued through IPPC licences, which must not be exceeded. In this study, EC_{50} values are reported. EC_{50} values are considered the most robust values for the estimation of the dose-response effect of the test substance (Isnard et al., 2001) and median EC_{50} values are usually employed for Quantitative Structure Activity Relationship, (QSAR) analysis (Cronin et al., 2003). A QSAR is a mathematical model, usually a statistical correlation, relating one or more quantitative parameters that can be derived from chemical structure to a quantitative measure of a property or activity, in this case an ecotoxicological end point.

4.2 Microtox Acute Toxicity Assay

As described in section 2.2, the acute Microtox toxicity assay was carried out on C_{60} and CB. Three separate assays were carried out on three separate occasions for each particle. On each occasion, a known reference toxicant, phenol, was employed to validate the test system prior to sample testing. The results of six phenol assessments carried out on separate occasions are shown in figures 4.1 and 4.2. The test system was deemed valid, as the EC_{50} values for each positive control test carried out were within the recommended acceptable range of 13.00-26.00ppm, (Azur Environmental Ltd, 1998). Once the reference test was validated, the samples were tested. The test protocol involved the exposure of the test species, *Vibrio fischeri,* to nine concentrations of each NP, within the range 0-833ppm and measurement of the percentage inhibition of light output of the test species was carried out. The principle behind this test being *Vibrio fischeri* naturally emit luminescence. Upon exposure to the test substance, if it is toxic to the species, i.e. if exposure kills the bacteria, the luminescence detected is lower when comparing to the untreated control sample. The Hill model, supported by the REGTOX software program, was used to calculate the EC_{XX} values obtained (Garric et al., 1990), as described in section 2.6 in chapter 2.

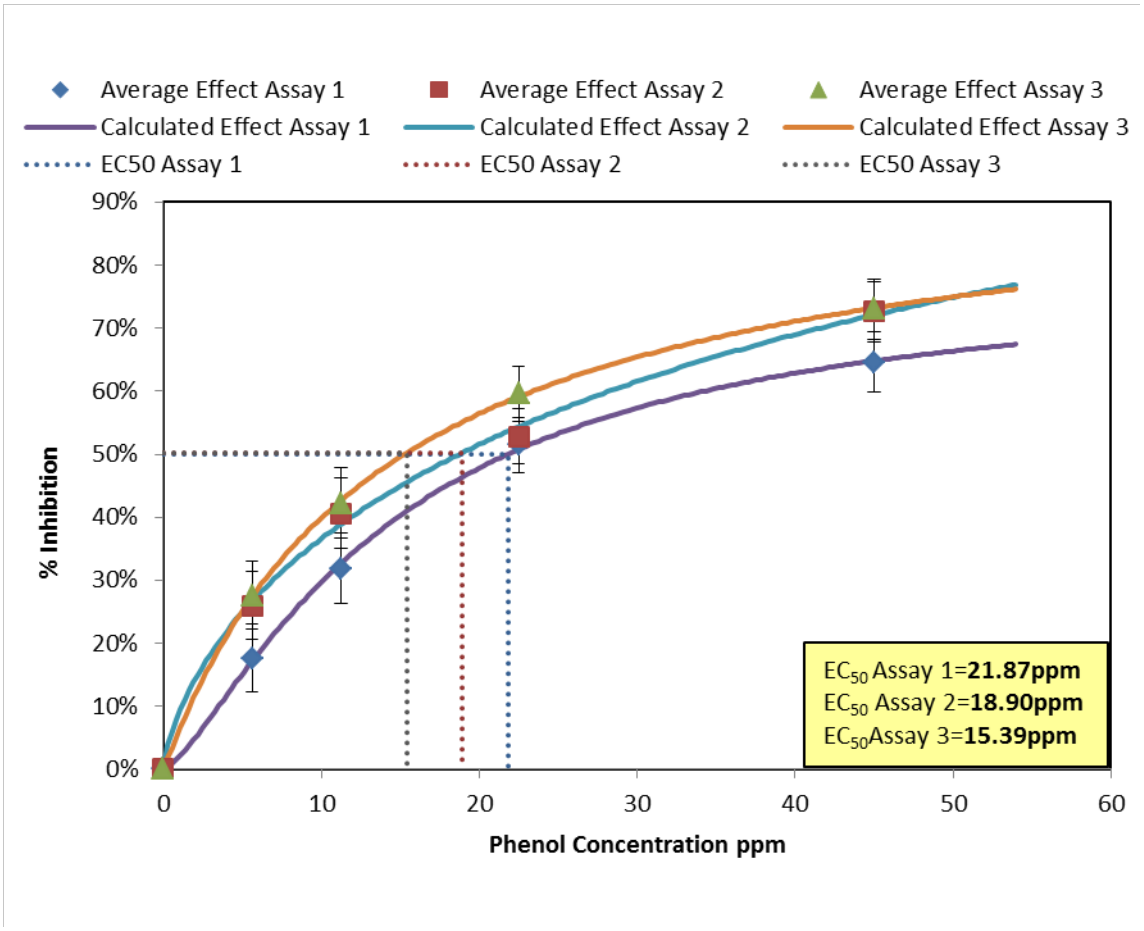


Figure 4.1: % Inhibition of Light output of *Vibrio fischeri* when exposed to varying concentrations of Phenol over 3 Assays.

As mentioned previously, once the reference test is carried out and the results fall within the range outlined by the Azur Environmental test protocol, test substance screening is carried out. As recommended in all toxicity tests, the test substance is screened three times on three separate occasions.

Figure 4.3 shows the results obtained for the assessment of C_{60}. It is evident that the C_{60} exposure did cause a high dose-response effect, which is seen to increase over time in terms of the inhibition of light output of the test species. It is important to note the increase in response from the 5 minute time point, to the 15 minute time point, which can be seen clearly when comparing the average effect recorded at both time points.

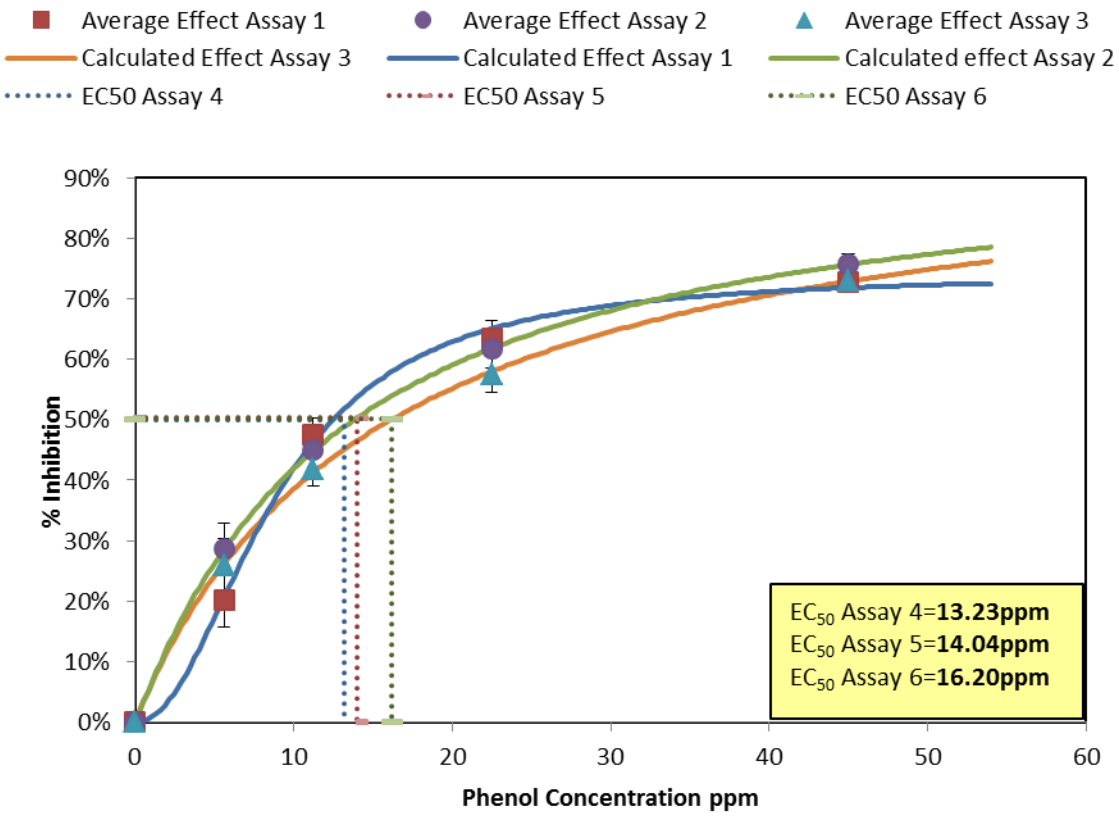


Figure 4.2: % Inhibition of Light output of *Vibrio fischeri* when exposed to varying concentrations of Phenol over 3 Assays

On assessing the DLS measurements taken for C_{60} over the first 2 time points, 5 and 10 minutes, C_{60} is shown to aggregate such that the particle size measured after 5 minutes was 241.6dnm and when comparing with the results obtained for the toxic effect of C_{60} to *Vibrio fischeri* in figure 4.3, its evident that up to 5 minutes exposure time did not cause a toxic response to the bacteria. After 15 minutes exposure time, toxicity is recorded, where in the DLS measurements at the 10 minute time point the particle distribution is seen to change, and 2 peaks are recorded. This suggests the aggregated particles initially exposed to the bacteria were not bioavailable and so toxicity was not observed, but as the exposure time increased toxicity was observed which correlated to the dispersion of aggregates recorded over time in the DLS measurements. An EC_{50} value could not be recorded at the 5 minute time point, as to do so would have been based on extrapolation of the data rather than on the actual concentration range used in the test. The responses at time points 15 and 30 minutes allowed for calculation of EC_{50} values, which highlights the importance of measuring the effect over time as well as on increasing doses as in this case toxicity was observed to increase after the 2nd time point. It is also evident that, for C_{60}, this test is reproducible when comparing the response measured by replicates in each test carried out, as shown by the standard error of the mean, the y error bars on figure 4.3. This is one of the species selection criteria in aquatic toxicity testing as discussed in chapter one; reproducibility of test systems. The test was carried out several more times than on three occasions, in order to investigate the reproducibility of this test for C_{60}.

A draw back identified here in using the traditional toxicological tests to assess NP toxicity is that a blank to test the suitability of the test material is not possible before carrying out the test as in the effect/interference of the NP on the fluorescence cannot be measured without exposing the material to the luminescent bacteria. One parameter that can be followed to assess the suitability of a test substance is the range of toxicity that is recommended for each test, which were considered during this study for each test.

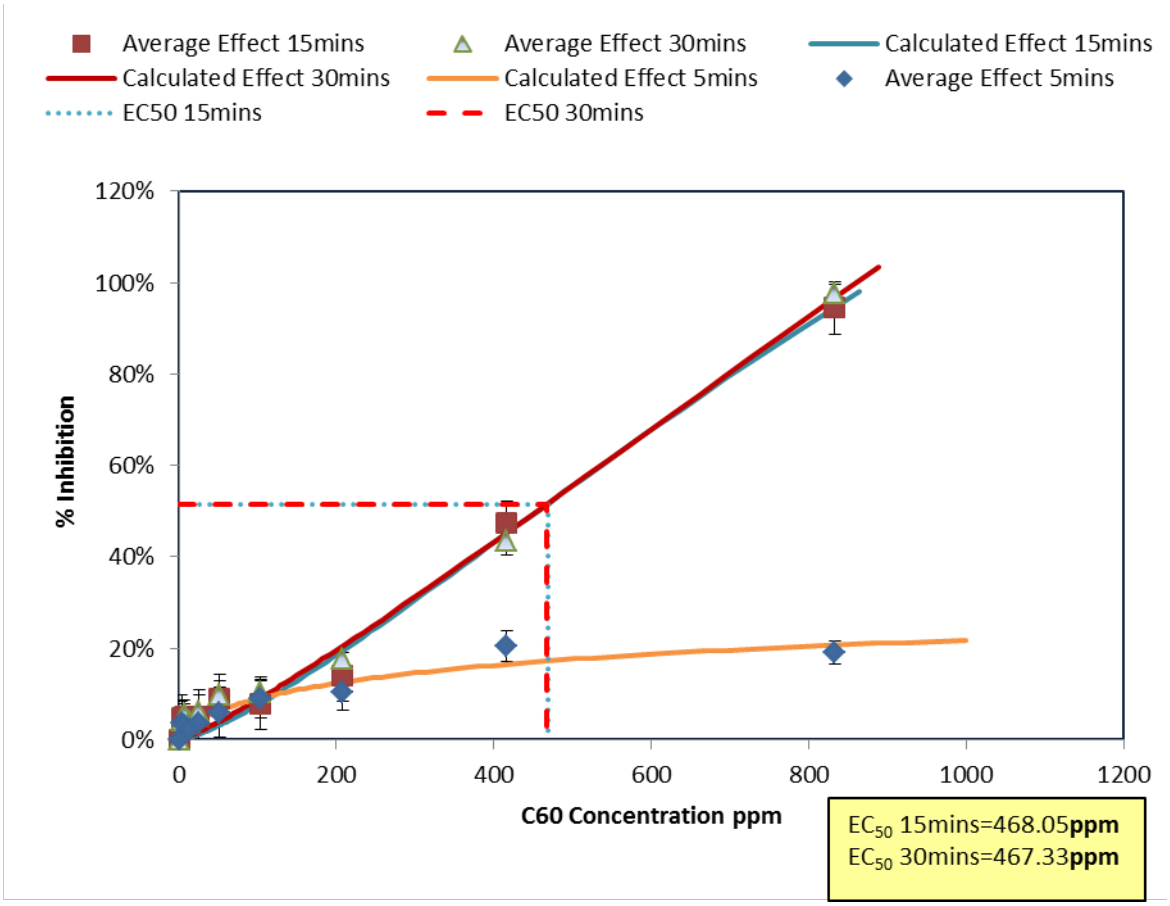


Figure 4.3: Average % Inhibition of light output of *Vibrio fischeri* of three assays when exposed to varying concentrations of C_{60} over 3 time points.

The challenge in finding repeatable test systems in the case of C_{60} and indeed many other carbonaceous NPs is an on-going issue that the nano ecotoxicological research community is attempting to address presently. Many believe it is due to the insolubility of the particle in test media, chemical structure, and surface modifications, (Engineered NP review of human and environmental safety Report, (ENRHES), 2011). Many methods utilise more rigorous dispersion techniques to overcome this obstacle, as reported in earlier nano ecotoxicological study methods, where particles were mixed for several months prior to testing, (Oberdorster, Oberdorster et al. 2005). Recent publications have, however, questioned these methods and indeed challenged the results obtained in these studies, highlighting the issue of real life exposure scenarios. Another challenge that is gaining attention in the scientific community is the development of suitable reference NPs for generic testing, (Engineered Nanoparticles, Review of Health and Environmental Safety final report, (ENRHES), 2011). Several ecotoxicological tests employ certain chemicals as standards. What the nanotoxicological community endeavour to do now is to create reference NPs that are on a par with the chemicals traditionally being employed in ecotoxicity tests that allow for accurate and reproducible assessments of test systems prior to sample testing. Figure 4.4 shows the percentage inhibition of light output recorded when *Vibrio fischeri* were exposed to nine concentrations of CB on three separate occasions. It is evident that the test system is reproducible for CB also, as there is a very low standard error of the mean for each replicate of the three tests carried out, which again highlights reproducibility of the test. From figure 4.4, it can be seen that increasing the concentration of CB resulted in an increase in the percentage of light that was inhibited, the highest concentration displaying the highest response. The EC_{50} values of 73.52ppm, 89.86ppm and 119.03ppm are for the three time points 5, 15, and 30 minutes respectively.

It is interesting to note that the toxic effect of CB is observed to decrease over time, suggesting the distribution of the CB in the test media is changing, which was shown with the CB DLS measurements. The reduction in the toxicity recorded may therefore be due to sedimentation of the CB. When comparing values obtained for both NPs tested, it is evident CB poses a greater threat to freshwater bacteria than C_{60}, requiring 73ppm CB to kill 50% of the population tested after 5 minutes exposure, compared to 468ppm in the case of C_{60} after 15 minutes exposure.

