THE ECOTOXICOLOGY OF CARBON NANOTUBES IN THE MARINE ENVIRONMENT

By

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ABSTRACT

With their high aspect ratio, strength, light weight and electrical conductivity single-walled carbon nanotubes (SWCNTs) provide properties of great interest to industry, and, consequently, are finding use in an ever increasing number of products and applications, which will eventually lead to their appearance in the environment. The toxicity of SWCNT, however, is controversial, mainly because of the inconsistency of results, owing to a lack of understanding of the behaviour of SWCNTs in environmental media. The present study explored the ecotoxicology of engineered SWCNTs and their interacton with other contaminants as well as their potential for trophic transfer.

A series of co-contaminant experiments was performed to understand the interaction of SWCNTs with dissolved metals at low-level and non-toxic concentrations of both. The results showed that any nano-scale effects were negated by the tendency of uncoated SWCNTs to agglomerate in seawater. However, SWCNTs, in combination with natural organic matter (NOM), remained suspended for long enough to become available to filter-feeding mussels. A potentiating toxicological effect was observed, expressed as DNA strand breaks obtained using the comet assay and oxidative stress, on divalent metals afforded by negatively charged SWCNT agglomerates in seawater at concentrations as low as 5µg L^{-1}. This is supported by the observation that SWCNTs alone were only toxic at concentrations ≥100µg L^{-1} and that the SWCNT-induced DNA damage was correlated with oxidative stress only in the absence of metals

The potential for trophic transfer was assessd using the green algae (*Tetraselmis suecica*). Light microscopical observations, confirmed by SEM and Raman spectroscopy, showed that SWCNTs adhered to the external algal cell walls and TEM results suggested internaization. A direct effect of SWCNT exposure on the algae was a significant decrease in chlorophyll *a* concentrations and cell viability.

Algea fed to mussels in the presence of SWCNTs led to a signifiacntly increased pseudofaeces production, suggesting selective feeding. However, histological sections of the mussel digestive gland following exposure showed evidence of SWCNTs containing algal and toxicological tests signs of DNA dmage and oxidative stress. In conclusion, the observed SWCNT-algal interaction may facilitate trophic transfer of SWCNTs up the food chain with potential consequences for human health. If these laboratory experiments are confirmed in the natural environment, the present results will have implications for the understanding of the role of carbon nanotubes in environmental metal dynamics, toxicology, and consequently, regulatory requirements.

Dedication

This thesis is dedicated to

The memory of my beloved late father,
Ahmed Al-Shaeri, 1932-2007
His words of inspiration and encouragement
in pursuit of excellence, still linger on.

My wonderful mother, Fatimah Al-Shaeri,
with love and eternal appreciation.
Her measureless support, encouragement, and constant love
has sustained me throughout my life.

My beloved wife, Areej, whose
love and support made this theis possible.

My beloved sisters and brothers
With love and support sustained me throughout my life.

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LIST OF ABBREVIATIONS

AAS	Atomic absorption spectrometry
ANOVA	One-way analysis of variance
BHT	Butylated hydroxytoluene
BMP	Breathing mode pattern
CA	Chromosomal aberration assay
CNTs	Carbon nanotubes
CPD	critical point drying
DiOC6	Dihexyloxacarbocyanine iodide
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DSS	DNA single strand
EB	Ethidium bromide solution
EDS	Energy dispersive spectroscopy
EDTA	Ethylenediaminetetraacetic acid
ELS	Electrophoretic light scattering
ENMs	Engineered nanomaterials
ENPs	Engineered nanoparticles
FC	Flow cytomerty
HBSS	Hank's Balanced Salt Solution
LM	light microscope
LMP	Low melting Agarose
LO	Longitudinal optical
MNT	micronucleus test
MWCNTs	Multi walled carbon nanotubes
NaOH	Sodium hydroxide
NGA	Normal Gel Agarose
NMs	Nanomaterials
NPs	Nanoparticles
NSMs	Nanostructured materials
PBS	Phosphate buffered saline
Pi	Propidium iodide
QDs	Quantum dots

RMB	Radial breathing mode
RMI	Residual metals impurities
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RRS	Resonance Raman scattering
SCE	Sister-chromatid exchange assay
SCGE	Single-cell gel electrophoresis
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
SOD	Superoxide dismutase
SRNOM	Suwannee river natural organic matter
SSB	Single strand DNA breaks
SWCNTs	Single walled carbon nanotubes
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TCS	Triclosan
TEM	Transmission electron microscope
TEP	Tetraethoxypropane
TO	Transverse optical
UV	Ultraviolet

1. INTRODUCTION

The presence of nanomaterials (NMs) in the environment is not novel (Petosa *et al*, 2010). Both carbon nanotubes (CNTs) and fullerenes (C_{60}) were discovered in 10,000-year-old polar ice cores by Esquivel and Murr (2004), in natural environment, they can be produced in hydrothermal vent systems (Luther and Rickard 2005; Navarro *et al.,* 2008), and during a volcanic eruption through processes like Aitken mode nucleation, in consequence of simultaneous emission of substantive nuclei (e.g. nitric and sulfuric acids) (Aitken 1884). However, it was not until the appearance of the release of man-made NMs and NPs in the environment (Biswas and Wu, 2005; Kumar, 2006), the entry of NMs into the environment is reviewed in section 1.2.

The emission of pollution from industrial, domestic and agricultural sources is able to damage ecosystems in the marine environment (Deudero *et al.,* 2009). One of these pollutant classes of recent concern is engineered carbon nanotubes (CNTs), a new man made structure of the carbon group, first developed in 1991 (Ren *et al.,* 2010). Since 1991, the research has focused on investigating the structure and properties of engineered CNTs. The first type of engineered CNT structure to be described was that of engineered multi-walled carbon nanotubes (MWCNT) (Ren *et al.,* 2010). In 1993, Iijima and Ichihashi described a second type of engineered CNTs with smaller diameter, the single walled carbon nanotube (SWCNT), which consists of one layer of carbon atoms (grapheme) rolled into a tube, with a length of up to 1 μm and a diameter range from 0.4 to 3.0 nm (Figure 1.1).

The use of engineered nanomaterials (ENMs) and engineered nanoparticles (ENPs) in an increasing number of industrial applications is leading to the release of ENMs and ENPs into the environment. Evaluating their risk to the environment requires an understanding of their environmental fate, bioavailability and toxicity (Krug, 2008). According to Fond and Meyer (2006) in the U.S alone, 2 million people regularly work with nanometre-diameter particles during development and production, or use NMs, NPs and associated consumer products. Although the inhalation of nanomaterials and air borne particles have attracted a lot of attention, mainly on the back of a need to understand the impacts of asbestos and coal dust on occupational health, very little is known about their possible environmental exposure to terrestrial and aquatic organisms, and also much less is known about their potential environmental fate (Kumar, 2006; Krug, 2008; Fond and Meyer 2006).

For the purpose of this section, the potential risks of ENMs to the environment will be discussed. The essential connotation beyond health and environmental risk assessments are that without exposure, there is no risk (Sahu and Casciano, 2009). 500 years ago Paracelsus realized that "All things are poison and nothing is without poison, only the dose permits something not to be poisonous" (Hunter, 2008). Risk is therefore defined as the probability of adverse effect due to exposure to a hazardous chemical, physical or biological agent (Christensen *et al.,* 2003). A change or impairment of biochemical function or pathological lesions as a consequence of organism exposure to a potentially hazardous substance can affect their respective environmental performance (Sahu, 2009) and can also be used as ecotoxicological biomarkers of exposure and effect (Hartl, 2002; Hartl *et al.*, 2005; Hartl *et al*., 2010). ENMs are categorized based on their shape, surface characteristics (area and charge), chemical composition, solubility and dispersion, properties that can influence their behaviour in a given medium, in terms of bioavailability and toxicity (Warheit *et al.*, 2007), as well as their potential to interact with other substances (e.g. metals) (Sahu, 2009; Al-Shaeri *et al.,* 2013). Abundant numbers, types and shapes of ENMs are being produced, including metallic, (e.g. nickel carbide, ruthenium, gold, germanium), quantum dots (CdSe, CdTe, PbSe, Pbs, InGaAs, ZnSe) and carbon-based ENMs (e.g. fullerenes, C_{60}, C_{70}, C_{78}, C_{84} and carbon nanotubes, and their respective toxicity will need to be assessed given the anticipated increase in application (Singh and Nalwa, 2007; Stone *et al.,* 2013). Likely exposure scenarios for most bulk substances can be predicted and modelled based on their physicochemical properties, behaviour in a given medium, bioavailability, duration of exposure and exposure concentration (Henderson *et al*, 1987). However, many of these conventional modelling techniques are not applicable to ENMs, because of the often very different behaviours exhibited by the same substances at the nanoscale (Stone *et al.,* 2014).

This section, will focus on identifying, reviewing and discussing the synthesis and characterization of single-walled carbon nanotubes (SWCNT) in terms of their residual metal impurities and their physiochemical properties, such as size, shape, surface properties and composition that all influence SWCNT behaviour, i.e. dispersion and agglomeration. This section also introduces relevant characterization techniques for SWCNTs in aqueous suspension, including transmission electron microscopy (TEM), Raman spectroscopy, dynamic light scattering (DLS), Zeta potential and Atomic absorption spectrometry (AAS). The fate of ENMs and NPs and their potential entry into the aquatic environment will be discussed as well as the possibility of their up-take

by organisms, in particular, aquatic biota (Long *et al.*, 2012). Releasing NMs such as SWCNTs into the aquatic environment and their presence outside or inside aquatic organisms may lead to disturbances in the ecosystem, and therefore, may have either acute or chronic effects on aquatic organisms (Wiench *et al.*, 2009) and will be reviewed here. The importance of using animals as bioindicators for ecotoxicological monitoring is mentioned and their advantages reviewed, particularly the bioindicators used in this research to monitor the toxicity of SWCNTs, i.e. mussels (*Mytilus edilus*) and algae (*Tetraselmis suecica*). In this chapter, the potential of the trophic web to trace the uptake of toxic contaminants by aquatic organisms, the pseudofaces and algal growth inhibition by SWCNTs is also discussed. The chapter also explains the main biomarker assays (cell viability, comet assay and oxidative stress) used in this project, and describes their applications in the field of toxicology and their advantages in determining and monitoring the potential toxicity of SWCNTs on mussel DNA damage and their effect on algal growth rate. Finally, aims and objectives of this project research are clearly outlined in section 1.8.

To my knowledge, however, prior to the commencement of this study, there were no reported *in vivo* studies involving SWCNT-induced DNA damage in marine organisms. Furthermore, it has been suggested that, especially in natural media, some instances of observed toxicity may also be in part caused by other pollutants sequestered by carbon nanomaterials so that the latter may be acting as pollutant vectors into cells (Christian *et al.*, 2008).

1.1 Synthesis and characterization of SWCNTs

The definition of nanomaterials has been generally agreed upon to include any material with at least one dimension smaller than 100nm (Royal Society and Royal Academy of Engineering, 2004). According to a recommendation on the definition of nanomaterial adopted by the European Commission (2011) “"Nanomaterials" are materials whose main constituents have a dimension of between 1 and 100 billionth of a metre.”

Different manufacturers making CNTS use a variety of synthesis methods, employing different catalytic metals and carbon sources leading to raw CNTs of varying purities and length, two characteristics important in determing the behaviour of CNTs and their potential health risk following exposure (Lam *et al.*, 2006). Currently, SWCNTs made by the HiPco or laser and are sold by Aldrich chemical company Inc, and the raw CNTs made by Rice's HiPco process were observed to have loose black clumps (Figure 1.1A-

D and 1.1E-H) (Lam *et al.,* 2006). The unprocessed HiPco SWCNT product contains 3.5% - 30% iron by weight (Chiang *et al.,* 2001; Baron *et al.,* 2007). However, according to Lam *et al.,* (2006) purified and raw SWCNTs products are sold by SES research (Houston TX) contain only 20-40% and ≤75% metals. Shvedove et al (2005) observed that samples of 9.7% purified CNTs SWCNT produced by the high-pressure CO disproportionation process (HiPco) (CNI, Houston, TX) contained 0.23% iron.

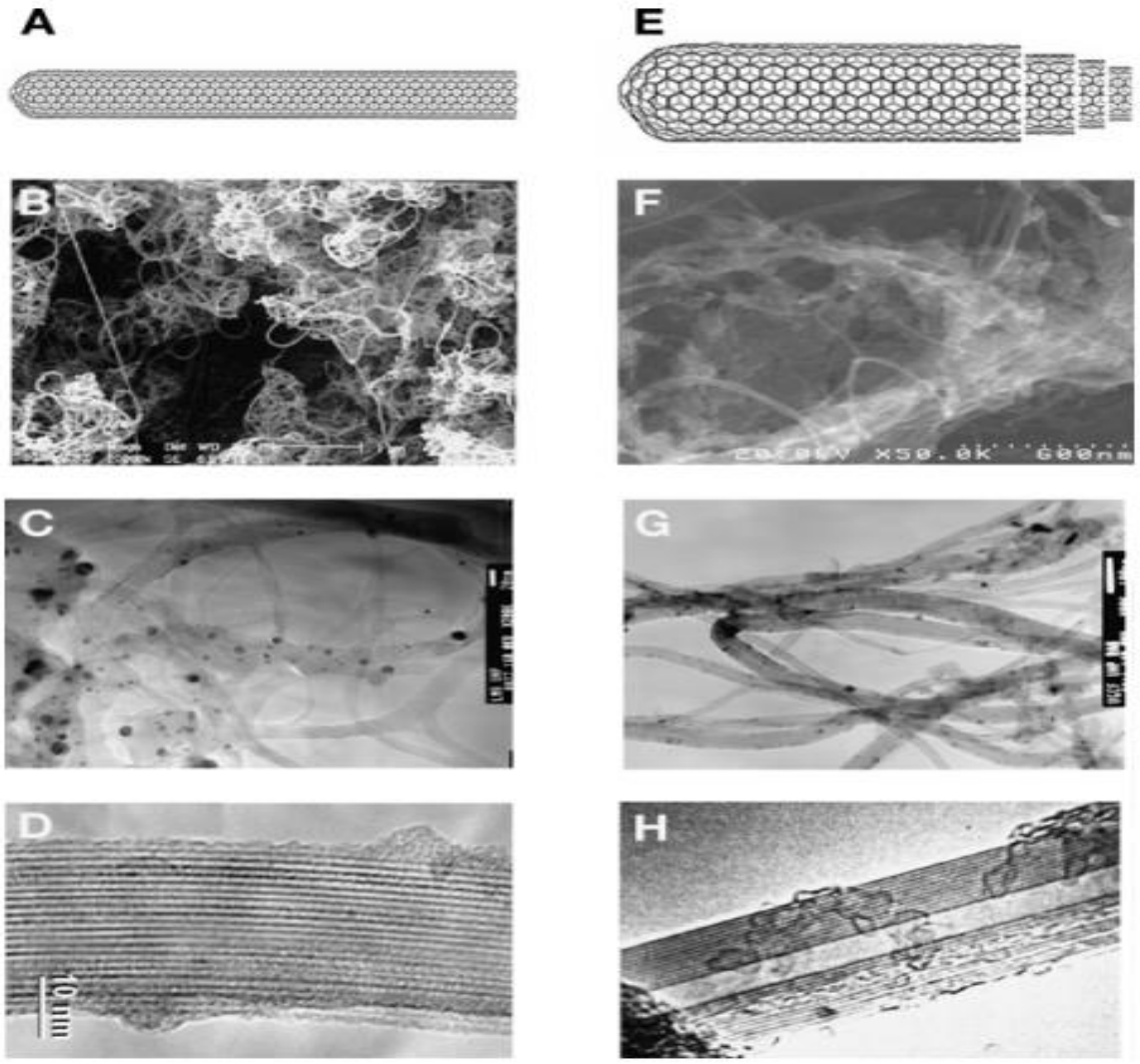

Figure 1.1 (A) to (D): SWCNTs; (E) to (H): MWCNTs. Scanning electron microscope (SEM) images show SWCNT (B) and MWCNT (F) aggregates; transmission electron microscope (TEM) images show raw SWCNT bundles (ropes) with metal nanoparticles (C), and individual multiwall tubes (G). High-resolution TEM images show a cross-section of a SWCNT bundle (D) consisting of >25 tubes and some amorphous carbon on the edges, and a longitudinal cross-section of a MWCNT (H) with an empty central cavity and ~20 walls on each side and some amorphous carbon (Nikolaev, 1999; Lam, 2006).

All SWCNT and MWCNT products contain residual metals (Arepalli *et al..,* 2004; Arepalli *et al.,* 2010). However, the metallic content in MWCNT products is much less than those in SWCNTS, which may contain up to 50% of metals by weight (Table 1.1) (Lam *et al.,* 2006; Kumar, 2006). SWCNTs look like a black powder and are sold in 'purified' form after the removal of most undesirable residual metallic impurities (RMI). SWCNTs produced using HiPco- and laser techniques have been shown to light-weight and easily transferred to the atmosphere (Baron *et al.,* 2007; Maynard *et al.,* 2004; Lam *et al.,* 2006). Although residual metals impurities (RMI) can be extracted from CNTs with nitric acid, the nitric acid produces carbonaceous impurities when it reacts with CNTs with the consequence of breaking the walls of the CNTs, the degree of damage and the amount of carbonaceous impurities are influenced by the concentration of oxidant and reflux treatment (Chin *et al.,* 2007). Residual metallic impurities (Fe, Si, Cu and Ni) have been observed in SWCNTs by Arepalli *et al.,* (2004), using a scanning electron microscope (SEM) and transmission electronic microscope (TEM), associated with energy dispersive spectroscopy (EDS) to assess the relative amounts of residual metal impurities and other non-carbon elements. The nano-scale and associated physiochemical properties have been implicated in numerous forms of toxic effects similar to asbestos (Berry *et al.,* 2005). However, not all nano-scale shapes are dangerous (Hart and Hesterberg, 1998). Nanosized particles were observed *in vivo* and *in vitro* to inhibit phagocytosis in rat when compared to non-nanosized particles (Renwick *et al.,* 2001). Rats were exposed to nanoparticles sized between 50nm-100nm, 4% to 7% were found in the spleen, liver, blood and bone marrow. However, particles larger than 100nm were not observed to reach the bone marrow, and particles larger than 300nm were not observed in the blood (Jani *et al.,* 1989; Kumar, 2006). It suggests that, the size of NPs plays a significant role in the up take process.

Although toxicologists are familiar with evaluating toxicity in both biological and chemical aspects, the study of (NMs) poses unique challenges, because nanomaterial size, shape, surface properties, composition and concentration, which affect dispersion and agglomeration are poorly understood at present, and may influence their toxicity (Sahu and Casciano, 2009). In Figure 1.2, some of the key physical properties of nanomaterials that may influence their behaviour in aqueous media are shown; particularly relevant here are surface charge and agglomeration.