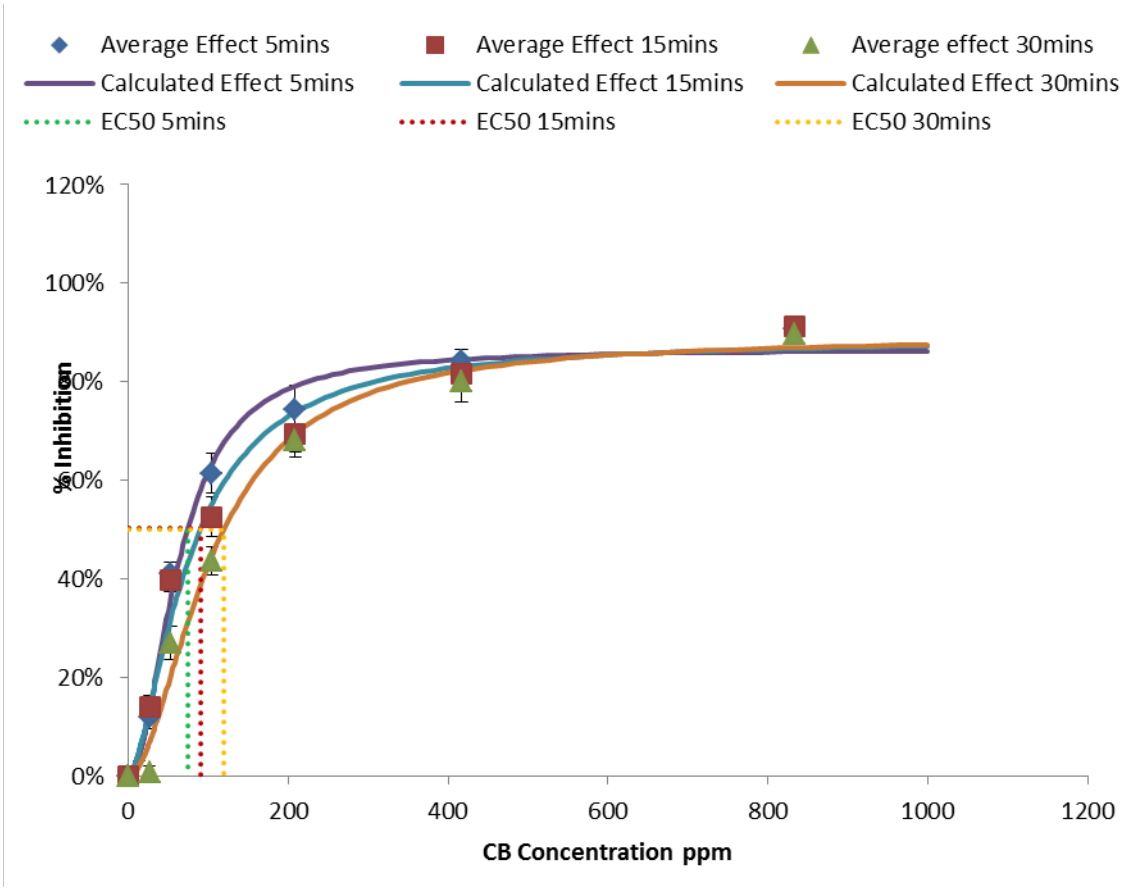


Figure 4.4: Average % Inhibition of light output of *Vibrio fischeri* of three assays when exposed to varying concentrations of CB over 3 time points

4.3 Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OCED test no. 201

The principle of this test as described in section 2.5.2 is that replicates of the test algae, *Pseudokirchneriella subcapitata*, are allowed to grow in media containing the test substance for 72 hours, after such time the number of algal cells per mL are counted manually using a haemocytometer counting chamber and a microscope. This value is then compared to the growth of the unexposed control over the same time period and the inhibition of growth caused by the test substance is calculated. Six test substance concentration points are used per test. In accordance with OECD guidelines, several range finding tests are carried out initially to determine a definitive concentration range that causes between 5 and 75% growth inhibition after the 72 hour exposure period. Once a definite range of concentrations are confirmed three definite tests are carried out which involve exposing *Pseudokirchneriella subcapitata* to three replicates of each concentration for 72 hours. For a test to be deemed valid, a 16 fold growth increase from the initial seeding density in the control must be observed. After 72 hours, algal growth begins to slow down, as it has entered a stationary phase of growth, primarily due to a reduction in nutrient levels in the medium and the presence of high amounts of waste materials, for this reason the test is limited to a 72 hour exposure time. Range finding studies were carried out for both NPs prior to definitive testing, as described above. Over the course of the range finding experiments for C_{60}, none of the tested concentrations elicited a 5-75% growth inhibition, the concentration range required to obtain a definitive range, as discussed above. In such a case the OECD guidelines state, when no 5-75% inhibitory concentration can be deduced from the range finding studies, the performance of a limit test is recommended.

A limit test is a set of six experimental replicates of an exposure concentration to a maximum concentration of 100ppm, (or a concentration equal to the limit of the test compounds solubility). Following OECD guidelines 201, reference toxicant testing was carried out in conjunction with NP assessment; guidelines for this test recommend reference testing twice a year. During the course of this study, reference testing was carried out as each set of test substances were being assessed, that is, three times. The most widely used reference toxicant for this study is potassium dichromate, whereby 6 concentrations of potassium dichromate are added to algal vessels in triplicate and algal cultures are allowed to grow for 72 hours, after which time algal growth is calculated. The test system is validated on observing a 16 fold increase of algal growth in the control and a dose response effect in increasing concentrations of potassium dichromate, that is, as the concentration of potassium dichromate is increased the observed % inhibition of growth is seen to increase. Figure 4.5 shows the data obtained from three reference toxicant studies carried out using potassium dichromate. On examining the figure, it is evident the test system was functioning accurately as shown by the dose-response effect recorded. An EC_{50} concentration of 1.38ppm was obtained which is found to be within the reported values for positive control tests for the algal growth inhibition of *Pseudokirchneriella subcapitata* (Nyholm et al., 1990).

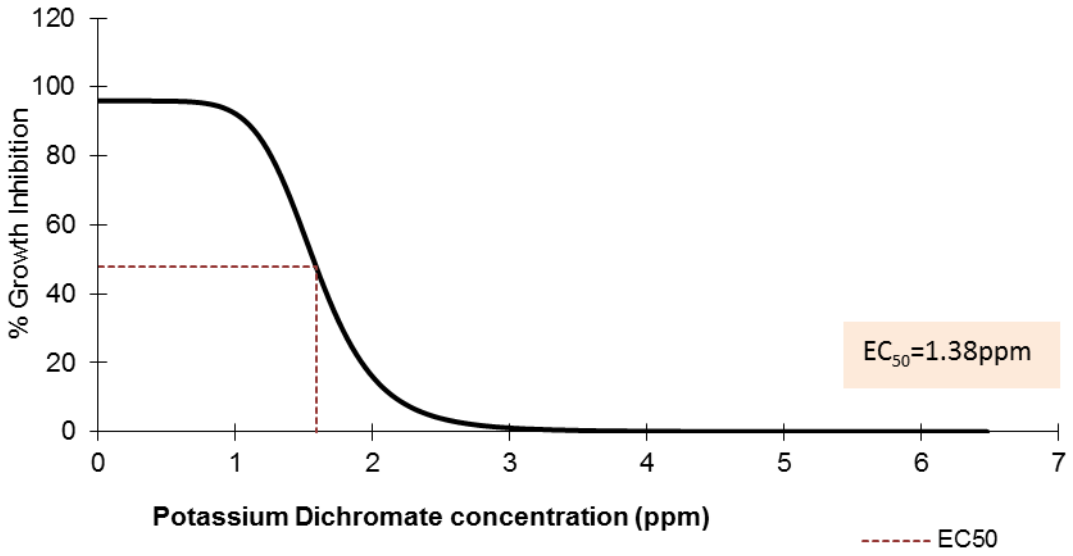


Figure 4.5: % Growth Inhibition of *P.subcapitata* when exposed to varying concentrations of Potassium Dichromate over three tests .

Figure 4.6 shows the results obtained from performing three limit tests for $C_{60,}$ as explained above, the measurement involved exposing *Pseudokirchneriella subcapitata* to six replicates of 100ppm C_{60} for 72 hours for each test, and determining the % growth inhibited by the test substance compared with the unexposed control replicates. These tests met the validity criteria as there was a minimum of a 16 fold increase in growth observed in the control vessels after the duration of the test, initial seeding concentrations being 10,000 algal cells per mL of vessel media. Manual cell counts of each test vessel were carried out to quantify the mean growth, cells/mL, (number of viable cells per mL post exposure), growth rate, and finally the % inhibition of the test vessels in comparison to the unexposed control. This calculation is explained in more detail in chapter 2, section 2.5.2.

From the results obtained, it can be suggested that C_{60} caused no significant growth inhibition of *Pseudokirchneriella subcapitata* following a three day exposure period. This result was expected, as from carrying out the range finding tests for C_{60} no observed effect level/concentration, (NOEL/C), could be established. NOEL is used widely in traditional ecotoxicological studies. It is an exposure level at which there are no statistically or biologically significant increases in the frequency or severity of any effect between the exposed population and its appropriate control, (U.S. Environmental Protection Agency, 2012). From this data, it can be suggested that C_{60} is not an eco-toxicological threat to the algae *P. subcapitata,* which is encouraging, as algae play a vital role in aquatic ecosystems, in that they are involved in nutrient cycling and O_2 production and are a food source for higher species along the food chain. However it is important to note that the inhibition recorded was from carrying out a limit test as a definitive range of concentrations could not be obtained, this may be due to the poor solubility of C_{60} in the test media as the limit test procedure suggests using a concentration point equal to the limit of the test substance solubility, as discussed in chapter 1 previous studies have endeavoured to overcome the poor solubility this by the use of solvents, prolonged stirring and sonication, sonication was used is this study to recreate the natural exposure environment as much as possible.

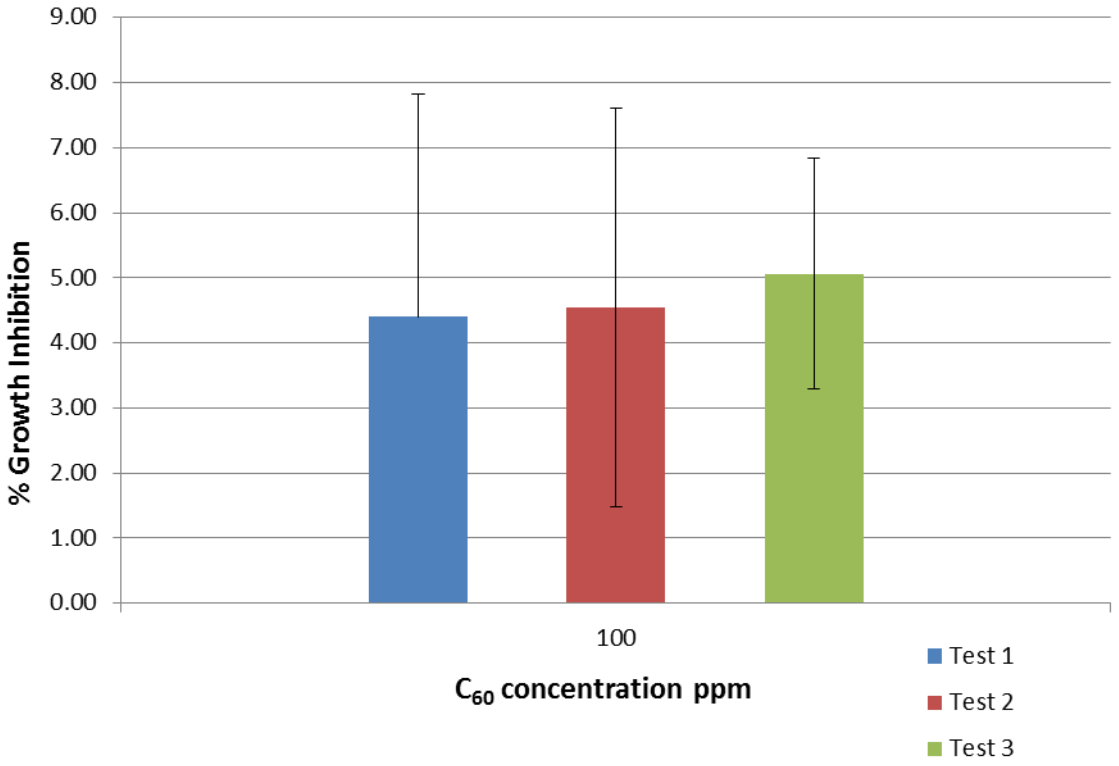


Figure 4.6: % Growth Inhibition of P. subcapitata when exposed to 100ppm C60 for 72 hours over three tests, showing standard deviation of 6 replicates per test.

Figure 4.7 displays the results of three independent tests carried to assess the effect of the NP CB on the algal species *Pseudokirchneriella subcapitata*. As for C_{60}, prior to definite testing being carried out, several range finding studies were performed, none of which produced an effect of between 5% and 75% inhibition of growth, as required by the OCED guidelines for definite test concentration points selection. However a dose response relationship could be observed when assessing a concentration range of 0-1ppm CB, figure 4.7. The EC_{50} values represented on Figure 4.7 are extrapolation of data that was obtained and was carried out to gauge the potential EC_{50} of CB.

However, it is imperative to highlight this value is not reflective of actual results obtained on the concentrations tested. Suggestions as to the unsuitability of this test to CB include possible shading effects and the particles being unbioavailable to the algal cells. This is discussed in more detail in the discussion.

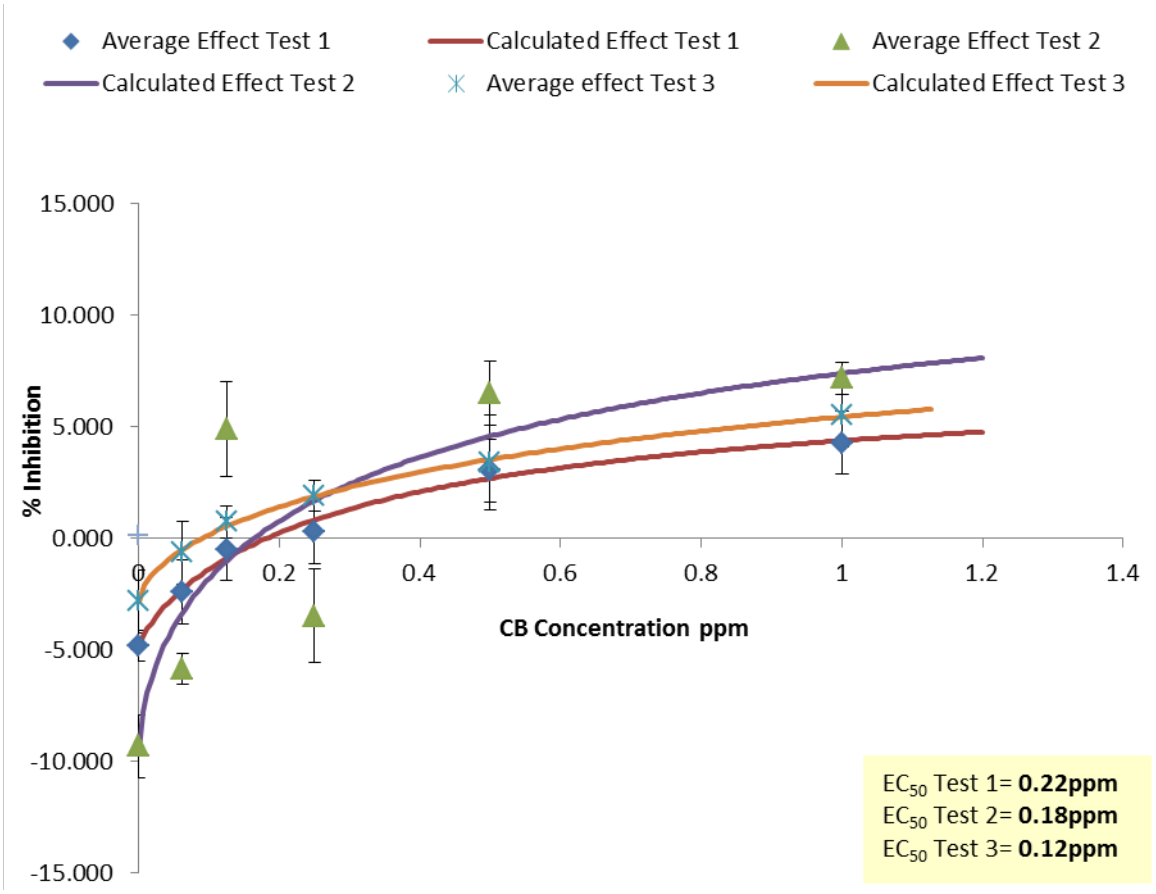


Figure 4.7: % Growth Inhibition of *P.subcapitata* when exposed to varying concentrations of CB for 72 hours over three tests.

From the results collected in assessing the toxicity of C_{60} and CB to *Pseudokirchneriella subcapitata* as already mentioned one questions the suitability of this test in screening NP toxicity, as in both sets of experiments the required range of concentrations to elicit an effect between 5 and 75% growth inhibition could not be ascertained. One possible reason for this is that the NPs were not bioavailable to the algal cells, due to the size of the algal cells, (2-3μm in width), and the formation of NP agglomerates in the algal media, which has been widely reported in toxicological studies assessing carbon based NPs in previous studies, (Casey, Davoren et al. 2007). One study assessed the bioavailability of CeO_2 to *P. subcapitata* cells by using a range of particle sizes and using the OCED test 201 method (Rodea-Palomares et al., 2011), 50nm particles were found to be the most toxic giving an EC_{50} of 2.4–29.6 mg/L, the order of toxicities of the remaining particle sizes were as follows; 25nm > 60nm, > 10nm.