Table 1.1 Synthetic process of SWCNTs include metals content (Kumar, 2006)

Test materials and characteristics	Manufacturer	Synthetic process	Metals content (%)
SWCNTs	Rice University, Houston, TX	Laser	Ni: 10
SWCNTs	Rice University, Houston, TX	HiPco	Fe: 29.9 Mo: 095 Ni: 0.8
SWCNTs purified	Rice University, Houston, TX	HiPco	Fe: 2.1
SWCNTs	CarboLex Inc., Lexington,KY	Electric arc	Ni: 26.0 Y:5.0 Fe: 0.5
SWCNTs purified	Carbon Nanotechnologies, Inc. Houston, TX	HiPco	Fe: 0.23
SWCNTs	Facultés University Notre-Dam de la paix in Namur, Belgium	CVD	Co: 0.95 Fe: ~1

In the current absence of insight into the effect of NMs on biological activity and ecosystems, the general guidance given is that characterization of NMs should be as complete as practicable, preferably under exposure conditions, as there is a strong likelihood that biological activity will depend on their physicochemical parameters (Oberdörster, 2005a; Thomas and Karluss, 2005;).

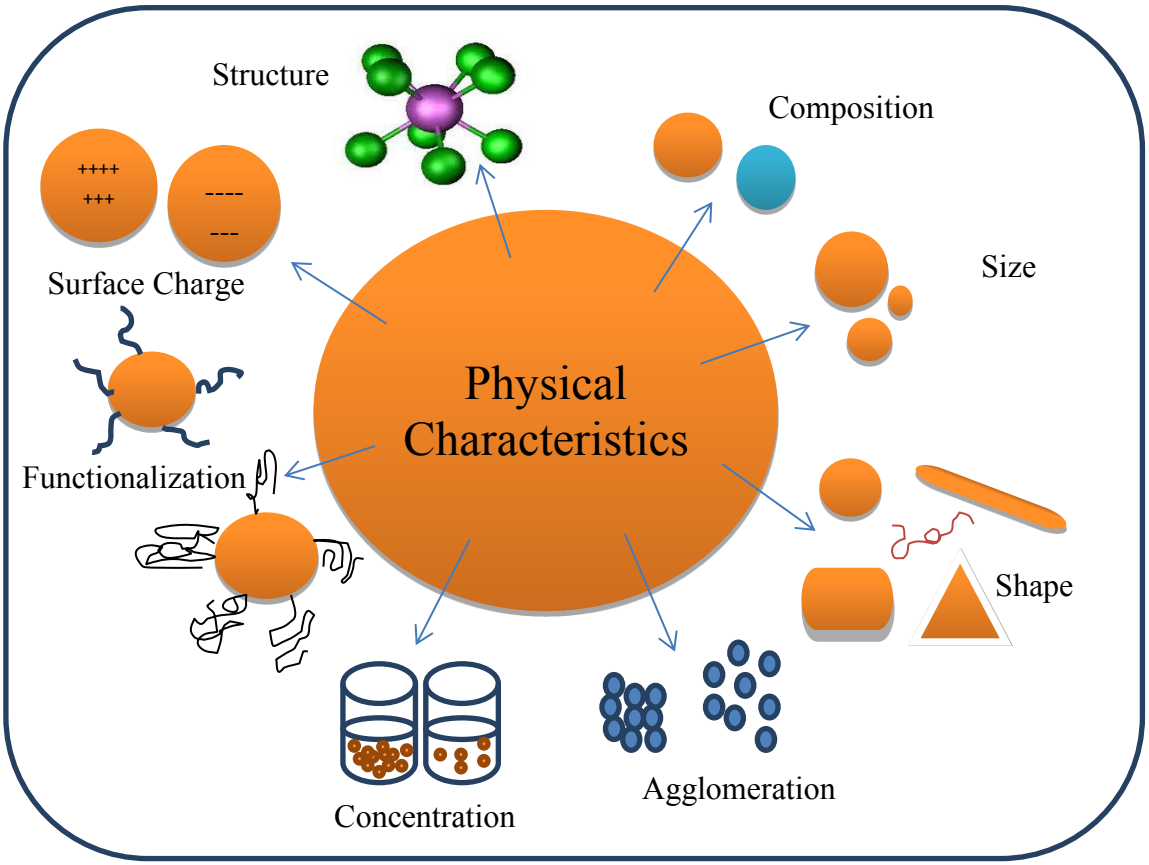

Figure 1.2 Key physical properties of nanomaterials.

Sahu (2009) lists parameter properties that have been pointed out and cited as essential or important for the characterization of NMs (Table 1.2). The understanding of the fundamental characterization of SWCNTs is considered essential to evaluate their potential toxicity and behaviour and how they may interact with other substances (e.g. metals) in biological systems.

Table 1.2 Important properties in material characterization for toxicity studies**.**

Property	**Importance for toxicity testing**	**Comments**
Particle size distribution	Essential	
Degree/state of agglomeration	Important	
Particle shape/shape distribution	Important	
Chemical composition/purity	Essential	
Solubility	Essential (where applicable)	
Surface properties		
Specific surface area/porosity	Essential	Surface roughness may be important
Surface chemistry/reactivity	Essential	In some cases may be the mechanism of toxicity (e.g. complement)
Surface adsorbed species	Important	
Surface charge/Zeta potential	Important (essential under aqueous conditions)	Especially in aqueous biological environment, may change according to the environment
Physical properties	Important	
Density	If applicable	
Crystallinity	If applicable	
Microstructure	If applicable	
Optical and electronic properties	If applicable	
Bulk powder properties	If applicable	May be important for dosimetery/exposure
Concentration	Essential	Can be measured as mass, surface area, or number concentrations.

1.1.1 Surface Charge

Besides particle size, the surface charge plays a dominant role in influencing the stabilization of particle dispersions in aqueous media. The charge and adsorption processes of submerged surfaces can be modified by natural media and are particularly pH-dependent, which plays a significant role in the behaviour of colloidal and particulate material (Krug, 2008). In the environment it has been observed that uncoated nanomaterials tend to form aggregates/agglomerates which may be eliminated or trapped and precipitated out of suspension (Farrē *et al..,* 2009). According to Lu and Chiu. (2006), the charge of SWCNTs becomes more negative with increasing pH, causing electrostatic attractions which make SWCNTs attract more anions (e.g. Zn^{+2}) from aqueous solution. Furthermore, Atomic Absorption Spectrometry (AAS) analysis showed that the observed removal of Zn^{+2} remained constant. Qiao and Aluru (2003) reported that CNTs with a negative surface charge, as can be observed at pH8, attract metal anions by the wall of the CNT. This suggests that SWCNTs may adsorb heavy metals and other charged contaminants and may increase toxicity to the point that these substances become more toxic than the ambient metal concentration alone. Chin *et al.* (2007) observed that, at pH3, the electrostatic repulsion between SWCNTs was limited and their surface charge was negative (-14.2 mV); however, highly negatively charged SWCNTs (~ -50 mV) were observed in water at pH 5 to 7.

The surface charge of NMs can be estimated through the measurement of the zeta potential that refers to charge density at the shear plane, which divides the fluid envelope associated with the NMs from the solution phase *(*Adamson and Reynolds, 1997; Kumar, 2006; Sahu and Casciano, 2009).

SWCNTs have unique electrical properties as well as physicochemical characteristics, because of their large micropore volume and high surface area, SWCNTs have been also considered to be perfect adsorbents (Chin *et al.,* 2007). The chemical interaction between the functional groups of CNT and metals ion has been reported as the major sorption mechanisms (Figure 1.3) (Rao *et al.,* 2007). Li *et al.* (2003) and Lu and Chiu. (2006) have shown that the removal of Cd^{+2} ions from water by SWCNTs are higher than those by activated carbons, and pH value (pH5.5-12) plays the main role in CNTs adsorbtion of Cd^{+2} and Zn^{+2}.

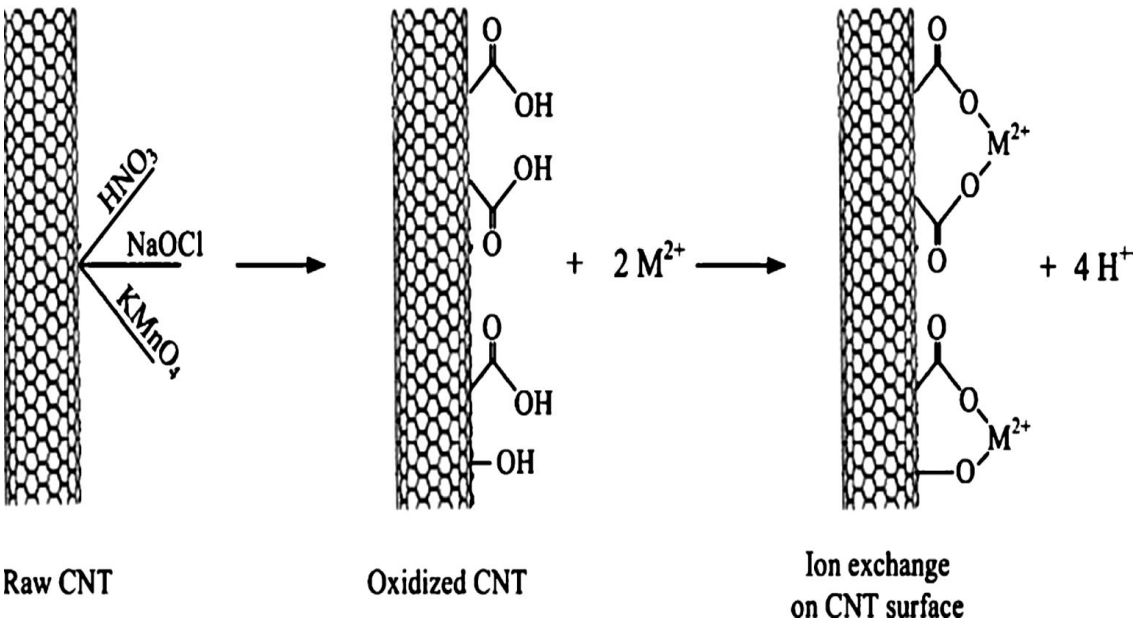

Figure 1.3 Schematic diagram of the major mechanism for sorption of divalent metal ions onto CNT surface (Rao *et al.,* 2007).