The results obtained in this test highlight the importance of assessing toxicity in several species, the biological makeup of the test species may inhibit the test being carried out effectively, as suggested in this study, the formation of NP aggregates in solution may render them unbioavailable to the algal cells and so assessment in this way is not possible. Alternative approaches and suggestions to address this issue are presented in Chapter 6.

4.4 Thamnocephalus platyurus screening

The first freshwater invertebrate that was exposed to C60 and CB was *Thamnocephalus platyurus.* As stated in chapter two, this assay is simple, cost effective and employs the larvae of the fairy shrimp, *(Thamnocephalus platyurus).* The procedure outlined in section 2.5.3 was followed exactly for the test set up. As the protocol requires, a large range of concentrations were investigated initially. The concentration range employed here was 1000ppm, 100ppm, 10ppm, 1ppm and 0.1ppm for each NP.

The procedure requires the operator to record the number of living and dead larvae for each concentration post exposure time of 24 hours and calculate the % mortality. Larvae are confirmed dead if they do not exhibit movement after 10 seconds of observation. A mortality of less than 10% in the control wells in each test indicates a valid result. Figure 4.8 displays the findings of exposing C_{60} to *Thamnocephalus platyurus* larvae for 24 hours; very low mortality rates were observed over the tested concentration range and as a result a definitive test could not be performed. The manufacturers of this protocol claim high sensitivity drawing comparison of the test species, with several other invertebrate species routinely employed in aquatic toxicology today (Micro-Bio Tests Inc. test protocol, 2012). The results obtained would suggest that C_{60} is not significantly toxic to aquatic species aquatic species outlined in manufacturers test protocol. However, further studies were performed and are described in the upcoming section on a second fresh water species *Daphnia magna* to further investigate this theory.

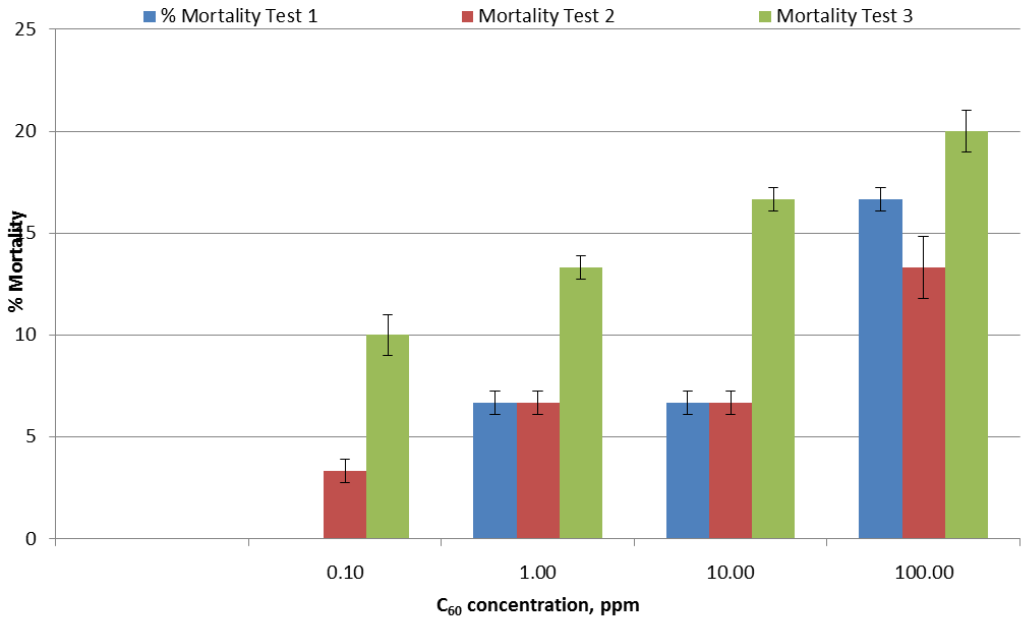


Figure 4.8: % Mortality of *Thamnocephalus platyurus* when exposed to varying concentrations of C_{60} over four tests.

In the case of CB, a range of concentrations that elicit over 50% mortality could be obtained which then allowed the performance of three individual definite studies and the calculation of EC_{50} values. The Hill model, supported by the REGTOX software program was used to calculate the EC_{50} values (Garric et al., 1990) Figure 4.9 shows the results obtained for these tests. When comparing the EC_{50} values one can deduce that for CB this test is reproducible. An average EC_{50} of 23.03 over the three tests was observed. Discussion of these results and comparison with additional ecotoxicological tests are described in the following chapter.

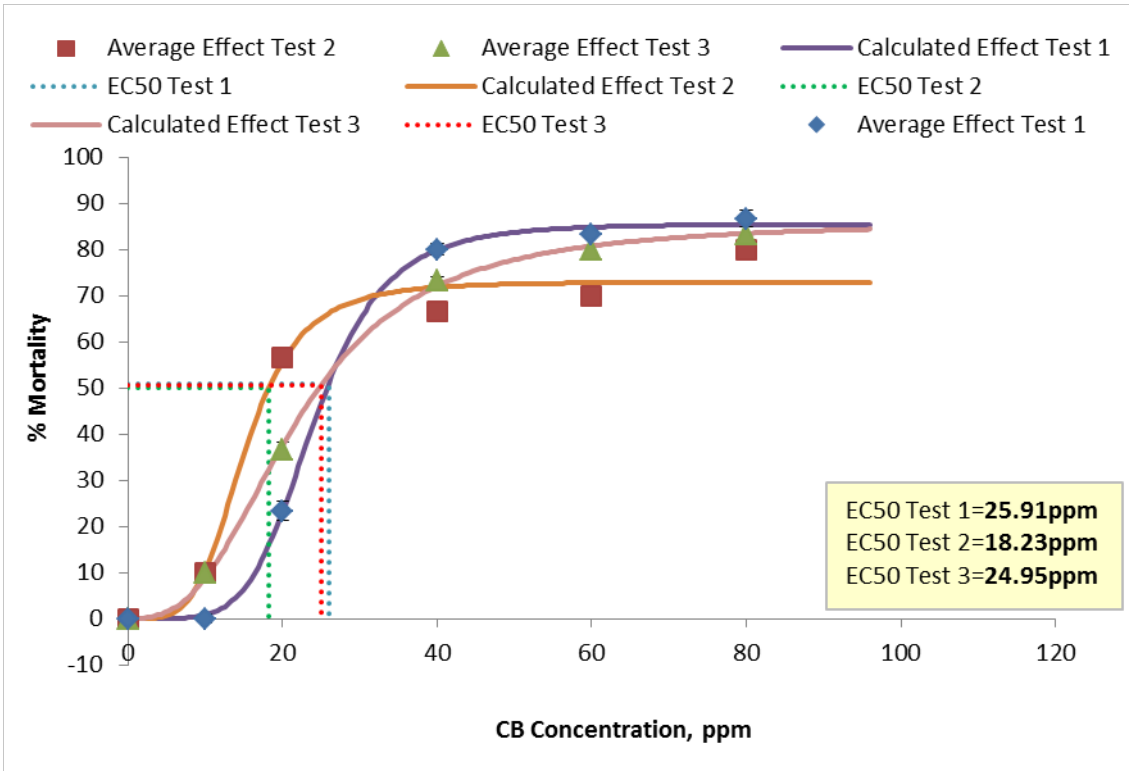


Figure 4.9: % Mortality of *Thamnocephalus platyurus* when exposed to varying concentrations of CB over 3 tests.

4.5 Findings of Daphnia magna Acute Immobilization Test, OECD test no. 202.

After completion of the *Thamnocephalus platyurus* screening, the results of which were discussed in the previous section, an additional freshwater invertebrate species was tested; *Daphnia magna.* Three independent experiments were carried for each NP. The OECD guidelines outlined in section 2.5.4 were adhered to when carrying out these experiments. The principle of the test is; 5 daphnia neonates, (offspring less than 24 hours old), are placed in daphnia media containing a specific concentration of the NP being tested. Concentration ranges are selected after performing range finding tests, OECD recommended concentrations for the range finding tests state that the highest concentration tested should preferably result in 100% immobilisation and the lowest concentration tested should preferably give no observable effect, no mortality to population tested. The test is run for 48 hours, as this is sufficient time for the neonates to undergo one moult, at which time they are considered most sensitive. The test is not run for longer to avoid the risk of starvation being a factor and having an effect on results. Throughout the exposure period, any unusual behaviour, such as immobilisation, inability to swim after 15 seconds, even if antennae are still moving, floating on the surface, or abnormal rotating or circling is recorded. After the required 48hour exposure period the number of dead neonates are determined. As with the *Thamnocephalus platyurus* test, they are confirmed dead if they do not show movement within 10 seconds of observation. Figure 4.10 shows the observed mortality in three individual tests following a 48 hour exposure to a wide range of C_{60} concentrations, a range finding study.

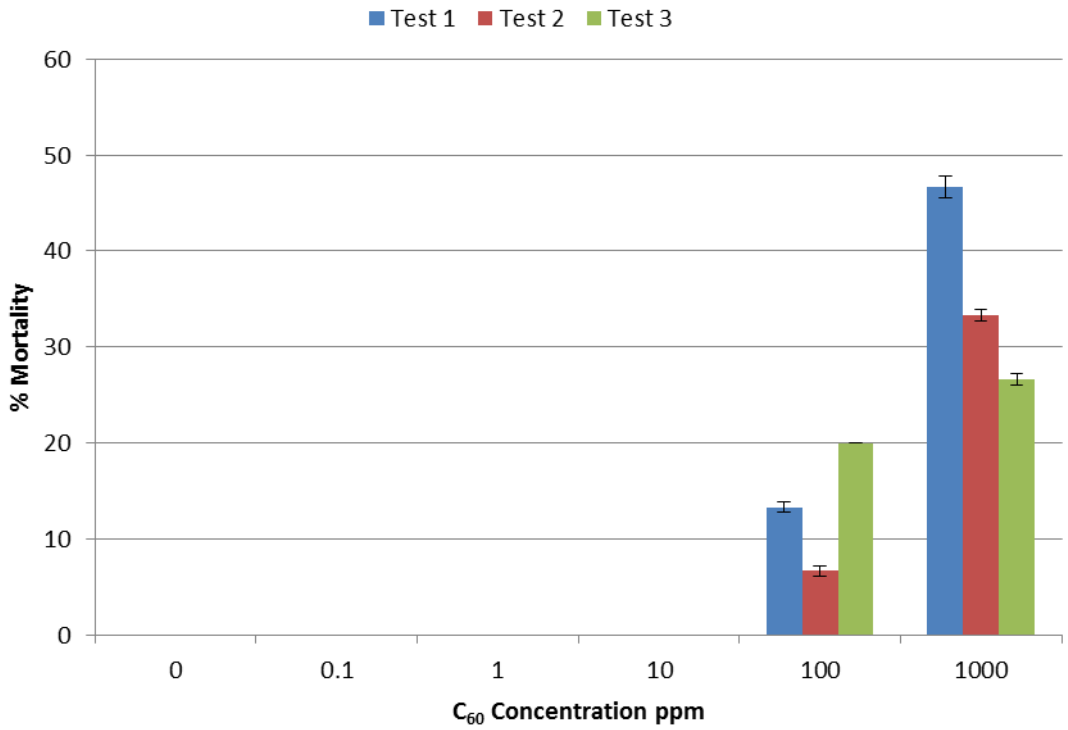


Figure 4.10: *Daphnia magna* mortality resulting from 48 hour exposure to C 60 in comparison to an unexposed control. Data represents the average of three independent experiments ± the standard deviation of replicates of each concentration.

As can be seen in figure 4.10, no mortality was observed at concentrations below 100ppm when assessing the results obtained for C_{60}. The highest recorded mortality at this concentration point was 20%, whereas at 1000ppm exposure 47% mortality was recorded. An EC_{50} value could not be obtained with this data due to the low mortality rates recorded. With the view to addressing the obstacles faced in the assessment of nanoparticle toxicity in the environment.

Several studies are now taking place to develop analytical methods to accurately measure the real concentrations of NPs in the environment (Paterson et al., 2011), in addition to the modelling of predicted environmental concentrations, (PEC'S), described by several groups, (e.g. (Gottschalk et al., 2009)). This is discussed in more detail in the discussion section and the following chapters. A concentration point of 1000ppm was included here for the purpose of validating the test system and comparing the result obtained with that of the 100ppm set of tests, following the OECD test recommendations, which stipulate that a range of test concentrations must include a concentration that elicits 100% mortality and 0% mortality. It should be concluded that these results suggest C_{60} is not toxic to the species *Daphnia magna*. However, it is also imperative to highlight here that this test is not very reproducible for C_{60}, as indicated by the significant variations in the mortality recorded for each concentration point when comparing the three tests carried out. This highlights the importance of carrying out multiple tests on a single species and indeed carrying out a comprehensive set of tests over a broad range of species, to fully evaluate toxicity, as toxicity has been proven in the past to being species specific, (SATL, 2011). Figure 4.11 shows the results obtained from three independent tests carried out to assess the toxicity of CB to *Daphnia magna*. As for the C_{60} tests, a series of range finding tests were carried out initially to find a concentration range that elicited a 10% mortality in the lowest concentration dose tested and a minimum of 70% mortality in the highest dose tested. On examining the graph, it is clear that CB elicits a dose response effect in the *Daphnia magna* acute immobilisation test, i.e. as the concentration of CB increases; the % mortality is also seen to increase.

The average effect points on the graph represent the % mortality of three replicates per concentration dose and are calculated from the number of dead neonates recorded post exposure in each independent test. The calculated effect shown on the graph was taken from the response obtained from inputting that data into the REGTOX software program using the Hill model to calculate the EC_{50} values. The EC_{50} values calculated for the three tests are low, when comparing to the 100ppm required to elicit a small response when assessing C_{60}, (11.49ppm, 15.12ppm & 18.72ppm), giving an average EC_{50} over the three tests of 15.11ppm, suggesting CB is highly toxic to *D. magna*, given that a concentration of 11.49ppm in test 1 for example, is a sufficient concentration to kill 50% of the *Daphnia* being tested.

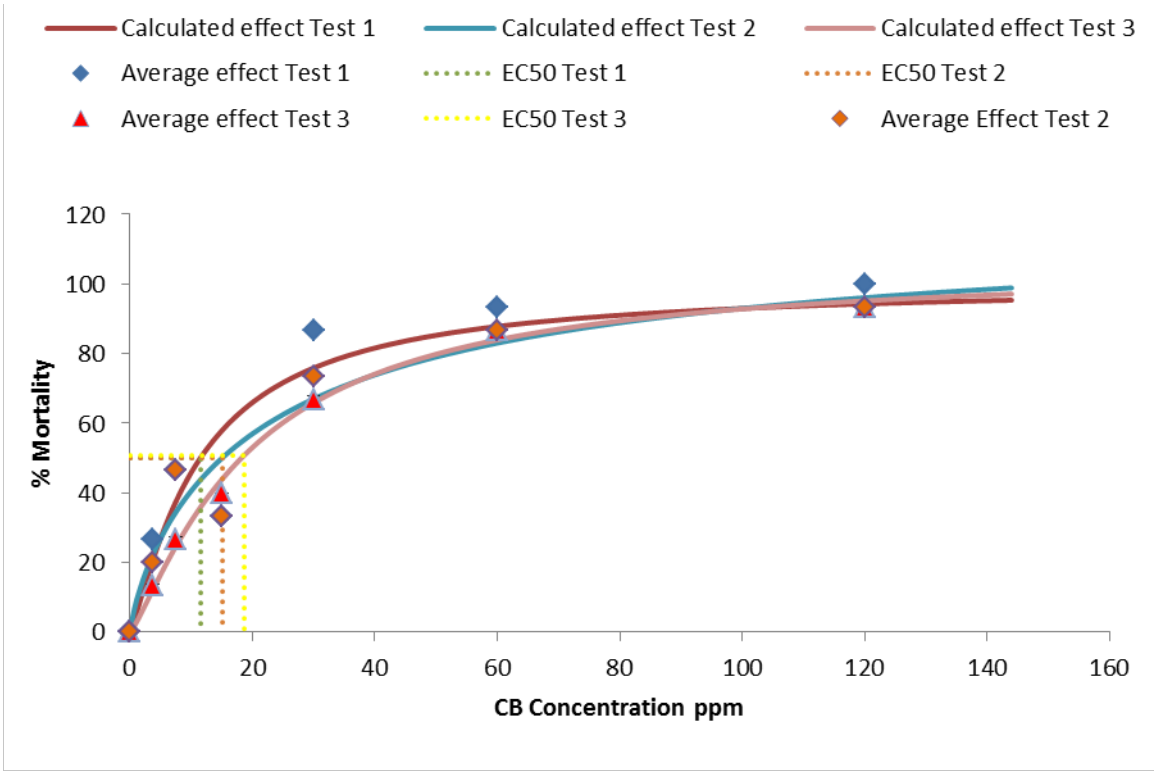


Figure 4.11: *Daphnia magna* mortality resulting from 48 hour exposure to CB in comparison to an unexposed control. Data represents the average of three independent experiments ± the standard deviation of the replicates of each concentration point

4.6 Chapter Summary

This chapter detailed the results obtained when assessing the aquatic toxicity of C_{60} and CB in several established aquatic toxicity tests, namely the Microtox acute immobilisation assay, freshwater Alga growth inhibition test, *Thamnocephalus platyurus* screening and the *Daphnia magna* Acute Immobilization Test. Table 4.1 summarises the results obtained when the aquatic toxicity of CB and C_{60} were assessed. From reviewing the results obtained, they suggest that CB is more toxic than C_{60} in the four species tested and that C_{60} elicited a toxic effect in an aggregated state. It is evident that assessing the ecotoxicity of C_{60} is difficult when using traditional ecotoxicological tests. For example, when employing the *Daphnia magna* acute immobilisation test, the mortality dose response required by the test procedure of <10% mortality in the lowest concentration dose tested and >70% mortality in the highest dose tested could not be obtained. However, this test system was shown to be suitable in assessing ecotoxicity of CB as a range of mortality as outlined in the test guidelines and EC_{50} values were obtained in each test carried out. Table 4.1 also shows that the algal growth inhibition test was not a suitable test system for the assessment of the ecotoxicity of these carbonaceous NPs, as the required range of % inhibition of growth over increasing concentrations could not be obtained for both particles. One possible suggestion for this being that the NPs form large aggregates in the algal media and therefore are not bioavailable to the algal cells. This is discussed further in chapter 6. The following chapter details the results obtained from the cytotoxicological assessment of the NPs using 2 fish cell lines and two cytotoxicological assays.

Table 4.1: Assessment of the aquatic toxicity of CB and C_{60}

Species Tested, Test Employed	Results obtained for **C_{60}**	Results obtained for **CB**
Vibrio fischeri: Microtox Acute Toxicity Test	15 min EC_{50} =468.05ppm 30 min EC_{50} =467.33ppm Ave EC_{50}=467.69ppm	5 min EC_{20}= 73.52ppm 15 min EC_{50}=89.86ppm 30 min EC_{50}=119.03ppm Ave EC_{50}=94.13ppm
Pseudokirchnerlla subcapitata: Alga growth Inhibition Test	Average Growth Inhibition of 4.67%* was recorded over 3 Tests.	Average Growth Inhibition of <5% * was recorded over 3 Tests.
Thamnocephalus platyurus: Acute Toxicity Test.	Definitive set of tests could not be performed, toxicity of less than 20% recorded.*	Test 1 EC_{20}= 25.19ppm Test 2 EC_{50}=18.23ppm Test 3 EC_{50}=24.95ppm Ave EC_{50}=22.79ppm
Daphnia magna: Acute Immobilization Test	% Mortality was lower than 20%*	Test 1 EC_{50}= 11.49ppm Test 2 EC_{50}=15.12ppm Test 3 EC_{50}=18.72ppm Ave EC_{50}=15.11ppm

*EC_{50} values could not be calculated with results obtained as doing so would be extrapolation of results.

When examining the results obtained for CB in the four tests carried out, as mentioned above three out of the four test systems employed were suitable for the assessment of the ecotoxicological effects of CB on the aquatic species tested. In these tests the results obtained met the criteria of each test, that is, the range of toxicity that is outlined in the test protocol to deem the test to be valid. From the three species tested it's evident that the most sensitive species was the *Daphnia magna*, which is in line with the reported sensitivity of *Daphnia magna*. As mentioned in chapter 2, one of the reasons it is used so widely in ecotoxicity studies, is due to the fact it is very sensitive to toxicants and therefore is a very useful test species in screening test substances for toxicity. The second invertebrate tested, the *Thamnocephalus platyurus* was also shown to be sensitive to exposure to CB, with an average EC_{50} over three tests of 22.79ppm, which is comparable to the concentration observed during the *Daphnia magna* screening; 15.11ppm. From comparing these results one would suggest that CB is toxic to fresh-water invertebrates and also fresh water bacteria. As the algal test results were not valid further plant studies would be recommended, higher plants could be used for this, such as the Lemna minor growth inhibition assay. Further recommendations for the testing of the ecotoxicity of NPs are discussed in chapter 6.

Chapter 5: Cytotoxicological Assessment of C_{60} and CB

5.1 Introduction

This chapter will detail the results of the remaining ecotoxicological tests carried out in assessing the effect of C_{60} and CB in the fresh water aquatic environment. These tests include the AB and NR cytotoxicity assays. Cytotoxicological assays are now used widely to assess toxicity of test substances due to the fact that they are simple to perform, rapid and reproducible. The development of *in vitro* assays for toxicity testing is increasingly important because of EU Directive 2010/63/EU on the protection of animals used for scientific purposes by the principle of Replace, Reduce and Refine the use of animals. Several assays have been developed and are now used widely to assess mammalian toxicity (Wakuri et al., 1993). However, there are relatively few assays developed specifically for fish cells lines. Although over 150 strains of fish cell lines have been developed, very few are used in cytotoxicity studies (Bols et al., 2005). Examples of fish cell lines that are being used in these bioassays include RTG-2 cells from the rainbow trout, PLHC-1 cells from the flathead minnow, both of which are employed in this study, BF-2 cells from bluegill sunfish, and STE cells from the steelhead trout embryo, (*Oncorynchus mykiss*).

5.2 In vitro Cytotoxic Evaluation

Two cytotoxicological end-points were used to assess the cytotoxicity of C_{60} and CB in two fish cell lines; PLHC-1 and RTG2. When choosing cell lines for cytotoxicological studies, it is recommended to select cell lines from differing tissues, as toxicity has be shown to be organ specific, showing varying degrees of toxicity of a test substance when exposed to several cell lines obtained from differing organs (Mori et al., 2000). To that end, RTG-2 cells and PLHC-1 were employed here; gonad tissue and liver tissue respectively. The cytotoxicological end points employed in this study were the Alamar Blue assay and the Neutral Red assay. Alamar blue is a proven cell viability indicator that uses the reducing power of living cells to convert resazurin to the fluorescent molecule, resorufin, the extent of the conversion is quantitatively measured, the results of which give an accurate indication of cell viability, the more toxic the substance the less the conversion is carried out and thus the absorbance recorded. The NR cytotoxicity assay is also a cell survival assay based on the ability of viable cells to incorporate and bind NR dye; toxic substances cause a decreased uptake and binding of NR, allowing spectrophotometric measurements of cell viability. Although accumulating specifically in lysosomes, NR retention is dependent on an intact plasma membrane, adequate energy metabolism and a functioning lysosome; thus the NR assay assesses impairment to all three cellular parameters (Bols, 2005). Cytotoxicity is expressed as a concentration dependent reduction of the uptake of NR after chemical exposure.

The following paragraphs and figures represent the data obtained in this study for the aforementioned 2 fish cell lines assessed by the 2 cell viability assays mentioned above. Data is expressed as a percentage of an unexposed control ± the standard deviation of three independent experiments carried out. Single factor ANOVA analysis of variance was carried out and, where possible, EC_{50} values were calculated using the software program MasterPlex 2010. The first cell line that was employed for cytotoxicological evaluation was the RTG2 cell line. The AB assay and NR assay were performed sequentially on the same plate as outlined in the procedure in section chapter 2.3.

5.2.1 NR cytotoxicity Results

The first cytotoxicological assay that was performed on the fish cell lines was the NR assay; this assay specifically assesses lysosomal activity in viable cells. NR measures a decrease in the number of lysosomes, due to cell death caused by the test substance. NPs are also taken up by the lysosomes which is a second effect measured here. The results of this assay for the RTG2 and PHLC-1 cell lines for C_{60} and CB are shown in the following graphs in this section.

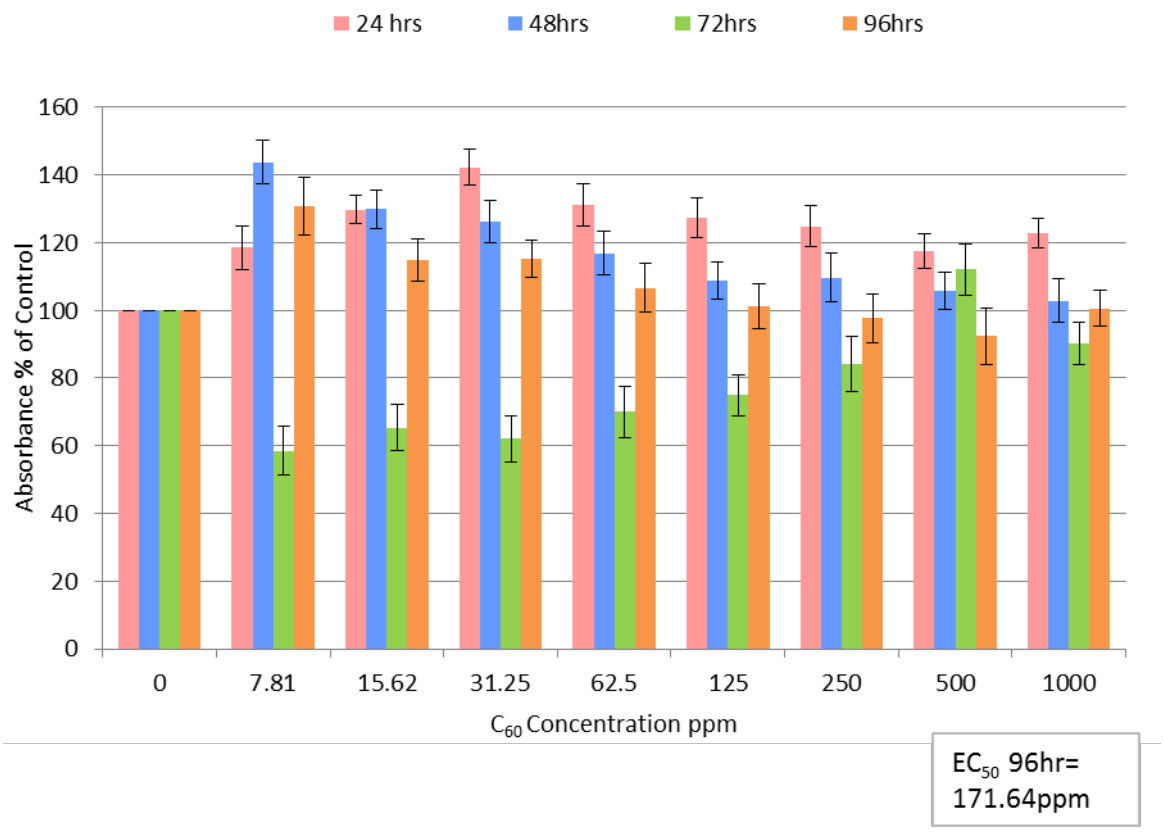


Figure 5.1: Observed cytotoxicity in RTG2 cells following 24 to 96 hour exposure to C60 as determined by the NR assay. Data is expressed as a percentage of an unexposed control ± the standard deviation of the three experiments. Single Factor ANOVA was run for this data giving a p value of 1.410394.

As can be seen in figure 5.1, exposure to C_{60} did appear to induce an associated cytotoxicological affect after 72 hours exposure and in certain 96 hour concentration points. An EC_{50} value of 171.64ppm was calculated for the 96 hour exposure replicates. However, this assay gave large errors with the associated toxicity. Furthermore, there appears to be an increase in viability, (or negative toxicity), predominately in the 24 and 48 hour exposures. Casey et al. 2007 have reported similar effects being recorded when using NR and other colorimetric assays in the presence of carbonaceous NPs.

These effects were determined to be absorptive interferences between the assays dye and the test species, specifically when assessing single walled carbon nanotubes. A similar effect may be occurring here with C_{60}. Figure 5.2 shows the results obtained from carrying out three independent studies to assess the effect of CB on RTG-2 cells using the NR assay. On examining the graph, it is clear that CB causes toxicity to RTG-2 cells, as there is a dose response effect observed. As the concentration of CB that the cells are exposed to is increased, the % conversion of resazurin to resorufin is seen to decrease when comparing to the unexposed control and the effect is also seen to decrease over time, giving an EC_{50} of 129.82ppm after 96 hours exposure.

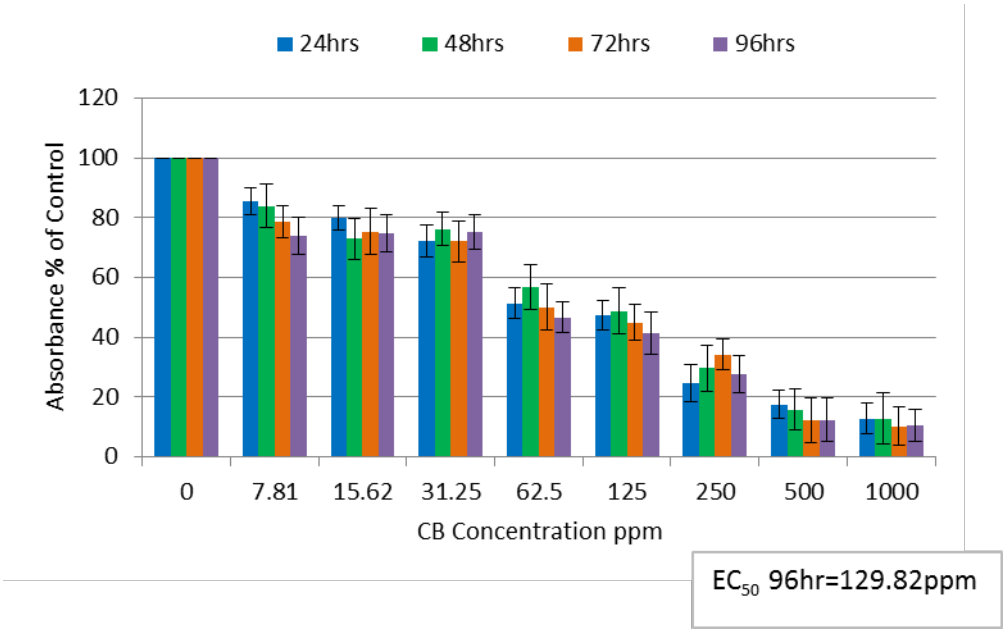


Figure 5.2: Observed cytotoxicity in RTG2 cells following 24 to 96 hour exposure to CB as determined by the NR assay. Data is expressed as a percentage of an unexposed control ± the standard deviation of the three experiments. Single Factor ANOVA was run for this data giving a p value of 0.993851.

Next, the NR assay was performed on the PLHC-1 cell line, (Figure 5.3 and Figure 5.4). As with the RTG2 cell line, an apparent increase in cellular viability was observed resulting from C_{60} exposure after 48 hours, as shown in figure 5.3, which again highlights the question of suitability for this assay for NP toxicity screening, in particular in the case of C_{60}. EC_{50} values could not be calculated for this data, as most points recorded negative toxicity responses. Results also correlate when comparing the effect of CB on the two fish cell lines during NR assessment. In both cell lines, CB is shown to cause a toxic effect increasing over time, (Figure5.4), an EC_{50} value of 61.48ppm was calculated post a 48 hour exposure period of PLHC-1 cells to CB.

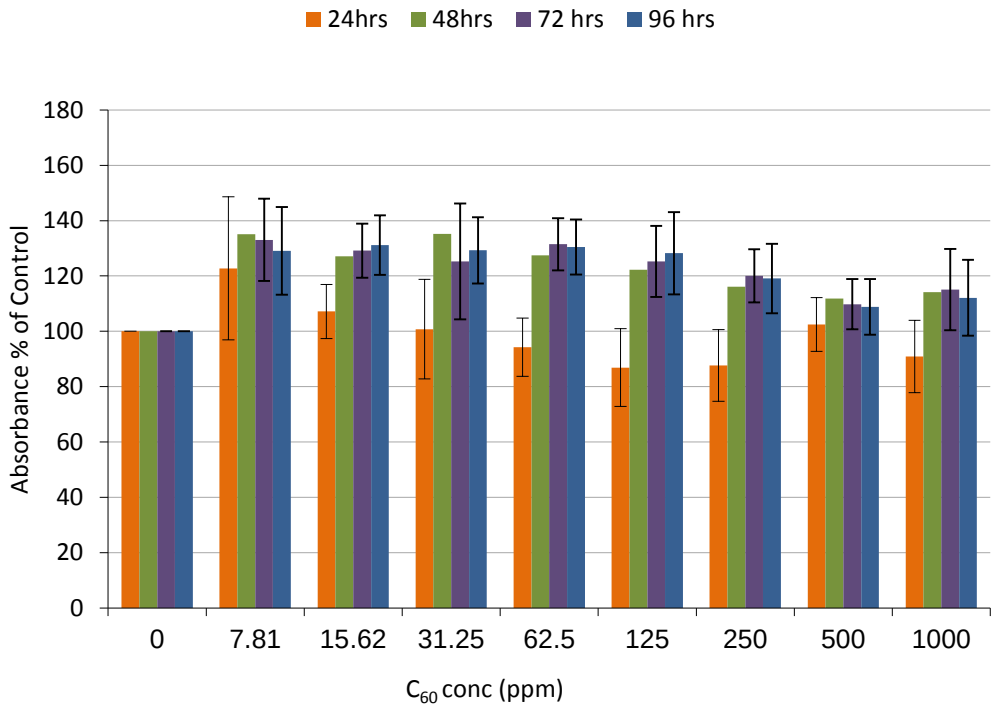


Figure 5.3 Observed cytotoxicity in PLHC-1 cells following 24 to 96 hour exposure to C60 as determined by the NR assay. Data is expressed as a percentage of an unexposed control ± the standard deviation of the three experiments. Single Factor ANOVA was run for this data giving a p value of 0.000312.