1.1.2 Raman spectroscopy

Raman spectroscopy has been successfully used for the detection and identification of SWCNTs, that both theoretically and experimentally, emit characteristic Raman spectra (Bandow *et al.,* 1998; Popov *et al.,* 2000). The first characteristic signature of SWCNTs are G-bands, arising from the optical vibration of two adjacent carbon atoms on the wall of the SWCNTs, which have a strong G^+ peak and a weaker G^- peak. The strong G^+ peak is identified with the longitudinal optical mode (LO) and correlated with vibrations along the nanotube, while the weak G^- peak appears at a lower frequency, identified with the transverse optical mode (TO) and is correlated with vibrations in the circumferential direction. The admixture of a small amount of a low frequency phonon mode normal to the graphene sheet is based on the relative down-shift ($\omega = \Omega$) between ω_G and ω_{G+} (Iliev *et al.*, 2000).

For metallic tubes, the G^+ mode is associated with the LO mode and the TO mode which fall at a lower frequency because of the strong electron phonon interaction (Baughman *et al.,* 1999; Bandow *et al.*, 1985; Dresselhaus *et al.*, 2007). The intensity of the Raman spectrum has been established as a high frequency (1500-1600 cm^{-1}), while the low frequency is between 140-250 cm^{-1}, and the zone centre in-phase radial breathing mode (RMB) is inversely proportional to the radius of the tube and is independent (Iliev *et al.*, 2000; Fujimori *et al.*, 2013) (Figure 1.4).

The second characteristic signature of a SWCNT is the appearance of a strong Raman feature at relatively low frequencies, correlated with RBM, where all the carbon atoms are vibrating in the radial direction in an asymmetry, totally symmetric, breathing mode pattern (BMP), unique to SWCNTs. According to Yowell *et al.* (2002) the frequency vibration ω_{RBM} has been identified with $1/d_t$ dm, thus supplying a characterization method for identifying the diameter distribution of SWCNTs. The strong dependence of the two characteristic Raman features on laser excitation energy E_{laser} is interpreted in terms of a resonance Raman scattering (RRS) process discovered earlier by two Brazilian scientists (Leite and Porto) when they were working at Bell Labs in the United states (Nikolaev *et al.*, 1999).

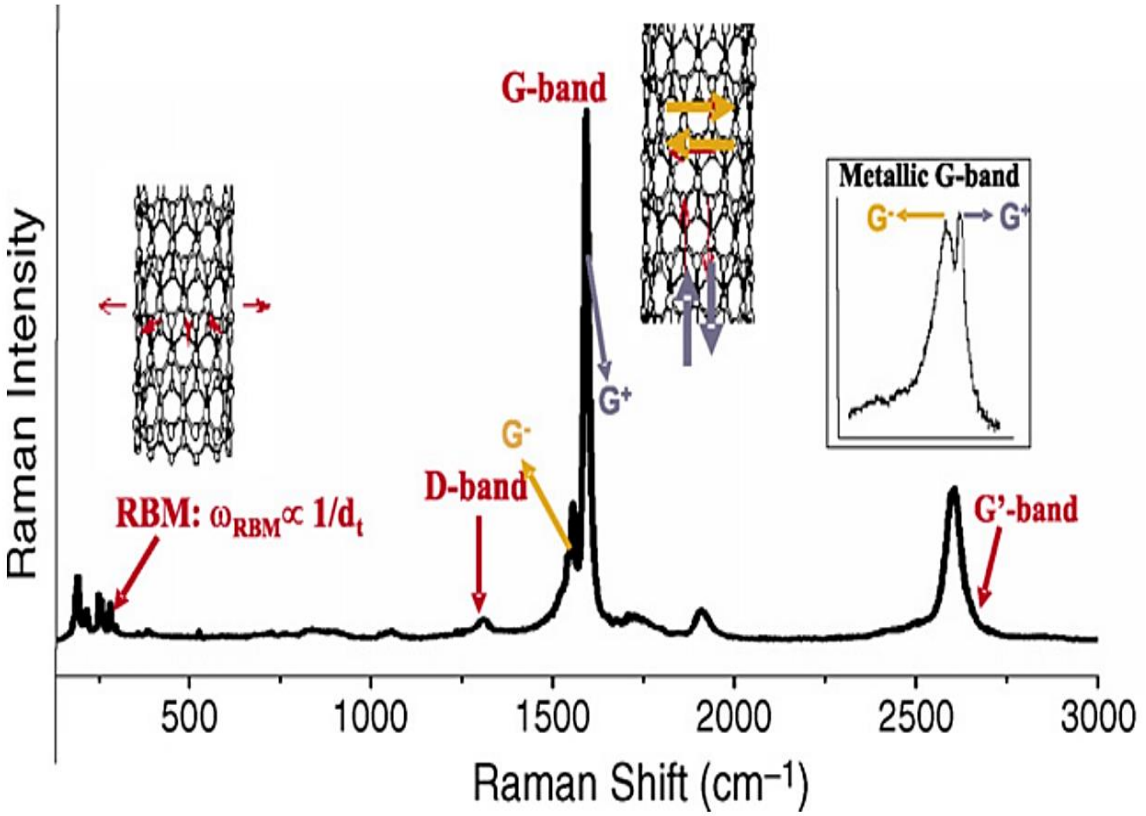

Figure 1.4 Raman spectra from a bundle of SWCNT samples, containing both SWCNTs and MWCNTs, showing the radial breathing mode (RBM, left inset), the G band for SWNTs in resonance (centre inset), and the G band line shape for SWCNTs in resonance with E_{laser} (right inset). Also featured is the G′ band which is the second order peak of the D band, but by symmetry, the G′ band is Raman allowed and is not defect-induced (Dresselhaus et *al.*, 2007).

1.1.3 Dynamic Light scattering (DLS)

In this part of the thesis, the accurate assessment of SWCNTs toxicity needs to be defined through measuring the DLS for SWCNTs agglomeration size. Dynamic light scattering (DLS) is used to measure the length distribution of nanotube in solution, whereas electrophoretic light scattering (ELS) generates the zeta potential, which gives data for the degree of dispersion (Sano *et al.,* 2001; Gao *et al.,* 2003; Lee *et al.,* 2005). Powers *et al*. (2006) reviewed the different methods of nanomaterial characterization and suggested DLS as a useful technique to assess particle size, size distribution, and the zeta potential (±) of NMs in solution. Since as early as 1975, DLS has been used as a simple method for measuring particle size in solution and analyzing their suspension stability. The measurment of small particle size frequencies in gases, liquid and solids has become simpler following the development of laser technology (light scattering) (Berne, 1976; Simakov and Tsur, 2007; Wu *et al..,* 2005). This technique yields a hydrodynamic diameter or radius calculated through the Stokes-Einstein equation from the aforementioned measurements (Murdock *et al.,* 2008). Below, the equation is given in the form most often used for particle size analysis:

$$D_h = \frac{K_B T}{3\pi\eta D_t} \qquad \textbf{Eq.1.1}$$

D_h is the hydrodynamic diameter.
D_t is the translational diffusion coefficient.
k_B is Boltzmann's constant.
T is thermodynamic temperature.
η is dynamic viscosity.

The need to characterize NPs in solution before evaluating the *in vivo* or *in vitro* toxicity is a high priority. Surface area and chemistry, particle composition, shape, size, size distribution and reactivity in solution are essential factors influencing toxicology (Murdock *et al.,* 2008), because physchemical properties, such as surface charge have been shown to play a major role in the toxicity of SWCNTs (Al-Shaeri *et al.,* 2013). Hund-Rinke and Simon (2006) research size effects and observed that 25nm TiO_2 particle were toxic to the green algae *Desmodesmus subspicatus* (EC_{50} 44mg L^{-1}), whereas 100nm TiO_2 particles were not. Helland *et al.* (2008) reported that, the large surface area of CNTs may cause other molecules to adhere and potentially pick up pollutants and transport these throughout the environment. Poland *et al.* (2008) have suggested that there may be some asbestos-like toxicity associated with SWCNTs. The

physicochemical properties of carbon CNTs are expected to be strongly dependent on nanotube dimension and their propensity to form bundles (Badaire *et al.,* 2004).

1.2 Entry of Nanomaterials into the Aquatic Environment

Many of NMs and NPs (e.g., SWCNTs, C_{60}) are de-stabilized or insoluble in water. However, their dispersion can improve and they can become soluble in water if coated with suitable molecules, such as proteins or peptides (Dieckmann *et al.,* 2003), and NPs can be rendered miscible in the water containing salts and humic acids (Fortner *et al.,* 2005). A recent study showed that humic acids in the form of 0.02% Suwannee river natural organic matter (SRNOM) are able to disperse SWCNTs in seawater (Al-Shaeri *et al.,* 2013). In an aquatic system there are many colloidal forms of natural organic material such as humic and fulvic acid, as well as inorganic silicates, carbonates, metal oxides, phosphates, hydroxides and sulphides. However, incidentally, it has been reported that NMs can be released into the environment and enter the aquatic system through anthropogenic activity and other industrial NMs waste, therefore, the physical properties of NM may affect the elimination, uptake and distribution of NMs by aquatic organisms. Some potential fates of NMs in aquatic environments are indicated in Figure 1.5.