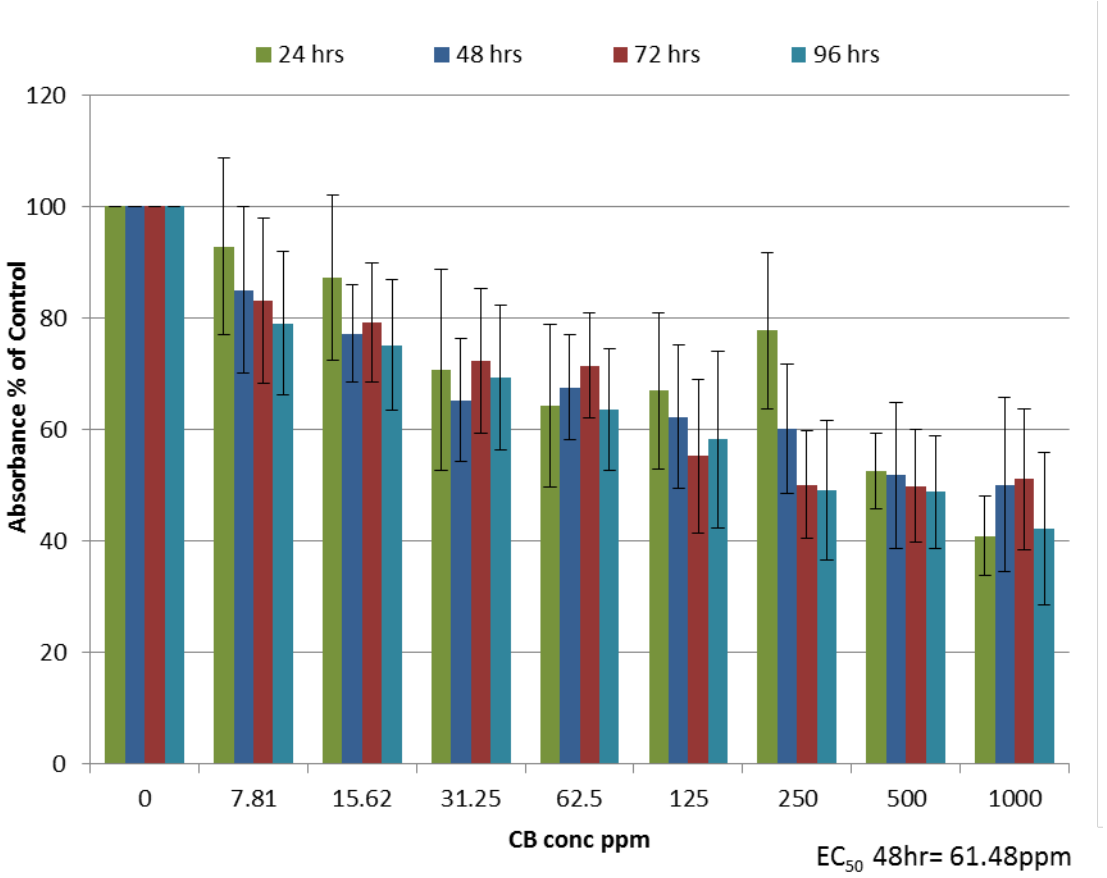


Figure 5.4 Observed cytotoxicity in PLHC-1 cells following 24 to 96 hour exposure to CB as determined by the NR assay. Data is expressed as a percentage of an unexposed control ± the standard deviation of the three experiments. Single Factor ANOVA was run for this data giving a p value of 0.844121.

5.2.2 AB Cytotoxicity Results

The second assay that was used to assess the NP cytotoxicity in the 2 fish cell lines was the AB assay. The following figures show the observed cytotoxic response to C_{60} and CB with the AB assay in the RTG-2 cells and PLHC1 calls following 24 to 96 hour exposures to a concentration range of 7.8 to 1000 ppm of each NP, (for all time points three independent experiments were performed).

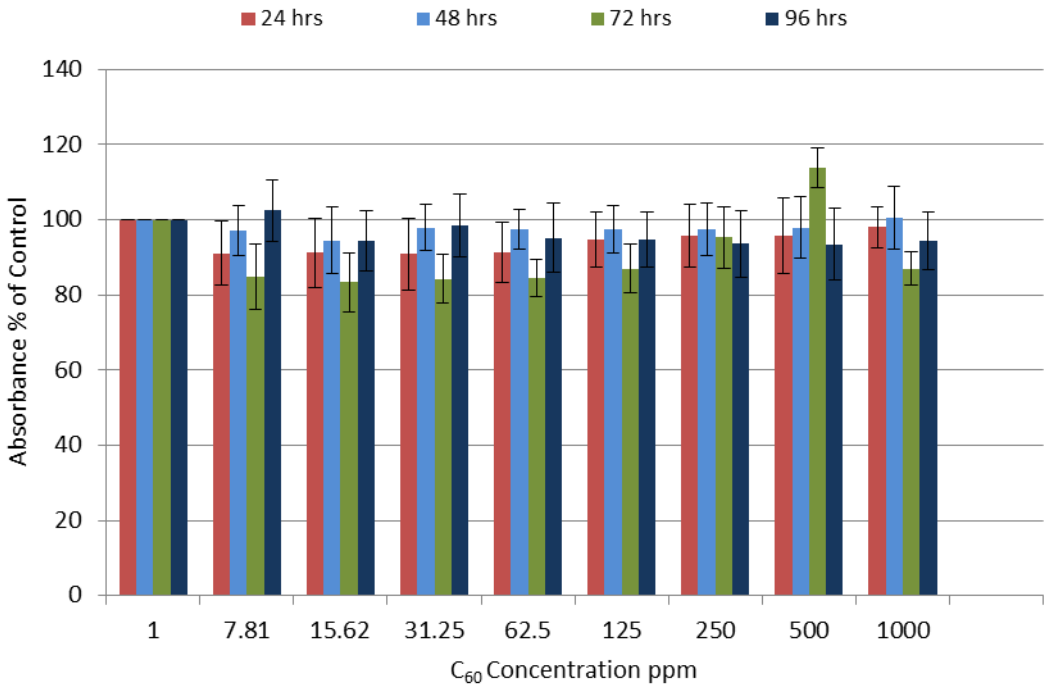


Figure 5.5: Observed cytotoxicity in RTG2 cells following 24 to 96 hour exposure to C_{60} as determined by the AB assay. Data is expressed as a percentage of an unexposed control ± the standard deviation of the three experiments, Single Factor ANOVA was ran for this data giving a p value of 0.0908.

AB is used to give an indication of the proliferation capacity of cell lines *in vitro* due to it having multiple sites of conversion within the cell, so in order for the dye to be reduced the cell must be viable, metabolically active and able to proliferate. Thus, the viability can be measured by quantatively measuring the conversion of the active component of the dye by viable cells to the product of the conversion. As can be seen in figure 5.5, there is a slight reduction in the levels of AB conversion at lower concentrations due to C_{60} exposure when compared to the unexposed controls. At the higher exposure concentrations of 250ppm and above, there is little difference observed between the exposure concentrations and that of the controls.

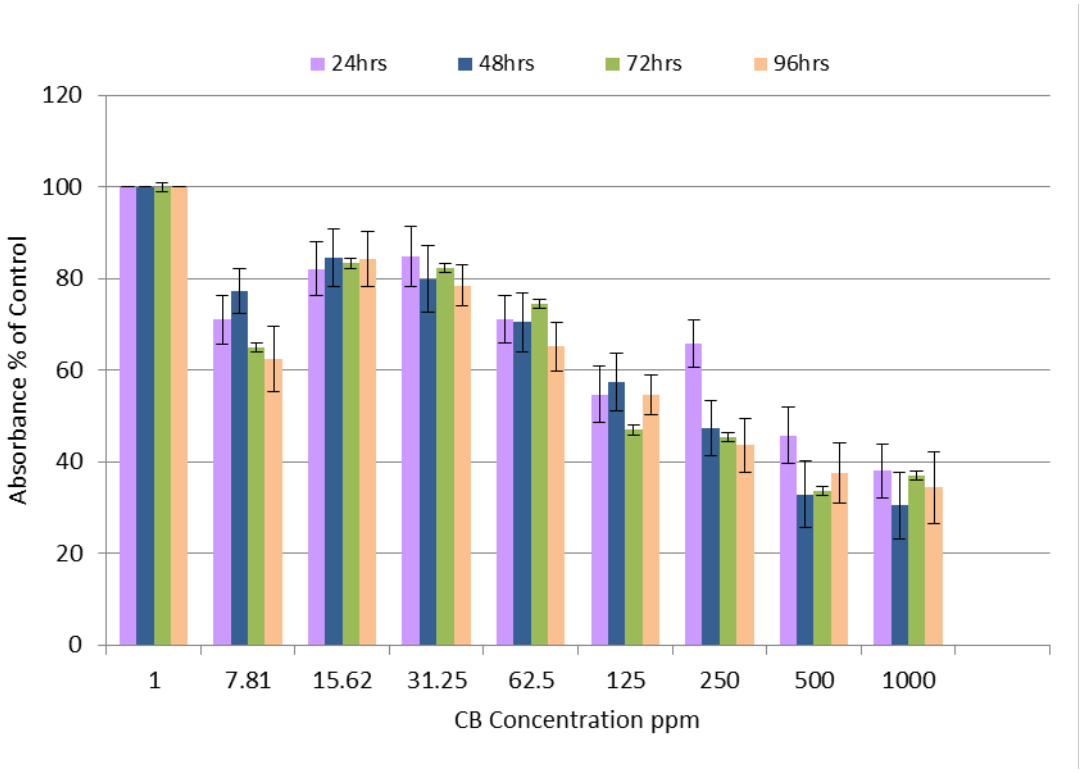


Figure 5.6: Observed cytotoxicity in RTG2 cells following 24 to 96 hour exposure to CB as determined by the AB assay. Data is expressed as a percentage of an unexposed control ± the standard deviation of the three experiments. Single Factor ANOVA was ran for this data giving a p value of 0.94713.

As such, this assay would suggest that C_{60} can elicit a small cytotoxic effect in RTG2 cells at lower concentrations. An EC_{50} value of 32.18ppm was calculated when inputting the data obtained into the Masterplex2010 program. As a concentration of >250ppm was required to elicit an effect where an EC_{50} could be determined, it is suggested that C_{60} is not toxic to RTG2 cells when using the AB assay to assess toxicity. In the case of CB toxicity assessment to RTG2 cells using AB, (figure 5.6) a clear dose response effect is recorded. The level of AB conversion is seen to be lowered in the presence of increasing concentrations of CB. There is an initial high effect, (77.18%) at concentration point 7.81ppm, which is then seen to recover at the concentration point of 15.62ppm, after which the effect is seen to gradually increase with concentration, as shown by reduction of AB conversion. From this data, it is evident that CB can elicit a toxic effect in RTG-2 cells, in concentrations as low as 7.81ppm. An EC_{50} value was calculated by for the 96 hour time point of 58.85ppm, which is quite high considering the rate of response observed. This is most likely due to the initial peak at the 7.81ppm concentration point, which highlights the importance of carrying out experiments of different concentration ranges to obtain a more accurate EC_{50} value calculated by the software program used to generate them.

In contrast to the RTG2 cell line, when the AB assay was performed on the PLHC-1 cells, there was an associated cytotoxicity resulting from a 24 hour C_{60} exposure, with cellular viability in concentrations above 62.5ppm reducing to 50% of the control, with an EC_{50} value of 29.76ppm. However, as can be seen in figure 5.7, this effect was not carried through to the 48 hour and subsequent exposures, where little or no reduction in viability was recorded at all tested concentrations. Conflicting cellular responses of this nature again could indicate the NP under test may be interfering with the endpoint being used to evaluate cellular viability.

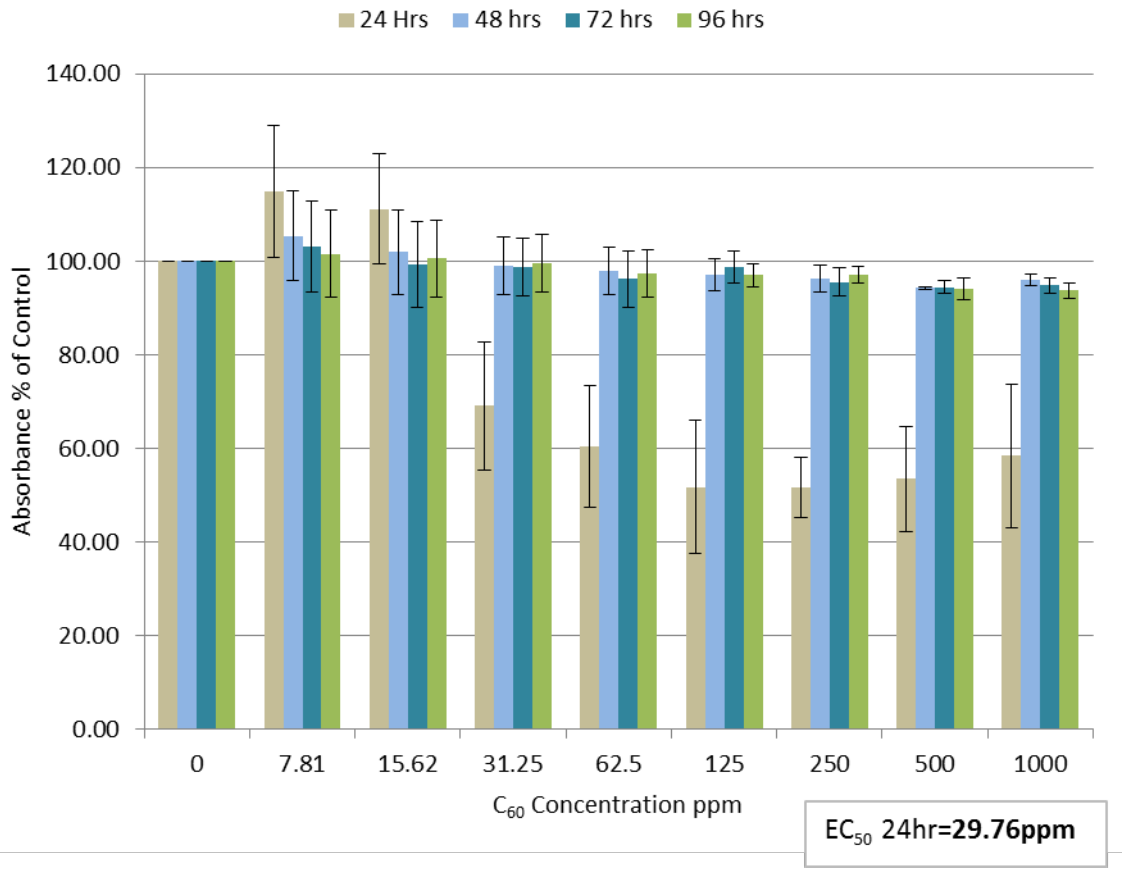


Figure 5.7: Observed cytotoxicity in PLHC-1 cells following 24 to 96 hour exposure to C_{60} as determined by the AB assay. Data is expressed as a percentage of an unexposed control ± the standard deviation of the three experiments. Single Factor ANOVA was ran for this data giving a p value of 0.379154.

The effect observed when assessing CB on PLHC-1 cells by using the AB assay shows a dose response effect over all time points, the lowest uptake of AB dye, (60% of control), being observed in the highest tested concentration set of 1000ppm and decreasing over the duration of the experiment, less than 40% of control after 96 hours exposure, (figure 5.8). An EC_{50} value of 46.99ppm was calculated after 48 hours exposure, the effect is not seen to increase significantly beyond that time point.