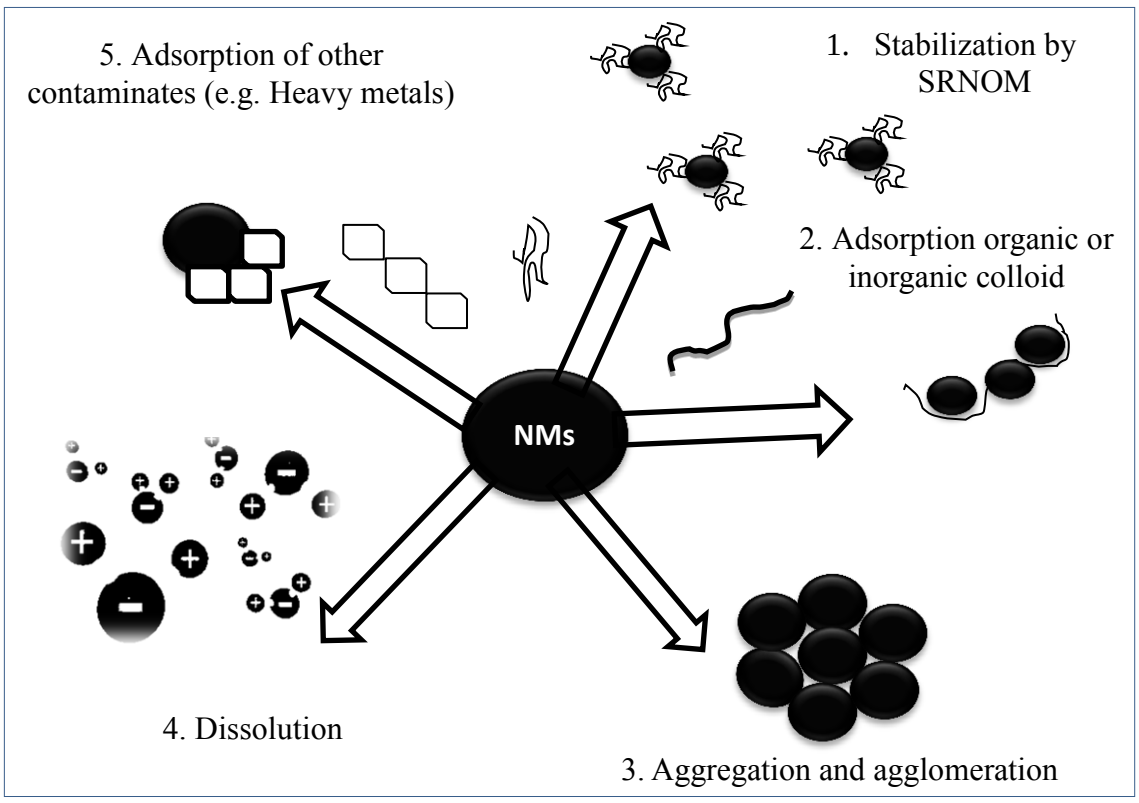

Figure 1.5 Shows the potential behaviour of NMs in aquatic environments.

According to EU environmental legislation (2011), there are three broad origins of NMs waste during their life cycle, with categorisation similar to conventional waste:

- Waste generated during the production of NMs;
- Waste generated during the usage of NMs;
- Waste generated during the end of life activities.

According to Sahu and Casciano (2009) cited in Emerging Nanotechnologies at the Woodrow Wilson International Centre, as of August 2008, there were 803 nanomaterials and nanoparticle products from the 21 countries had been listed; the majority of products (502) were in fitness applications and health sector , followed by personal care (153), clothing (115), and sunscreens (33). The database also listed 56 in consumer electronic applications and 80 products used in food and beverages. Meller and Nowack (2008) reported that nanomaterial production still unknown, however, it estimates ranging from 500 metric tons per year for carbon nanotube (CNTs) and silver nanoparticles, to 5000 metric tons per year for titanium dioxide**.** The concentrations in sediments (U.S.) will presumably rise between 2008 and 2012 from 0.2 to 0.5µg/kg for CNT, from 1.8 to 5.7µg/kg for nano-ZnO, from 0.2 to 0.6 mg/kg for nano-TiO2 and from 0.7 to 2.2µg/kg for nano-Ag, while the concentrations in sludge-treated soil decreased from 0.1 to 0.4µg/kg for CNT, however, increase the concentrations from 6.8 to 22.3 µg/kg for nano-ZnO, from 0.1 to 0.5 mg/kg for nano-TiO2 and from 2.3 to 7.4 µg/kg for nano-Ag. For Europe and the U.S., relatively high concentrations of ENMs were found on soil treated with sewage sludge (Gottschalk *et al.,* 2009). However, recent estimates for global production of CNTs are in the range 11-10,000 tons per year (Yang and Westerhoff, 2014).

The background level of nanomaterials can vary widely (Sahu and Casciano, 2009**),** but in the surface waters, the typical values were assessed as ranging from mass concentration 10µg L^{-1} to 500µg L^{-1} and number concentrations of 10^6 to 10^7 particles with a diameters below 100 nanometre per ml (Rosse and Loizeau, 2003). Releases of SWCNTs in aerosol form to the surrounding atmosphere during production have been shown to be less than 53µg/m^3 **(**Maynard *et al.*, 2004). As mentioned above the production, use and disposal of CNTs will inevitably lead to their appearance in air, soil and eventually water., where they can be taken up by aquatic organisms, such as green algae (*Chlorella sp*.), leading to a plasmolysis and cell wall breakage (Wiesner *et al.*, 2006; Petosa *et al.*, 2010; Long *et al.*, 2012).

1.3 Single walled carbon nanotubes (SWCNTs) and their potential toxicity

In vivo toxicity studies on aquatic organisms using carbon nanomaterials in general and carbon nanotubes in particular are still relatively rare, probably because of the difficulties in solubilizing an essentially hydrophobic material in water and subsequent substance delivery. The few examples of *in vivo* SWCNT toxicity studies in aquatic organisms include the occurrence of organ-specific oxidative stress and impaired ionic regulation in the gills of rainbow trout (Smith *et al.,* 2007), 96hrs acute toxicity tests with *Daphnia magna* resulting in concentration-dependent mortality (Roberts *et al.,* 2007), and impaired survival and growth in amphipods, freshwater mussels, and Chironomus larvae (Mwangi *et al.,* 2012). Technically, uncoated or nonfunctionalized nanomaterials are not dissolved but rather dispersed in their respective media (Handy *et al.,* 2008a) and often require an organic surfactant or dispersant to prevent excessive agglomeration (Smith *et al.,* 2007). The toxicity of uncoated carbon nanomaterials, including SWCNTs, is controversial, mainly because of the inconsistency of results (Buzea *et al.,* 2007; Gonzalez *et al.,* 2008) and because observed toxic effects have also been attributed to residual metal impurities from the synthesis process (Oberdörster *et al.,* 2005) or the dispersants used (Handy *et al.,* 2008b; Oberdörster *et al.,* 2004). In some instances SWCNTs have been shown to induce reactive oxygen species and depletion of antioxidant defences (Pacurari *et al.,* 2008); other authors have not observed oxidative stress following SWCNT exposure (Jacobsen *et al.,* 2008). Concentrations of at least 500µg L^{-1} of uncoated SWCNTs were sublethal to marine mussels (Woods *et al.,* 2009; Al-Shaeri *et al.,* 2013), and have also been observed by other authors to be nonlethal to the copepod *Amphiascus tenuiremis* at concentrations as high as 10mg L^{-1} (Templeton *et al.,* 2006). The genotoxic potential of engineered nanomaterials has been extensively reviewed (Singh *et al.,* 2009). Examples of nanomaterials-induced DNA damage *in vivo* include the freshwater mussel *Elliption complanata* exposed to cadmium-telluride quantum dots (Gagné *et al.,* 2008) and *D. magna* and *Chironomus riparius* larvae exposed to cerium oxide nanoparticles (Lee *et al.,* 2009).