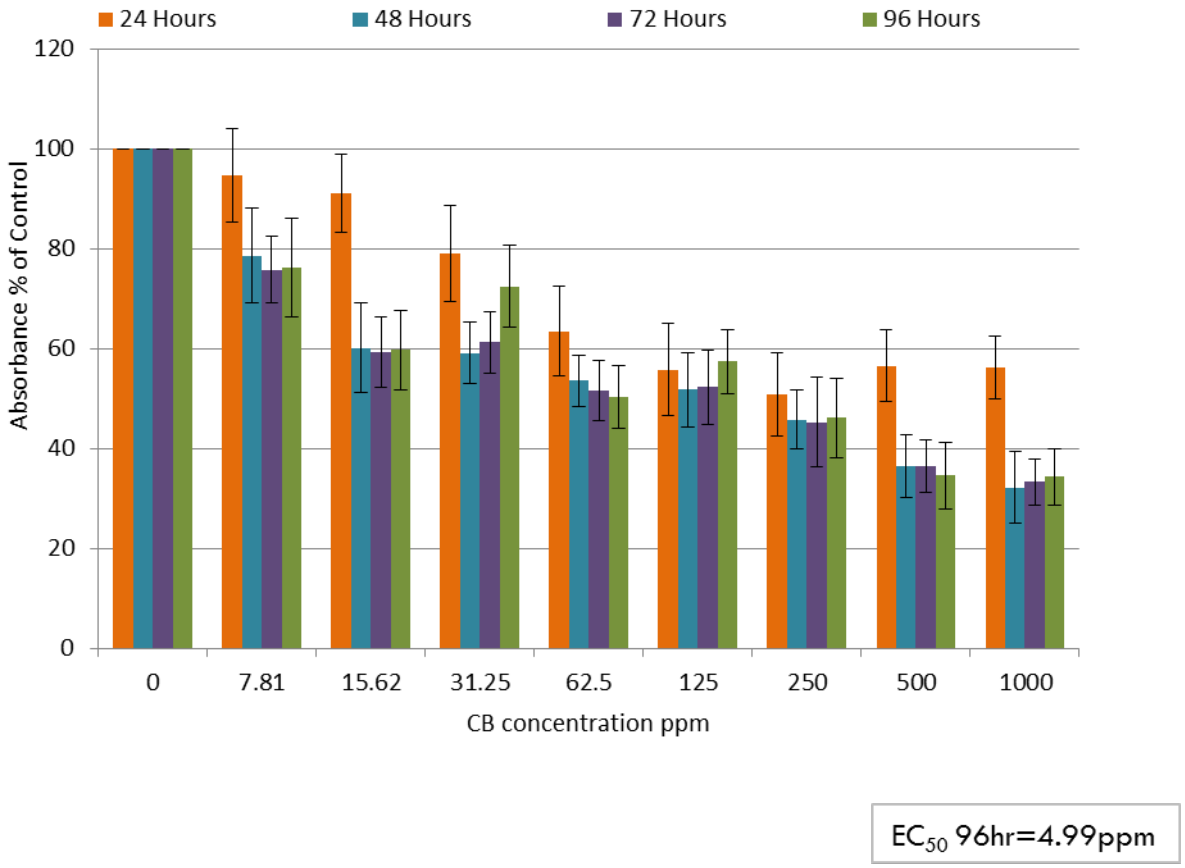


Figure 5.8: Observed cytotoxicity in PLHC-1 cells following 24 to 96 hour exposure to CB as determined by the AB assay. Data is expressed as a percentage of an unexposed control ± the standard deviation of the three experiments. Single Factor ANOVA was ran for this data giving a p value of 0.120076.

5.3 Chapter Summary

In vitro cytotoxicity testing was performed on two fish cell lines RTG2 and PHLC-1 with the aid of two cytotoxic indicator dyes namely AB and NR. Examining the results obtained for both cell lines when assessing toxicity with the NR assay suggests the results are inconclusive in the case of C_{60} showing no toxicity but would appear conclusive for CB, showing a clear toxic response. The results obtained would suggest that CB is considerably more toxic than C_{60} to both the PLHC-1 and RTG-2 cell lines with the NR assay. This is also seen when examining the results of the AB assay. No significant toxicity was observed when assessing C_{60}, or the results obtained were inconclusive. However, toxicity was recorded for CB but this was not significant. Table 5.1 summaries the results obtained in the assessment of CB in the NR and AB assays for the 2 fish cell lines employed.

Table 5.1: Results of Cytotoxicity screening of CB with the NR & AB Assays in PLHC-1 & RTG2 cells.

NR Assay		AB Assay	
CB		CB	
RTG2 cells	PLHC1 cells	RTG2 cells	PLHC1 cells
EC_{50} 96 hr 129.82ppm	EC_{50} 48hr 61.48ppm	EC_{50} 96 hr 58.85ppm	EC_{50} 48 hr 46.99ppm
p=0.947130	p=0.844120	p=0.947130	p=0.120076

When comparing the results obtained for each cell line in both assays, it suggests that the cell line and assay used may affect the results obtained, showing different sensitivities, when comparing the EC_{50} values obtained for both assays. In the RTG2 cell line for example, the EC_{50} value could be calculated after 96 hours exposure in both assays, and this is also seen for the PLHC1 cell line. An EC_{50} value is obtained after 24 hours exposure, suggesting that the results are possibly dependent on the cell diving time and not exclusively on the effect of the NP being tested. This is discussed in more detail in the following chapter, which discusses the results obtained in this study and lists future work recommendations for the ecotoxicological assessment of NPs in the hope of overcoming the obstacles that studies have encountered to date.

Chapter 6: Detailed Discussion and Future Work Recommendations

6.1 Summary of findings

The work presented aimed to assess the ecotoxicity of C_{60} and CB in the hope of gaining a greater understanding on how carbonaceous ENPs could affect the aquatic environment. Initially, a physico-chemical characterisation of the NPs was performed which involved; particle size measurements, (DLS), surface area, (AFM, and BET) and zeta potential measurements. Ecotoxicological assessment of C_{60} and CB was then carried out by employing a battery of bioassays that represent several trophic levels of the aquatic environment. Four separate species were selected; *Vibrio fischeri, Pseudokirchneriella subcapitata, Thamnocephalus platyurus*, and *Daphnia magna.* After this, two cytotoxicological assays, (NR & AB assays) using two fish cell lines; PLHC-1 and RTG-2 were carried out. The following paragraphs discuss the details of the results obtained in these tests carried out and suggest recommendations for future work in the study of the ecotoxicity of NPs in the aquatic environment. Powers et al., 2006 highlighted the importance of particle characterisation prior to toxicological assessment, stating that it allows for a better understanding of the properties of the NPs and how these properties are likely to affect biological systems.

DLS is the most commonly employed method for particle characterisation, mainly due to the fact that the measurement is carried out in aqueous media, which is of particular relevance when carrying out size measurements in toxicological assessments, as with all of the tests carried out in this study, as listed above, require suspension of the test compound in aqueous media. A higher surface area implies more of the particles surface exposed and a higher surface reactivity, the premise upon which NPs are engineered. However, this also changes the surface chemistry and surface charge, which determines how a substance will interact with other substances, for example in which solvents it will dissolve. (Royal Commission on Environmental Pollution, 2008). Surface charge also affects whether particles will remain dispersed or will aggregate and agglomerate in a specific medium. An aggregate is a cluster of particles held together by strong chemical bonds, while agglomerates are ruled by weaker forces, like hydrogen bonds or van der Waals forces and are defined as loose accumulations, (Schulze et al., 2009). As reported in chapter 3, the principle findings of the physiochemical assessments carried out during this study were that both particles were observed to form aggregates after 10 minutes, confirmed by the DLS measurements. CB produced larger aggregates, (250nm) in comparison with $C_{60,}$ (150nm) as shown in both the DLS measurements as well as the AFM measurements. In terms of the surface area measurements, CB was also determined to have a larger specific surface area, ($4.94m^2/g$) in comparison to the surface area recorded for C_{60} which was $1.13m^2/g$. This would suggest that CB would potentially be more reactive in its local environment and potentially more toxic, as it has a higher effective area available to interact with ions/proteins in its environment.

Zeta potential measurements recorded during this study indicated that C_{60} was stable in the deionized water suspension; the CB zeta potential indicated that CB did not form a stable suspension. A recent review by Kalantzi et al., 2014, discussed the techniques commonly employed in the physical characterisation of NPs and suggested the use of Nanoparticle Tracking Analysis, (NTA), to measure the scattering generated from particles undergoing Brownian motion, similar to the principle of DLS. In addition to AFM, other microscopy techniques are suggested such as high-resolution TEM, (HRTEM), where the image of the sample is constructed by measuring the interaction of the sample with the beam. Another method research groups are including in their characterisation assessments is, Asymmetric Flow FFF, (AF4), which is a variation of Flow Field-Flow Fractionation, (Flow FFF), (Kalantzi et al., 2014). In AF4, particles are separated in a cross-flow channel based on their diffusivity, where larger particles are immobilized in an expanding channel faster than the smaller ones, fractionating particles and macromolecules having diameters down to 2 nm at concentrations that range from 103 to 1010 g/mol.

Table 6.1 summarises the data obtained in the toxicological assessment studies. In the case of the four species tested; *Vibrio fischeri, Pseudokirchneriella subcapitata, Thamnocephalus platyurus*, and *Daphnia magna*, significant toxicity by C_{60} exposure was not detected. It should be noted that concentrations up to the limit of what is considered to be environmentally significant, 100pmm, as outlined in the OCED test guidelines 201 and 202, (2002), were assessed. This questions the suitability of these tests for C_{60} toxicity screening.

In comparison a study conducted by Seda et al., 2011 in assessing the toxicity of C(70)-gallic acid to *D. magna* reported an LC_{50} of 0.4 ± 0.1 mg/L. Apart from this study little or no other studies have been carried out to assess the toxicity of NPs to C_{70} or other fullerenes, this is largely due to the fact that C_{60} is the most widely used fullerene, but studies to include other fullerenes should be considered in future studies (Burger et al., 2009).The primary cause for this is the difficulty in obtaining a suspension of NPs in test aqueous medium. Other reports have highlighted this issue also and have endeavoured to overcome it by using the following methods; the suspension of the NPs in solvents, sonication and prolonged stirring. Solvents such as THF have been used as a carrier solvent, which is subsequently removed by evaporation before suspension of the NMs in water. Such a study was carried out by Oberdörster et al., 2004, who reported that C_{60} induced oxidative stress in the brains of largemouth bass exposed to 0.5 ppm after 48 hours exposure. However, there is evidence that residues of THF can remain in clusters of fullerene particles (Andrievsky et al., 2002) causing additional toxicity, ((Henry et al., 2007), (Spohn et al., 2009)).

Table 6.1: Summary of toxicity data collected.

Toxicity Test	**Concentrations (ppm)**		**Effect Calculated**	
	C60	CB	C60	CB
Microtox Acute Toxicity Test	0-833ppm	0-833ppm	EC_{20}5mins=**754.69ppm** EC_{50}15mins=**468.05ppm** EC_{50} 30mins=**467.33ppm**	EC_{50} 5mins=**73.52ppm** EC_{50} 15mins=**89.86ppm** EC_{50} 30mins=**119.03ppm**
P. subcapitata Growth Inhibition Test (OECD 201)	Limit Test 100ppm	0-1ppm	<6% Inhibition observed in three replicates	<10% Inhibition average of three replicates
Thamnotox Acute Toxicity Test	0-100ppm	0-80ppm	Test inconclusive, results not reproducible	Average EC_{50} of three tests=**23.03ppm**
D. magna acute Immobilisation Test (OECD 202)	0-1000ppm	0-120ppm	Test inconclusive, results not reproducible	Average EC_{50} of three tests=**15.11ppm**
AB Assay on RTG2 cells	0-1000ppm	0-1000ppm	EC_{50} 24hr=**32.18ppm**	EC_{50} 96hr=**29.32ppm**
AB Assay on PLHC-1 cells	0-1000ppm	0-1000ppm	EC_{50} 24hr=**30.22ppm**	EC_{50} 48hrs=**46.99ppm**
NR Assay on RTG2cells	0-1000ppm	0-1000ppm	EC_{50} 72hr= 171.64ppm	EC_{50} 96hr=**115.38ppm**
NR Assay on PLHC-1 cells	0-1000ppm	0-1000ppm	No EC_{50} value could be obtained	EC_{50} 96hr=**61.48ppm**

The other two methods of NP preparation reported are sonication for 30 minutes, (which was the method used in this study), and stirring in deionized water for an extended period of time. Sonicated fullerenes were reported to induce mortality in 48 hour exposures, with an LC_{50} 48 hour of 7.9ppm when assessing *Daphnia magna* (Lovern et al., 2006). It is important to take into consideration that there was great variation and no clear dose response recorded during this study. When the fullerenes were suspended by prolonged stirring, no significant mortality was observed within a 48 hour exposure. In contrast to what was observed for the same concentration range when the THF method was used, an LC_{50} value of 800ppb was recorded after the 48 hour exposure (Zhu et al., 2006). This was a follow up study to the Oberdörster study in 2004 using the same fish species tested, largemouth bass and C_{60} concentrations but differing in the NP preparation method used. When comparing the results obtained from these studies in assessing the toxicity of C_{60}, it is imperative to note that the NP preparation step impacted the level of toxicity measured and in the case of using solvents, may have directly increased the toxicity recorded, which was not exclusively caused by the test NP. (Hyung, Fortner et al. 2007) highlighted the possible unknown risks of the effects of insoluble C_{60} in aqueous environments by showing that, in the presence of dissolved ozone, C_{60} aggregates produced water-soluble fullerene oxide species, thus highlighting some of the possible real life scenarios when C_{60} is released into an aquatic environment.

Another such study focusing on real life risk assessment of fullerenes in the aquatic environment was carried out by (Xie, Xu et al. 2008), who performed thorough physico-chemical assessments of C_{60} in the presence of two aquatic NOM; Suwannee River humic acid and fulvic acid, which resulted in the disaggregation of the C_{60} aggregates, leading to changes in particle size and morphology and potentially the transport and fate of C_{60} in the environment. In the case of *D. magna* exposure, other studies have reported degradation and/or adsorption of the NP to the exposure vessel. Sedimentation is also an observed effect when carrying out these studies, and was observed during the course of the *D. magna* exposures presented in this study. One suggested way to overcome this issue is by using the static renewal test, wherein *D. magna* neonates are exposed to a fresh solution of the concentration of test sample at a defined time interval, either by transferring the test organisms from one test chamber to another, or by replacing all or a portion of solution in the test chambers. The organisms are often fed in these tests to keep them in a healthier state, (U.S. Environmental Protection Agency, 2002). However, the most accurate method to ensure that the concentration of a test substance is kept at the required level is the flow-through method, wherein test chambers are provided continuously with fresh solution or suspension to be tested to keep the concentrations stable. However, this requires a large volume of test solution consumption and the specific apparatus required for a flow-through system which can be expensive to set up.

The following paragraphs discuss in more detail the results obtained in the assessment of the four species listed; *Vibrio fischeri*, *Pseudokirchneriella subcapitata*, *Thamnocephalus platyurus*, and *Daphnia magna*, and also the cytotoxicological assessment. Results of these were presented in chapters 4 and 5. The majority of the published studies carried out to assess C_{60} toxicity in bacteria were carried out with a view to assessing the anti-microbial activity of C_{60}, as several NPs are used due to their anti-microbial capabilities. One such study carried out by Fang et al. in 2007 to assess the effects of C_{60} on *Bacillus subtillis* and *Pseudomonas putida*, reported a minimal inhibitory concentration, (MIC), for bacterial growth of 0.5-0.75ppm and 0.25-0.5ppm respectively. There are few publications describing the results of the effects of C_{60} on *Vibrio fischeri*, as tested in this study. One such study carried out by (Velzeboer et al., 2008) did not observe any affects in the light emitted by *Vibrio fischeri*, after 30 minutes exposure to 1ppm C_{60}. However, it is important to note that all of these studies included the use of the solvent THF, which, as discussed previously in this chapter, has been reported to interfere with test results. In the case of this study, % light inhibition readings and EC_{50} values could be obtained for C_{60} and CB; 467.33ppm and 119.33ppm respectively after 30 minutes exposure. The EC_{50} concentrations obtained are high, meaning that the toxicity measured by these NPs to the bacterial species *Vibrio fischeri* is low, which correlates with previous studies claiming low toxicity levels of NPs in bacteria, (Velzeboer et al., 2008).

For both C_{60} and CB, the algal growth inhibition test failed to produce dose response effects. This issue has also been reported previously in assessing NP and soil sample toxicity in algal cultures (Velzeboer et al., 2008). Suggestions for the unsuitability of this test to assess NP toxicity include turbid test solutions, obtained after sample preparation, such as sonication. Turbidity in the samples may affect growth by shading the algae added to the test vessels from the light source and thus indirectly inhibiting algal growth. Shading studies are widely carried out in ecology studies of plants assessing the effect of plant growth in specified degrees of shade (Blackman et al., 2012). The results recorded here are inconclusive for both NPs when using the algal growth inhibition test and suggest the test system was not suitable for the assessment of the ecotoxicity of C_{60} and CB. Turbidity of samples was observed after sample preparation in this test also, which is a potential cause of the test being inconclusive. There are presently very few published reports on the effects of fullerenes on algal cultures, possibility due to the issue highlighted. One study reported by Baun *et al*., 2008 gave results of a 30% inhibition of algal growth in 90ppm exposures. However, no dose-response relationship could be established. To overcome the fact that the turbid suspensions interfered with optical density measurements (Aruoja et al., 2009) developed a chlorophyll extraction protocol in which 200µl of ethanol was added to each 50µl algal culture sample on a 96-well plate, instead of using the culture vessels, as per OECD method 201, and as employed for this study. The plate was then shaken for 3 hours in the dark and the fluorescence was measured with a microplate fluorimeter.

The limit of this method is that the exposure time of 3 hours does not allow for cell growth, although it could provide acute toxicity results which could be used as an initial screen test, after which a more advanced plant species such as lemna minor could be tested, which is a very commonly used plant in ecotoxicology studies, (OECD Lemna Growth Inhibition test guideline 221, July 2002, Drost et al., 2007). Very few reports that have assessed NP toxicity have included *Thamnocephalus platyurus* in the battery of tests carried out, which is surprising, as *Thamnocephalus platyurus* assessment is one of the OECD recommended test invertebrate species in fresh water toxicity studies. *D. magna* is the other, which has traditionally been the species of choice. Numerous studies have been published using this species and it is still extensively being used in ecotoxicity studies due to its ease of culture, it allows for an acute study and is reported valuable in aquatic biomonitoring (Park et al., 2010). Also, due to the fact that the sequencing of the *Daphnia pulex* genome was published in 2007 (Colbourne et al., 2011), it allows for development of proteomic studies with *D. magna.* One known study that included *Thamnocephalus platyurus* was carried out by (Patra, Ma et al. 2011). Notably, however, the focus was primarily assessments of particle size change and not toxicity. The test involved exposing *Thamnocephalus platyurus* to aqueous suspensions of fullerenes, at 3mg/L and 6mg/L. Fullerene ingestion was then assessed after the exposure time of 1 hour. The recorded ingestions were burdens of 2.7+/-0.4mg/mg and 6.8+/-1.5mg/mg net weight respectively.

Thin-section TEM images of aqu/C_{60}-exposed *T. platyurus* showed the formation in the gut of fullerene agglomerates, (5–10mm) that were an order of magnitude larger than the suspended fullerene agglomerates. Upon excretion, the observed fullerene agglomerates were in the 10 to 70mm size range and settled to the bottom of the incubation wells, in contrast to the control polystyrene microspheres, which dispersed after depuration. *Thamnocephalus platyurus* assessments carried out in this study yielded inconclusive results in the case of C_{60}, most probably due to the formation of C_{60} agglomerates in the test media resulting in the C_{60} not being bioavailable to the test species. In comparison to the test described above, where samples were stirred for 100 days, samples were sonicated for 30 minutes in this study, as the real life comparisons with extended stirring methods are now being questioned. CB assessment was reproducible and did give a dose response effect when exposing *Thamnocephalus platyurus* to CB, (0-80ppm), an EC_{50} value of 23.03ppm was recorded after carrying out three independent tests. An EC_{50} of 15.11ppm (average over three tests), was observed after 48 hours exposure to a range of CB concentrations, (0-120ppm), to *D. magna,* the full details of which are provided in chapter 4. Previous studies have recorded in samples of 100ppm, 40% of the total animals could not be accounted for. That is, they were not easily distinguishable from the agglomerates of CB NPs and no LC_{50} values could be calculated (Rosenkranz, et al. 2009).

OECD Test 202, by which the present *D. magna* methods were adhered to, recommend reference testing for international ring test studies but they are not compulsory for each test or set of tests carried out. In this context, a suggestion of further study would be to include positive controls alongside particle exposures, such as SDS or cadmium chloride, as proposed by U.S. Environmental Protection Agency, (EPA, 2002). Using reference toxicants would ensure a better evaluation of the health and sensitivity of the test organisms, particularly if long term exposure, (21 days), chronic tests are being carried out. After the battery of ecotoxicological tests were carried out, two cytotoxicity assays, AB and NR were employed to assess the effect of C_{60} and CB on two fish cell lines. The C_{60} cytotoxicity studies were deemed inconclusive, as the assays yielded spurious results due to suspected absorptive interferences from the C_{60}, which has been previously reported (Casey et al., 2007). This was particularly evident in the assessment of C_{60} using the NR assay in PLHC-1 cell line. This was also observed when assessing single wall carbon nanotubes (Worle-Knirsch, et al. 2006) interference of the CNTs with reagents used in the MTT assay, and false positive effects due to that interference were the conclusions of that study. This study showed that the interaction of SWCNT's and the MTT reagent resulted in the formation of formazan crystals after the reduction of MTT, SWCNTs bind these crystals and stabilise their chemical structure and as a consequence, these crystals cannot be solubilised. These crystals were not formed with water soluble tetrazolium salts used in comparable assays such as WST-1, XTT or INT.

Both articles highlight the need for standardizing methods to ensure results are comparable. The majority of the cytotoxicity screening assays are based on fluorescence or absorbance measurements post toxicant exposure and incubation with a colorimetric indicator dye. Interactions between SWCNT and other carbon-based nanomaterials with a number of these indicator dyes, such as MTT, NR and AB, two of which were employed in this study, have been identified (Herzog et al., 2007). It is suggested that these interactions are due to physisorption of dye molecules through van der Waals forces which lead to quenched fluorescence and/or absorbance of the dyes. This is very significant as it can lead to false readings and misinterpretation of particle toxicity. Indeed the variations in observed toxicity and large error bars experienced in the *in vitro* studies of this project would suggest that these assays may not be appropriate for quantitative assessment of NPs toxicity and an alternative endpoint must be employed in the future studies. Not with standing this, there are published reports that also declare no cytotoxicity on cell cultures resulting from exposures to C_{60}, (Levi et al., 2006). An alternative method of assessing the *in vitro* toxicological effects would be to employ the Clonogenic assay. The procedure for the clonogenic assay has recently been adapted from (Puck et al., 1956) where cells are treated after plating to be used in toxicological screening of ENPs (Herzog et al., 2007).

This assay involves harvesting exponentially growing cells, seceding into 6-well microplates. Their ability to form colonies in the presence of a suspected toxicant is monitored. Resultant colony formation post exposure is carried out on a Clonogenic counting system ColCount®. As this protocol does not rely on any colormetric dyes, the absorptive interferences encountered with the AB and NR assays should be overcome. A further suggestion would be the use of other fish cell lines, other than the two employed in this study. One such study carried out by (Okamura, Watanabe et al. 2002), used the fish cell line CHSE-sp, derived from Chinook salmon; *Oncorhynchus tshawytscha* embryos in order to assess the toxicity of marine antifouling compounds; copper pyrithione, diuron, Irgarol 1051, KH101, Sea-Nine 211, and zinc pyrithione. The *in vitro* acute toxicity (24 hour EC_{50}) of the six compounds to these fish cells was evaluated using the AB assay to determine cell viability. The suspension-cultured fish cells were found by the authors to be suitable for the screening of such chemicals using the AB assay. To confirm this, EC_{50} values were correlated with the results of *in vivo* chronic toxicities, (28-day LC_{50}) to juvenile rainbow trout, *Oncorhynchus mykiss.* Mori et al., 2000, reported the use of suspension fish cell lines to avoid the issue of slow growth rates of adherent fish cells that are commonly employed in ecotoxicological studies (Mori, *et al*., 2000). This is a further suggestion in future cytotoxicological assessment of NPs. In the case of CB, cytotoxicological testing seems relevant as from the results obtained in this study it was possible to assess CB toxicity on the 2 cell lines employed in both viability assays. Dose-response relationships were recorded and EC_{50} calculations were obtained with the resultant data.

From analysing the results obtained from the characterisation studies and toxicity studies carried out on the two carboneous NPs that were the focus of this study, namely C_{60} and CB, it is evident that any ecotoxicological study attempting to assess the risk of NP release and effect in the aquatic environment must include both particle characterisation and toxicological assessments. The examination of the results gathered for each toxicity test should include particle solubility and size considerations. However examining the results collated in this study suggests CB is significantly more toxic to aquatic environments than C_{60}. As discussed above and as widely reported, in relation to the tests carried out for C_{60}, sample preparation remains challenging in order to produce reliable, reproducible results in the ecotoxicological assessment of C_{60}. One of the current research areas in this field is that of the provision of reference Nano materials, similar to those used in chemical toxicity studies, to be used in conjunction with test NPs. However, due to the aforementioned reasons, this is currently proving difficult. One possible suggestion for use as a NP reference material was CB (Stone et al., 2010), and indeed in the assessments carried out here, it is reasonable to suggest that CB is a suitable reference material as results were obtained for all but one, of the toxicity assessments, and were reproducible. Other authors make the following recommendations for future toxicity studies in the aquatic environment; the development of standardized short-term (lethality) invertebrate tests, which would allow for testing mechanisms for ENP toxicity and uptake, such as the uptake and depuration of gold NPs in *D. magna* described by Skjolding *et a*l., 2014.

Several studies have found that *D. magna* filter the ENPs from the water and the digestion tract becomes completely filled with NPs, visualised by microscopy, such studies include the electron microscopy of iron oxide NPs in *D. magna* carried out by (Kwon, Nho et al. 2014), who showed the digestive tract filled with the iron oxide NPs using electron microscopy. At the same time, aggregates of ENPs adhere to the outer surfaces of the animals, which has been less reported and is an important factor in mobility being affected (Baun et al., 2008). OCED test 202 is a standardised test for *D. magna* assessment of chemicals. The nanotoxicology research community sees a requirement for a specific test that addresses the issues that have occurred to date in the assessment of NPs in *D. magna* toxicity as well as other traditional ecotoxicological tests. Baun et al., 2008 also identified the requirement for long-term low exposure invertebrate tests focused on chronic endpoints. Very few studies have carried out these tests. As discussed above, aggregation, chemical speciation and composition of NPs change in water, due to preparation methods, and test media should be considered. As the bioavailability of NPs is expected to be related to speciation and aggregation state, it is not straight forward to predict whether increased concentrations of NPs in aqueous suspensions will lead to higher organism exposure. A chronic 21 day exposure test exists for *D. magna.* However, the same issues that occur in the acute 48 hour exposure test would need to be addressed here also to provide an accurate reliable chronic low exposure test. Very few bioaccumulation studies of NPs are currently available.

These are the corner stone of traditional ecotoxicological assessments of whole food webs such as an aquatic habitat. The fact that the current NPs are predicted to persist in the environment and the feeding traits and habits of many invertebrates such as filter feeders will remain unchanged, calls for research on the bioaccumulation behaviour of NPs in invertebrates are warranted. In addition, the use of sediment tests should be emphasized. Sediments are identified as a main sink of NPs in the aquatic environment due to the expected aggregation upon discharge. In conclusion, this study was carried out to assess the aquatic toxicity of C_{60} and CB by conducting tests to gain a greater understanding of the structure and behaviour of NPs in aqueous media as well as the effects of the particles in traditionally used toxicity tests to evaluate the suitability of these tests in relation to Nanotoxicology studies. Tests carried out were assessed individually and compared to relevant current literature available in this field. In the case of C_{60}, sample preparation issues point to the cause of inconclusive findings. Alternatives were suggested and would be advised for future C_{60} aquatic toxicology studies. Results obtained for the assessment of CB were more promising in the respect of producing repeatable, accurate NP assessments using traditional ecotoxicological tests and examination of the results presented here would suggest that CB proves toxic to the aquatic environment. In addition to the suggestions of alternative toxicological tests, a more detailed physiochemical assessment was suggested where the methods employed in this study could be broadened to include assessment of the particles in test method solutions also.

A number of additional characterisation techniques were also discussed in the view to completing a more comprehensive physiochemical profile of C_{60} and CB. It is the hope of the author that this work added to the knowledge gaps that exist currently in the toxicity of NPs in the aquatic environment and that the research scientific community responds to the challenges that are evident in this field, in the hope of successfully developing accurate risk assessments that can be used to protect the aquatic environment from the threat of NP.

Bibliography

Alexis, F., E. Pridgen, et al. (2007). “Factors affecting the clearance and biodistribution of polymeric nanoparticles. 7th International Symposium on Polymer Therapeutics”. Valencia, Spain, Amer Chemical Soc.

“Auqatic Toxicity Testing in Ireland”, Shannon Aquatic Toxicity Laboratory Annual Report 2011, funded by Enterpride Ireland, available online: http://www.envirocentre.ie/includes/documents/Aquatic%20Toxicity%20Testing %20in%20Ireland% 202011%20ver%205.pdf.

Azur Environmental MicrotoxOmni™ Software for Windows® 95/98/NT user manual, Pages 1-35, http://www.sdix.com/uploadedFiles/Content/Products/Water_Quality _Tests/MicrotoxOmni-Software-forWindows.pdf.

ATCC product sheet: PLHC-1 (ATCC® CRL-2406™): *Oeciliopsis Lucida*, topminnow.

Al-Nasiry, S., N. Geusens, et al. (2007). "The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells." Human Reproduction **22**(5): 1304-1309.

Andrievsky, G. V., V. K. Klochkov, et al. (2002). "Comparative analysis of two aqueous-colloidal solutions of C-60 fullerene with help of FTIR reflectance and UV-Vis spectroscopy." Chemical Physics Letters **364**(1-2): 8-17.

Aruoja, V., H. C. Dubourguier, et al. (2009). "Toxicity of nanoparticles of CuO, ZnO and TiO2 to microalgae *Pseudokirchneriella subcapitata*." Science of the Total Environment **407**(4): 1461-1468.

Augustin, M. A. and Y. Hemar (2009). "Nano- and micro-structured assemblies for encapsulation of food ingredients." Chem Soc Rev **38**(4): 902-912.

Barlow, P. G., K. Donaldson, et al. (2005). "Serum exposed to nanoparticle carbon black displays increased potential to induce macrophage migration." Toxicology Letters **155**(3): 397-401.

Baun, A., S. N. Sorensen, et al. (2008). "Toxicity and bioaccumulation of xenobiotic organic compounds in the presence of aqueous suspensions of aggregates of nano-C-60." Aquatic Toxicology **86**(3): 379-387.

Bols, N. C. and L. E. J. Lee (1991). "Technology and Uses of Cell-Cultures from the Tissues and Organs of Bony Fish." Cytotechnology **6**(3): 163-187.

Bouwmeester, H., I. Lynch, et al. (2011). "Minimal analytical characterization of engineered nanomaterials needed for hazard assessment in biological matrices." Nanotoxicology **5**(1): 1-11.

Brant, J., H. Lecoanet, et al. (2005). "Aggregation and deposition characteristics of fullerene nanoparticles in aqueous systems." Journal of Nanoparticle Research **7**(4-5): 545-553.

Canesi, L., C. Ciacci, et al. (2010). "In vitro effects of suspensions of selected nanoparticles (C60 fullerene, TiO2, SiO2) on Mytilus hemocytes." Aquatic Toxicology **96**(2): 151-158.

Casey, A., M. Davoren, et al. (2007). "Probing the interaction of single walled carbon nanotubes within cell culture medium as a precursor to toxicity testing." Carbon **45**(1): 34-40.

Cataldo, F. (2010). "Interaction of C-60 fullerene with lipids." Chemistry and Physics of Lipids **163**(6): 524-529.

Chaudhry, Q., M. Scotter, et al. (2008). "Applications and implications of nanotechnologies for the food sector." Food Addit Contam Part A Chem Anal Control Expo Risk Assess **25**(3): 241-258.