In marine or estuarine, aquatic biota can take up CNTs (Templeton *et al.,* 2006; Oberdörster *et al.,* 2006; Smith *et al.,* 2007; Roberts *et al.,* 2007). Although the mechanism by which NPs or NMs are taken up by gill epithelium is not clear, the two major routes of NMs uptake are (1) across the gill epithelium and (2) across the epithelium of the digestive tract or the hepatopancreas, the site of nutrient absorption

and storage in invertebrates (Sahu and Casciano, 2009). Adult medaka (*Oryzias latipes*) were exposed to 10 mg L^{-1} fluorescent latex NPs, showed 39.4nm in diameter accumulated in gills of see-through medaka by Kashiwada (2006). Similarly, crabs exposed to 0.160g of TiO_2 for 25 days accumulated up to 0.74µg/g of nano-TiO_2 in the gill (Zhang *et al.,* 2006), while within 12 hours, glass wool (silicon dioxide) NPs were taken up by the gill of blue mussel *Mytilus edulis* (Koehler *et al.,* 2008). The likelihood of NMs and NPs uptake by invertebrates may therefore depend on their feeding strategies (Sahu, 2009). A variety of NPs have been shown to be ingested by invertebrates, such as daphnia and mussels, through their filter-feeding (Lovern and Klaper, 2006; Roberts *et al.,* 2007; Koehler *et al.,* 2008). Some studies have shown that NMs can be toxic to aquatic organisms, such as fish or *Daphnia*, as well as unicellular organisms, such as protozoa or bacteria (Zhu *et al..,* 2006). SWCNTs were taken up from sediment by the oligochaete *Lumbriculus variegatus,* but they were not easily incorporated into the tissues (Petersen *et al.,* 2008). NMs have been shown to cross the membranes of various mammalian cell types (Lynch *et al.,* 2006; Rothen-Rutishauser *et al.,* 2006; Smart *et al.,* 2006), and protozoans to take up CNTs (Nowack and Bucheli, 2007). Eggs of *Oryzias latipes* were shown to take-up latex NMs and the gills and the intestine of adult fish accumulated NMs, which were were also discovered in the testes, brain, blood and liver of fish (Kashiwada, 2006; Smith *et al.,* 2007). Rainbow trout (*Oncorhynchus mykiss*), were affected by SWCNTs, which were shown to have neuro-toxic and respiratory effects, where concentrations were less or equal to 500µg L^{-1} (Blaise *et al.,* 2008). The result from exposure to SWCNTs in sediment did not affect the burrowing behaviour of *Arenicola marina* into clean sediment and SWCNTs did not affect their feeding behaviour during the exposure time (Galloway *et al.,* 2010). Smith *et al.* (2007) observed a break down of social groups owing to mortalities in *O. mykiss* exposed to SWCNT (100µg L^{-1}, 250µg L^{-1} and 500µg L^{-1}) and solvent contros (150µg L^{-1} sodium dodecyl sulphate, SDS). On day 4, excessive mucus secretion was observed containing black granules identified as, SWCNTs (Smith *et al.,* 2007). This was carried out with 100µg L^{-1}, 250µg L^{-1} and 500µg L^{-1} SWCNTs, and a solvent control. On day 7, the behaviour of the fish was more aggressive in the solvent control than in the tanks with SWCNTs. By day 7 the fish had died in the highest concentrations 500µg L^{-1}, the nuclear morphology of liver cells, with condensed nuclear bodies showed changes at 250µg L^{-1} SWCNTs, whereas all fish in the 100µg L^{-1} SWCNTs treatment showed inflammation with fusion of intestinal villi in some areas, as well as atrophy and erosion

of the mucosa. Furthermore, the blood haemoglobin of *O. mykiss* decreased after exposure to 500µg L^{-1} SWCNTs.

Although there were some effects on plasma Na^+ and K^+ from SWCNTs or solvent exposure, there were no effects on the plasma osmolarity (Smith *et al.,* 2007). Na^+/K^+-ATPase activity was increased in the gill and intestine after exposure to SWCNTs but no effects were observed in whole brain homogenates (Smith *et al.,* 2007). Mouchet *et al.* (2008) found that SWCNT concentrations higher than 120mg L^{-1} induced a significant hatching delay of 52-72hrs in zebrafish embryos. In terms of concentrations, it was observed that there was no effect on mortality rates of the shrimp *Thamnocephalus platyurus* (24 hours), and the zebra plant *Haworthia attenuata* (96 hours) and the copepod *Amphiacus tenuiremis* (28-35 days) which were exposed to the highest concentrations of SWCNTs (10mg L^{-1}) (Templeton *et al.,* 2006; Leeuw *et al.,* 2007; Blaise *et al.,* 2008).

Galloway et al. (2010) reported DNA damage in coelomocytes collected from *Arenicola marin*a, which were exposed *in vivo* to 0.003g kg^{-1} SWCNTs. Studies have also demonstrated that SWCNT exposure resulted in a reduction in cell viability (Tejral *et al.*, 2009). Test concentrations of SWCNTs as low as 0.580µg L^{-1} caused significant effects on the development of *Daphnia magna* (Baun *et al.*, 2008). However, coating SWCNTs with phosphor lipoproteins appeared to protect *Daphnia magna* from exposure, as demonstrated by reduced mortality rates (Cattaneo *et al.*, 2009). Some observed effects on test biota were dependent on the size of the SWCNTs, showing a decreased success of life cycle moulting in *Daphnia* (Oberdörster *et al.,* 2006), and carbon black nanoparticles showed an effect on reproduction, by reducing the fertilization success of the macoralgae *Fucus serratus* (Klaine *et al.,* 2008).

1.4 Animals as bioindicators for ecotoxicological monitoring

A bioindicator can be defined as ‘‘a species or group of species that readily reflects the abiotic or biotic state of an environment, and represents the impact of environmental change on a habitat, community, or ecosystem, or is indicative of the diversity of a subset of taxa, or of the wholesale diversity, within an area’’ (Mcgeoch, 1998). The fundamental concept of bioindication has been broadened within the past 25 years to address emerging environmental issues (Hodkinson and Jackson, 2005).

A large number of studies have suggested using invertebrates and algae as bioindicators for assessing toxicity values of chemicals and for indicating other environmental impacts (Forsberg *et al.*, 1988; Dallinger 1994; Paoletti and Bressan 1996; Jonsson and Jonsell 1999; Cortet *et al.*, 1999; Moritz *et al.*, 2001; Reyers *et al.*, 2000; Norden and Appelqvist 2001; Taylor and Doran, 2001; Hodkinson and Jackson, 2005).

Hodkinson and Jackson (2005) discussed aquatic animals as bioindicators and posed a fundamental question: what can invertebrates bioindicate? The answer to that question was provided by Mcgeoch (1998) who divided bioindicators into three types; firstly, environmental indicators (changes in physicochemical environment); secondly, ecological indicators (impacts of factors on ecosystems) and thirdly, biodiversity indicators (habitat assessment for conservation).

1.4.1 Mussels as bioindicators for monitoring of trace ecotoxic substances

Monitoring of trace toxic substances in the aquatic environment has been well understood by using biological indicators (mussels or other bivalves), because they have many advantages, so they are ordinarily preferred for this purpose (Tanabe *et al.*, 1987). These comprise their broad geographical distribution, easy sampling, sessile lifestyle, wide range of tolerance to salinity, resistance to high accumulations of chemicals, being sufficiently long-lived to allow the sampling of distinct annual cohorts and having a high filtration rate which favours the uptake and bioconcentration of toxic chemicals. Many national and international monitoring programmes (e.g. Musselwatch) use bivalves as bioindicators, particularly of trace metals (Tanabe *et al.*, 1987; Goldberg *et al.*, 1978; Phillips, 1980; Cowan, 1981). Consequently, mussels are one of the most commonly used marine species for monitoring metal pollution (Goldberg *et al.*, 1983; Ostapczuk *et al.*, 1997).

The genus *Mytilus* in comprised of three species *Mytilus edilus, Mytilus gallorovincialis* and *Mytilus trossulus*, displaying genic and subtle morphological differences, complicated by hybridization (Innes and Bates, 1999; McDonald *et al.*,1991). *Mytilus edulis* has a cosmopolitan distribution in coastal waters and particularly common (Suchanek, 1981), especially in intertidal beds on rocky shores (Gosling, 1992). Contact hybridization zones between *M. edulis* and *M. galloprovincialis* have been located in south western England and France (Tanabe, 1987; Toro *et al.*, 2004). *M. edulis* is abundantly distributed in Scotland, and is particularly common at Cramond, Edinburgh,

Firth of Forth, from where specimens were collected for the present study (Al-Shaeri *et al.,* 2013).

1.4.2 Algae as bioindicator for monitoring of trace ecotoxic substances

A large number of studies with algae, very important indicator organisms for ecotoxicological monitoring, have been published and reviewed in Schwab *et al.,* (2011). The determination of potentially toxic substances in sea water is difficult, because their concentrations are very low. However, marine plants, including algae, accumulate contaminants with concentration factors of 1000 or more (Ostapczuk *et al.*, 1997). The brown alga *Fucus vesiculosus* is an excellent bioindicator of metal pollution**,** accumulating metals from the elements from the surrounding water (Forsberg *et al.*, 1988). Most of the binding or interacting of elements with algae can probably be attributed to ion exchange on the cell walls which contain a number of compounds correlated with anionic groups, including polysaccharides, polyphenols and proteins (Forsberg *et al.,* 1988; Ostapczuk *et al.*, 1997). Once these elements (TiO_2 NPs) adhere to their external surface, they are then conveyed passively or actively via the algal cell wall resulting in growth inhibition (Hartmann *et al.*, 2010). Based on their importance in the ecotoxicological monitoring, the alga *Tetraselmis suecica* was used in this study, and the toxicological effect of CNTs on algae is clearly explained in section 1.6.

1.4.3 Trophic transfer of potentially toxic trace elements in aquatic organisms

Phytoplankton is an important food source and feed additive in the commercial rearing of many aquatic organisms (Borowitzka, 1997). Flagellated green algae *Tetraselmis suecica,* are widely used as favourite food for bivalves, shrimp and larvae (Wikfors *et al.,* 1996; Muller-Feuga *et al.,* 2003).

The primary mechanisms controlling the accumulation elements in aquatic food webs must be understood sufficiently (Mason *et al.,* 1996; Soto *et al.,* 2013). The fate and effect of trace element accumulation in aquatic environments is of concern to ecologists as well as of interest in food web dynamics (Reinfelder *et al.,* 1998).