Cheng, J. P., E. Flahaut, et al. (2007). "Effect of carbon nanotubes on developing zebrafish (Danio rerio) embryos." Environmental Toxicology and Chemistry **26**(4): 708-716.
Cheng, X, Jun Zhong, Jie Meng, Man Yang, Fumin Jia, Zhen Xu, Hua Kong, Haiyan Xu. (2011). "Characterization of Multiwalled Carbon Nanotubes Dispersing in Water and Association with Biological Effects" Journal of Nanomaterials.
Cleveland, J. P., "Theory of phase imaging". Appl. Phys. Lett. 1998, **72,** 2613-2615.
Colbourne, J. K., M. E. Pfrender, et al. (2011). "The Ecoresponsive Genome of Daphnia pulex." Science **331**(6017): 555-561.
Costa, F. R., M. Saphiannikova, et al. (2008). "Layered double hydroxide based polymer nanocomposites." Wax Crystal Control: Nanocomposites, Stimuli-Responsive Polymers **210**: 101-168.
Cronin, M. T. D. and T. W. Schultz (2003). "Pitfalls in QSAR." Journal of Molecular Structure-Theochem **622**(1-2): 39-51.
Daughton CG. Non-regulated water contaminants: emerging research. Environ Impact Asses Rev 2004;**24:**711–32.
Day, R., Quinn, G. (1989) "Compasisons of treatments after an analysis of variance in Ecology". Ecological Monographs Vol 54 (4) 433-463.
Fang, S. C., A. J. Mehta, et al. (2012). "Residential Black Carbon Exposure and Circulating Markers of Systemic Inflammation in Elderly Males: The Normative Aging Study." Environ Health Perspect **120**(5): 674-680.
Fortner, J. D., D. Y. Lyon, et al. (2005). "C60 in water: nanocrystal formation and microbial response." Environmental Science & Technology **39**(11): 4307-4316.
Fortner, J. D., D. Y. Lyon, et al. (2005). "C-60 in water: Nanocrystal formation and microbial response." Environmental Science & Technology **39**(11): 4307-4316.
Fubini, B., M. Ghiazza, et al. (2010). "Physico-chemical features of engineered nanoparticles relevant to their toxicity." Nanotoxicology **4**(4): 347-363.
Garric, J., B. Migeon, et al. (1990). "Lethal Effects of Draining on Brown Trout - a Predictive Model Based on Field and Laboratory Studies." Water Res **24**(1): 59-65.
Geiser, M, Kreyling, W. G. (2010). "Deposition and biokinetics of inhaled nanoparticles". Particle and Fibre Toxicology. 2010, **7:**2.
Gottschalk, F., T. Sonderer, et al. (2009). "Modeled Environmental Concentrations of Engineered Nanomaterials (TiO2, ZnO, Ag, CNT, Fullerenes) for Different Regions." Environmental Science & Technology **43**(24): 9216-9222.
Gottschalk, F., T. Sonderer, et al. (2009). "Modeled environmental concentrations of engineered nanomaterials (TiO(2), ZnO, Ag, CNT, Fullerenes) for different regions." Environmental Science & Technology **43**(24): 9216-9222.
Hackley, V. A. and J. D. Clogston (2011). "Measuring the Hydrodynamic Size of Nanoparticles in Aqueous Media Using Batch-Mode Dynamic Light Scattering." Characterization of Nanoparticles Intended for Drug Delivery **697**: 35-52.
Handy, R. D., R. Owen, et al. (2008). "The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs." Ecotoxicology **17**(5): 315-325.
He, F., Zhao, D., 2005. Preparation and characterization of a new class of starch-stabilized bimetallic nanoparticles for degradation of chlorinated hydrocarbons in water. Environ. Sci.Technol. **39**, 3314-3320.
Henry, T. B., F. M. Menn, et al. (2007). "Attributing effects of aqueous C60 nano-aggregates to tetrahydrofuran decomposition products in larval zebrafish by assessment of gene expression." Environ Health Perspect **115**(7): 1059-1065.
Herzog, E., A. Casey, et al. (2007). "A new approach to the toxicity testing of carbon-based nanomaterials - The clonogenic assay." Toxicology Letters **174**(1-3): 49-60.

Hoet, P. H. M., A. Nemmar, et al. (2004). "Health impact of nanomaterials?" Nature Biotechnology **22**(1): 19-19.
Hyung, H., J. D. Fortner, et al. (2007). "Natural organic matter stabilizes carbon nanotubes in the aqueous phase." Environmental Science & Technology **41**(1): 179-184.
Hwang, H.M., Foster, G.D. "Characterization of polycyclic aromatic hydrocarbons in urban stormwater runoff flowing into the tidal Anacostia River,Washington, DC, USA. Environmental Pollution **140** (2006) 416e426.
Isnard, P., P. Flammarion, et al. (2001). "Statistical analysis of regulatory ecotoxicity tests." Chemosphere **45**(4-5): 659-669.
Kaszuba, M., J. Corbett, et al. (2010). "High-concentration zeta potential measurements using light-scattering techniques." Philosophical Transactions of the Royal Society a-Mathematical Physical and Engineering Sciences **368**(1927): 4439-4451.
Keene, A. M., D. Peters, et al. (2012). "Tissue and cellular distribution of gold nanoparticles varies based on aggregation/agglomeration status." Nanomedicine (Lond) **7**(2): 199-209.
Kim, K. T., M. H. Jang, et al. (2010). "Effect of preparation methods on toxicity of fullerene water suspensions to Japanese medaka embryos." Science of the Total Environment **408**(22): 5606-5612.
Knauer, K., Sobek, A., Bucheli, T.D., 2007. Reduced toxicity of diuron to the freshwater green alga *Pseudokirchneriella subcapitata* in the presence of black carbon. Aquat. Toxicol. **83**, 143e148.
Köhler AR, Som C, Helland A, Gottschalk F. Studying the potential release of carbon nanotubes throughout the application life cycle. J Clean Prod. 2008;**16**:927–937.
Kreyling, W. G., J. J. Godleski, et al. (1990). "Invitro Dissolution of Uniform Cobalt Oxide Particles by Human and Canine Alveolar Macrophages." American Journal of Respiratory Cell and Molecular Biology **2**(5): 413-422.
Kroto, H. W., A. W. Allaf, et al. (1991). "C60 - Buckminsterfullerene." Chemical Reviews **91**(6): 1213-1235.
Kwon, D., H. W. Nho, et al. (2014). "X-ray and electron microscopy studies on the biodistribution and biomodification of iron oxide nanoparticles in *Daphnia magna*." Colloids Surf B Biointerfaces **122C**: 384-389.
Lam, C. W., J. T. James, et al. (2004). "Pulmonary toxicity of single-wall carbon nanotubes in mice 7 and 90 days after intratracheal instillation." Toxicological Sciences **77**(1): 126-134.
Lecoanet, H. F. and M. R. Wiesner (2004). "Velocity effects on fullerene and oxide nanoparticle deposition in porous media." Environmental Science & Technology **38**(16): 4377-4382.
Levi, N., R. R. Hantgan, et al. (2006). "C60-fullerenes: detection of intracellular photoluminescence and lack of cytotoxic effects." J Nanobiotechnology **4**: 14.
Lim, J., S. P. Yeap, et al. (2013). "Characterization of magnetic nanoparticle by dynamic light scattering." Nanoscale Res Lett **8**.
Lovern, S. B. and R. Klaper (2006). "*Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C-60) nanoparticles." Environmental Toxicology and Chemistry **25**(4): 1132-1137.
Lovern, S. B., J. R. Strickler, et al. (2007). "Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C-60, and C(60)HxC(70)Hx)." Environmental Science & Technology **41**(12): 4465-4470.
Markovic, Z. and V. Trajkovic (2008). "Biomedical potential of the reactive oxygen species generation and quenching by fullerenes (C60)." Biomaterials **29**(26): 3561-3573.

McCunney, R. J., H. J. Muranko, et al. (2001). Carbon Black. Patty's Toxicology, John Wiley & Sons, Inc.
Metanawin, T., T. Tang, et al. (2011). "Cytotoxicity and photocytotoxicity of structure-defined water-soluble C60/micelle supramolecular nanoparticles." Nanotechnology **22**(23): 235604.
Monopoli, M. P., F. B. Bombelli, et al. (2011). "NANOBIOTECHNOLOGY Nanoparticle coronas take shape." Nat Nanotechnol **6**(1): 11-12.
Moore, M. N. (2006). Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environment H:9 International , **32**, 967-976.
Mueller, N. C. and B. Nowack (2008). "Exposure modeling of engineered nanoparticles in the environment." Environmental Science & Technology **42**(12): 4447-4453.
Mwangi, J. N., N. Wang, et al. (2012). "Toxicity of carbon nanotubes to freshwater aquatic invertebrates." Environmental Toxicology and Chemistry **31**(8): 1823-1830.
Mytych J., Maciej, W. "Nanoparticle Technology as a Double-Edged Sword: Cytotoxic, Genotoxic and Epigenetic Effects on Living Cells". Journal of Biomaterials and Nanobiotechnology, 2013, **4**, 53-63.
Induction of the gene encoding macrophage chemoattractant protein 1 by Orientia tsutsugamushi in human endothelial cells involves activation of transcription factor activator protein 1.
Infect. Immun.
Infect Immun 2002 Sep;70(9):4841-50
Nam-Hyuk Cho, Seung-Yong Seong, Myung-Sook Huh, Na-Hyun Kim, Myung-sik Choi, Ik-sang Kim. (2002) "Induction of the Gene Encoding Macrophage Chemoattractant Protein 1 by Orientia tsutsugamushi in Human Endothelial Cells Involves Activation of Transcription Factor Activator Protein 1". Infect Immun. 70(**9**): 4841–4850.
Navarro, E., A. Baun, et al. (2008). "Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi." Ecotoxicology **17**(5): 372-386.
Nel, A., T. Xia, et al. (2006). "Toxic potential of materials at the nanolevel." Science **311**(5761): 622-627.
Nel, A. E., L. Madler, et al. (2009). "Understanding biophysicochemical interactions at the nano-bio interface." Nat Mater **8**(7): 543-557.
Nowack, B. and T. D. Bucheli (2007). "Occurrence, behavior and effects of nanoparticles in the environment." Environmental Pollution **150**(1): 5-22.
Noy, A., D. V. Vezenov, et al. (1997). "Chemical force microscopy." Annual Review of Materials Science **27**: 381-421.
Nyholm, N. (1990). "Expression of Results from Growth-Inhibition Toxicity Tests with Algae." Arch Environ Contam Toxicol **19**(4): 518-522.
Oberdorster, E. (2004). "Manufactured nanomaterials (fullerenes, C60) induce oxidative stress in the brain of juvenile largemouth bass." Environ Health Perspect **112**(10): 1058-1062.
Oberdorster, G., E. Oberdorster, et al. (2005). "Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles." Environ Health Perspect **113**(7): 823-839.
Okamura, H., T. Watanabe, et al. (2002). "Toxicity evaluation of new antifouling compounds using suspension-cultured fish cells." Chemosphere **46**(7): 945-951.
Park, K. S., J. Tae, et al. (2010). "Characterization, in vitro cytotoxicity assessment, and in vivo visualization of multimodal, RITC-labeled, silica-coated magnetic nanoparticles for labeling human cord blood-derived mesenchymal stem cells." Nanomedicine **6**(2): 263-276.
Paterson, G., J. M. Ataria, et al. (2011). "The toxicity of titanium dioxide nanopowder to early life stages of the Japanese medaka (Oryzias latipes)." Chemosphere **82**(7): 1002-1009.

Patra, M., X. Ma, et al. (2011). "Changes in agglomeration of fullerenes during ingestion and excretion in *Thamnocephalus platyurus*." Environmental Toxicology and Chemistry **30**(4): 828-835.
Pelaz B, Charron G, Pfeiffer C, Zhao Y, de la Fuente JM, Liang XJ, Parak WJ, Del Pino P.
Pletikapic, G., V. Zutic, et al. (2012). "Atomic force microscopy characterization of silver nanoparticles interactions with marine diatom cells and extracellular polymeric substance." J Mol Recognit **25**(5): 309-317.
Powers, K. W., S. C. Brown, et al. (2006). "Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation." Toxicological Sciences **90**(2): 296-303.
Praetorius, A., M. Scheringer, et al. (2012). "Development of Environmental Fate Models for Engineered Nanoparticles-A Case Study of TiO2 Nanoparticles in the Rhine River." Environmental Science & Technology **46**(12): 6705-6713.
Raja, P. M., J. Connolley, et al. (2007). "Impact of carbon nanotube exposure, dosage and aggregation on smooth muscle cells." Toxicology Letters **169**(1): 51-63.
Rakowska, M. I., D. Kupryianchyk, et al. (2012). "In situ remediation of contaminated sediments using carbonaceous materials." Environmental Toxicology and Chemistry **31**(4): 693-704.
Rao, A., M. Schoenenberger, et al. (2007). "Characterization of nanoparticles using Atomic Force Microscopy." Proceedings of the International Conference on Nanoscience and Technology **61**: 971-976.
Rem, #233, et al. (2012). "Environmental Nanoparticles Interactions with Plants: Morphological, Physiological, and Genotoxic Aspects." Journal of Botany **2012**: 8.
Rodea-Palomares, I., Karina Boltes, Francisca Fernández-Piñas, Francisco Leganés, Eloy García-Calvo, Javier Santiago, Roberto Rosal (2011). "Physicochemical characterization and ecotoxicological assessment of CeO2 nanoparticles using two aquatic microorganisms". Toxicological Sciences, Oxford University Press. 135-145.
Rosenkranz, P., Q. Chaudhry, et al. (2009). "A comparison of nanoparticle and fine particle uptake by *Daphnia magna*." Environmental Toxicology and Chemistry **28**(10): 2142-2149.
Ruoff, R. S., R. Malhotra, et al. (1993). "Anomalous solubility behaviour of C60." Nature **362**(6416): 140-141.
Sayes, C. M., A. M. Gobin, et al. (2005). "Nano-C-60 cytotoxicity is due to lipid peroxidation." Biomaterials **26**(36): 7587-7595.
SEDA, B, PU-CHUN KE, ANDREW S. MOUNT, STEPHEN J. KLAINE. (2012). "TOXICITY OF AQUEOUS C70-GALLIC ACID SUSPENSION IN *DAPHNIA MAGNA*". Environmental Toxicology and Chemistry, Vol. **31**, No. 1, pp. 215–220.
Shih, Y. H. and C. H. Lin (2012). "Effect of particle size of titanium dioxide nanoparticle aggregates on the degradation of one azo dye." Environmental Science and Pollution Research **19**(5): 1652-1658.
Sing, K. S. W., D. H. Everett, et al. (1985). "Reporting Physisorption Data for Gas Solid Systems with Special Reference to the Determination of Surface-Area and Porosity (Recommendations 1984)." Pure and Applied Chemistry **57**(4): 603-619.
Smedes F. Sampling and partition of neutral organic contaminants in surface waters with regard to legislation, environmental quality and flux estimations. Int J Environ Anal Chem 1994;**57**:215–29.
Smith, C. W., S. H. Wender, et al. (1969). "Growth and Free Proline Content of Tobacco Callus and Hela Cells Exposed in Vitro to Rubber Dust and Carbon Black." American Industrial Hygiene Association Journal **30**(4): 402-+.

Spohn, P., C. Hirsch, et al. (2009). "C60 fullerene: a powerful antioxidant or a damaging agent? The importance of an in-depth material characterization prior to toxicity assays." Environmental Pollution **157**(4): 1134-1139.
Stone, V., B. Nowack, et al. (2010). "Nanomaterials for environmental studies: Classification, reference material issues, and strategies for physico-chemical characterisation." Science of the Total Environment **408**(7): 1745-1754.
Tang, J., Xiong, L., Wang, S., Wang, J., Liu, L., Li, J., Yuan, F., Xi, T. "Distribution,Translocation and Accumulation of Silver Nanoparticles in Rats". Journal of Nanoscience and Nanotechnology, Volume **9**, Number 8, pp. 4924-4932.
Torres, V. M., M. Posa, et al. (2011). "Solubilization of fullerene C60 in micellar solutions of different solubilizers." Colloids Surf B Biointerfaces **82**(1): 46-53.
Velzeboer, I., A. J. Hendriks, et al. (2008). "Aquatic ecotoxicity tests of some nanomaterials." Environmental Toxicology and Chemistry **27**(9): 1942-1947.
Wakuri, S., J. Izumi, et al. (1993). "Cytotoxicity Study of 32 Meic Chemicals by Colony Formation and Atp Assays." Toxicology in Vitro **7**(4): 517-521.
Wang, D. C., K. Y. Chen, et al. (2011). "AFM membrane roughness as a probe to identify oxidative stress-induced cellular apoptosis." J Biomech **44**(16): 2790-2794.
Wang, J. Y. and C. A. Quarles (2003). "Temperature dependence of the lifetime spectrum of rubber-carbon black composites." Radiation Physics and Chemistry **68**(3-4): 527-529.
Warheit, D. B., C. M. Sayes, et al. (2008). "Health effects related to nanoparticle exposures: Environmental, health and safety considerations for assessing hazards and risks." Pharmacology & Therapeutics **120**(1): 35-42.
Warheit, D. B., T. R. Webb, et al. (2006). "Pulmonary instillation studies with nanoscale TiO2 rods and dots in rats: Toxicity is not dependent upon particle size and surface area." Toxicological Sciences **91**(1): 227-236.
Weiss, J., E. A. Decker, et al. (2008). "Solid lipid nanoparticles as delivery systems for bioactive food components." Food Biophysics **3**(2): 146-154.
Worle-Knirsch, J. M., K. Pulskamp, et al. (2006). "Oops they did it again! Carbon nanotubes hoax scientists in viability assays." Nano Letters **6**(6): 1261-1268.
Wurl O, Obbard JP. A review of pollutants in the sea-surface microlayer (SML): a unique habitat for marine organisms. Mar Pollut Bull 2004;**48**:1016–30.
Xie, B., Z. Xu, et al. (2008). "Impact of natural organic matter on the physicochemical properties of aqueous C60 nanoparticles." Environmental Science & Technology **42**(8): 2853-2859.
Zhu, S., E. Oberdorster, et al. (2006). "Toxicity of an engineered nanoparticle (fullerene, C60) in two aquatic species, Daphnia and fathead minnow." Mar Environ Res **62 Suppl**: S5-9.