Blue mussels use their gills to filter out food and other particles suspended in seawater. The filtered water of mussels is released back into the environment. The mucous material on their gills retains materials including detritus, contaminants, tiny organisms and suspended sediment matter, which are sorted and either rejected as pseudofaeces

(see below) or ingested and accumulated in tissues (Figure 1.6). Nanoparticles can be transferred through planktonic crustaceans as food from primary producers such as algae to secondary consumer such as fish, mussels and others to the next trophic level and even to humans (Baun *et al.*, 2008a). The potential trophic transfer of CNTs has been recognized as a major knowledge gap (Zhu *et al.*, 2010).

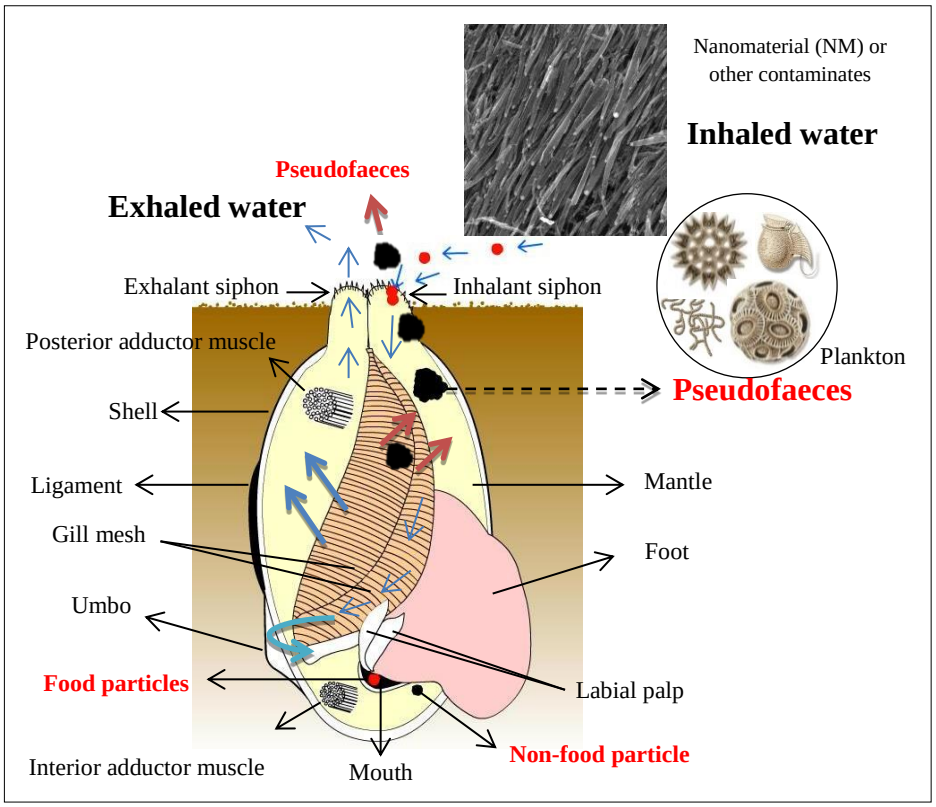

Figure 1.6 The mechanisms of uptake of plankton contaminants and other particulate matter by gill filtration in mussels. Image adopted from (Heather, 2001).

A stable invertebrate community, consisting of the metazoan food web and microbial loop, has been recognized as a fundamentally important component of productive aquatic ecosystem. The trophic transfer of quantum dots (QDs) have been observed to be taken up by ciliates from the aqueous phase and subsequently transferred to grazing rotifers (Holbrook *et al.*, 2008).

Werlin *et al.* (2011) in Figure 1.7 observed trophic transfer and biomagnification of CdSe QDs during 24 h period in the protozoan *Tetrahymena thermophila* feeding on the bacterium *Pseudomonas aeruginosa*.

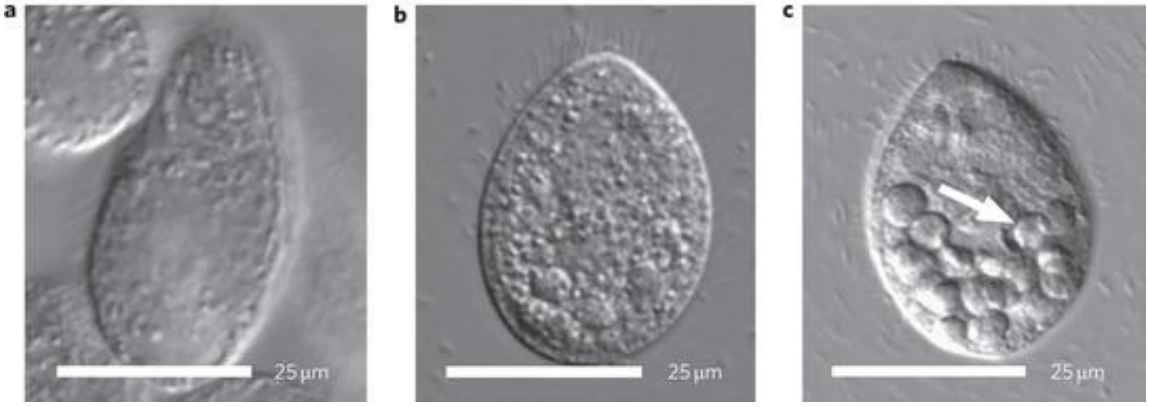

Figure 1.7 *Tetrahymena* cells after 24hrs culture with (a) control *Tetrahymena thermophila* cells, b and c CdSe Qd-grown in *Psedumonas aeruginosa* bacteria. Source (Werlin *et al.*, 2010).

1.4.3.1 Pseudofaeces

Particle selection in filter-feeding bivalve molluscs is well established (Defossez and Hawkins, 1997). Various criteria have been proposed, including particle shape, size, density, motility and chemical cues, such as algal ectocrines (Bayne *et al.*, 1977; Ward and Targett, 1989), but the suggested physical factors (shape, size, density and motility) may also affect capture and selection efficiency (Ward and Shumway, 2004).

Defossez and Daguzan (1996) reported that the previous physical factors suggestion is complicated by the inability to confirm complete separation of particles within bio-deposits, in order to facilitate a comparison with available food particles. However, for the first time, Defossez and Hawkins (1997) observed conclusively that particles are preferentially rejected as pseudofaeces prior to ingestion, and the particle size is considered a significant criterion.

In the past, technical difficulties hampered the studies of particle selection by organisms ranging in size from small heterotrophic flagellates and ciliates of a few micrometres to adult bivalve molluscs (Cucci *et al.*, 1985). However, the preferential feeding in bivalves fed mixtures of unicellular algae was confirmed by using the technique of "flow cytometry" (Cucci *et al.*, 1985; Yentsch *et al.*, 1984; Shumway *et al.*, 1986). Bivalves were fed suspended matter composed of the alga *Tetraselmis suecica* and inorganic particles of ashed sediment and it was observed that pseudofaecal mucus production and the overall rejection rate was partially inversely correlated with the organic matter composing the feeding suspension (Urrutia *et al.*, 2001). Many bivalves have been observed to select particles on the basis of size alone (Winter, 1978), and

Kiørboe *et al*., (1980) and other studies suggested that, bivalves can select preferentially algae from a mixture of algae and silt particles.

1.5 Algal growth Inhibition by SWCNTs

The concentration of phototrophic biota depends on a number of variables such as, temperature, light conditions, grazing pressure, chemical composition of the water, and nutrients (Håkanson and Viktor V, 2002).

As carbonaceous CNTs are opaque, it has been often speculated that shading may explain the reduced cell viability of phototrophic biota exposed to these nanomaterials (Baun *et al.,* 2008; Wie *et al.,* 2010). A number of studies have reported that NPs and CNTs can adhere to algal cell surfaces, and hence restrict light accessibility to the algal cells, subsequently reducing photosynthesis and resulting in growth inhibition (Hund-Rinke and Markus, 2006; Kwok *et al.,* 2010). Schwab *et al.* (2011) reported that, in the presence of concentrations >1.3 -25 mg L^{-1} CNTs, the majority of algal cells (*Chlorella vulgaris* and *Pseudokirchneriella subcapitata*) were located inside of CNTs agglomerates, the agglomeration of CNT causes a shading effect, which led to a reduced growth rate within the first 72hrs. For instance, in the experiment with *C. vulgaris,* 50 mg L^{-1} agglomerated CNT caused growth reductions of 34, 82, 94, and 93% after 24, 48, 72, and 96 hours, respectively, whilst 50 mg L^{-1} agglomerated CNTs caused growth reductions of only 11% in *P subcapitata.*

1.6 Biomarkers

The committee on Biological Markers (mainly in biomedical terms) of the National Research Council (NRC) formally proposed the definition of a biomarker as "indicators signalling events in biological systems or samples following chemical exposure" and presented a three point classification for utilizing biomarkers in toxicity determination, firstly, biomarkers of exposure (the biologically active concentration and exposure period or internal dose); secondly, biomarkers of effect (adverse effects); and thirdly, biomarkers of susceptibility (susceptible populations or individuals) (Henderson *et al.,* 1987). However, definitions have since been introduced to include non-human and environmental systems, including populations, communities and characteristic organisms that respond in measurable ways to changes in the environment (Adams, 1990) and a subsequent inclusion of critical ecotoxicological perspectives (e.g. genetic diversity and latency) have been added to the biomarker definition by Depledge,

(1994); Depledge et al. (1993). For the purpose of this study, a suite of biomarkers and toxicity endpoints relevant in aquatic biota will be reviewed.

1.6.1 Cell viability (Flow cytometry)

Cell viability as a biomarker is important in cytotoxicity studies; there are several accepted assays of viability that utilize the exclusion of certain dyes by live cell membranes (Jones and Senft, 1985). The cytotoxicity of Triclosan (TCS) on haemocytes of the freshwater bivalve Zebra mussel (*Dreissena polymorpha*) was assessed as an early indicator of subsequent genotoxicity by Binelli *et al.* (2009). However, the above techniques are rather labour in tensive and time consuming.

Flow cytometry (FC) is a powerful technique for the rapid analysis of single cells in a mixture, by means of light-scattering and fluorescence measurements (Díaz *et al.*, 2010), and significantly, has impacted biotechnology, in particular cell count and identification used in bioprocess control and prediction for the development of more accurate kinetic models (Rieseberg *et al.*, 2001; Díaz *et al.*, 2010). Besides microbial bioprocesses, flow cytometry has been used to assess the cell viability of mussel haemocytes and gill cells (Al-Shaeri *et al.*, 2013) and to assess other bioprocesses with mammalian cells (Shapiro, 2003).

The key feature of analytical flow cytometry is its ability to measure ~50.000 cells per second, so it has been considered an excellent standard for quantifiable data, used to identify and count cell viability in heterogeneous samples (Di Carlo *et al.*, 2010; Díaz *et al.*, 2010), compared to a traditional method Trypan blue which can only measure or count a small number of cells in a haemocytometer chamber (Freshney, 1987). A very wide range of cellular parameters can be measured with flow cytometry and through the use of fluorescence, light-scattering and absorbance measurements on stained cells (e.g. Propidium iodide (Pi)) and unstained cells (Rieseberg *et al.*, 2001; Papadimitriou *et al.*, 2007). The laser beam illuminates the cell populations at a single cell level, which emit a fluorescence and/or scattering signal (in the case of use of fluorescent dyes), correlated to functional and/or structural cell parameters (Shapiro, 2003).

A flow cytometer can be described as an "automatic microscope" with the advantages of sensitivity, objectivity, precision and high throughput (Shapiro, 2003; Veal *et al.*, 2000). Based on their cytometric characteristics, some flow cytometres are able to separate cell subsets physically (sorting), grouping subpopulations with similar

properties (mode enrichment) within a mixed population for further applications or to single out individual cells (mode cloning) (Shapiro, 2003; Veal *et al.,* 2000; Papadimitriou *et al.,* 2007) (Figure 1.8). In this research the cell viability (vital, dead cells) was used to measure the haemocytes and gills cells of mussels, viable algal cells and algal content of psuedofaeces by using a newly developed flow cytometry technique.

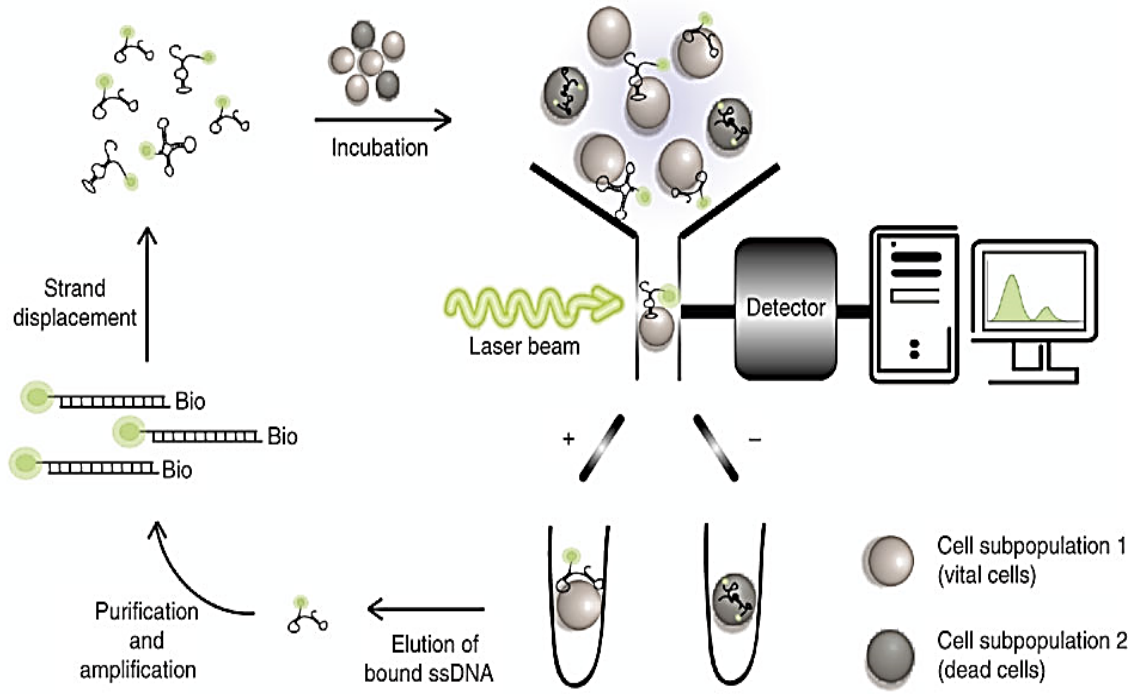

Figure 1.8 Flow cytometry procedure for determining vital/dead cells, using two fluorescence detectors and one forward light scatter (Mayer *et al.,* 2010).

1.6.2 Comet assay

Among the molecular components of the cell, DNA is considered as one of the most important targets of environmental stress in both terrestrial and aquatic organisms. The effect on the integrity of DNA may lead to the occurrence of mutations, and subsequently, may cause diseases such as cancer in vertebrates, and other irreversible toxic effects known as the 'genotoxic disease syndrome' in invertebrates (Sina *et al.,* 1983; Kadhim and Parry, 1984; De Flora *et al.,* 1991; Kurelec, 1993; Bailey *et al.,* 1996; Steinert, 1999).

The single-cell gel electrophoresis (SCGE) assay for detecting single-strand DNA breaks (SSB) in individual cells was first described by Östling and Johnson (Anderson *et al.,* 1998; Wilson *et al.,* 1998; Fairbairn *et al.,* 1995), and the Comet assay subsequently developed by Singh *et al.* (1988). Cells are embedded in agarose, lysed

and the DNA allowed to partially unwind in alkaline buffer, electrophoresed and single strand DNA breaks (SSB) are identified by DNA staining and measured by fluorometry (Rojas *et al.,* 1999). Evaluation of DNA damage has been examined by using a variety of techniques, for instance, the micronucleus test (MNT), sister-chromatid exchange assay (SCE) and chromosomal aberration assay (CA) (Kim and Hyun, 2006). However, the Comet assay is considered as the most commonly used current method for detecting DNA damage (Klobucar *et al.,* 2003), because it is a sensitive, rapid and inexpensive method for measuring DNA strand breaks. Furthermore, the Comet assay has a high sensitivity enabling the assessment of individual cells (Lee and Steinert, 2003). Although the majority of studies analysed DNA damage in mammalian cells, as the Comet assay or SCGE was developed for vertebrate cells, ecologists have also adopted and developed the Comet assay for invertebrate cells (Wilson *et al.,* 1998; Mitchelmore and Chipman, 1998) and DNA damage in the cells of aquatic organisms such as marine invertebrates has also been analyzed (Cotelle and Ferard, 1999; Coughlan *et al.,* 2002; Lee and Steinert, 2003).

The Comet assay has been used in many applications to assess compound-induced genotoxicity, irradiation damage (UV; X-rays and solar radiation) and to detect the effects of environmental mutagens, Failure to repair DNA lesions can initiate a cascade the biological reactions with potential consequences at cellular, organ, whole animal and finally at the community and population levels. DNA damage may occur through a number of DNA damage responses or agents (Figure 1.9) (Mitchelmore and Chipman, 1998; Lee *et al.,* 1999; Lee and Steinert, 2003). In a variety of aquatic organisms, DNA damage has been observed associated with abnormal development, growth and reduced survival of embryos, larvae and adults (Steinert, 1999; Lee *et al.,* 1999).

The increased use and refinement of the Comet assay for environmental purposes made the assay an effective tool for bio-monitoring (McCarthy and Shugart, 1990; Belpaeme *et al.,* 1996; Coughlan *et al*., 2002; Hartl *et al*., 2010). DNA damage has been used to assess the genotoxicity effect of water-borne pollutants on both marine and freshwater bivalves (Nacci *et al.,* 1996; Pavlica *et al.,* 2001; Coughlan *et al.,* 2002; Hartl *et al.,* 2004). The method for Comet assay was adapted by Wilson *et al.,* 1998 to investigate single cell suspensions obtained from the gill and digestive gland in mussels and Rank (1999) also used the Comet assay to detect DNA damage in haemolymph and gill cells. The assay has since been further refined by Hartl *et al.* (2010) and Singh & Hartl (2012). The relationship between DNA integrity and susceptibility to oxidative stress in

mussels (*Mytilus galloprovincialis*) was investigated by Frenzilli *et al.* (2001), who observed the significantly higher levels of DNA damage and oxidative stress in mussels collected from inner parts (the highly eutrophicated Orbetello Lagoon) compared to specimens from more external sites.

A number of other studies have used the Comet assay as a method for detecting and monitoring the effects of DNA damage in both marine and freshwater animals (Pandrangi *et al.*, 1995; Nacci *et al.*, 1996; Mitchelmore and Chipman, 1998; Belpaeme *et al.*, 1998; Wilson *et al*., 1998; Sumathi *et al*., 2001; Frenzilli *et al.*, 2001; Hartl *et al.*, 2004; Hartl *et al.*, 2007; Hartl *et al.*, 2010; Al-shaeri *et al.*, 2013). In addition to being a sensitive way of measuring genotoxic damage in different cell types, the Comet assay has also been found to be simple, quick and reliable (Collins *et al.*, 1997).

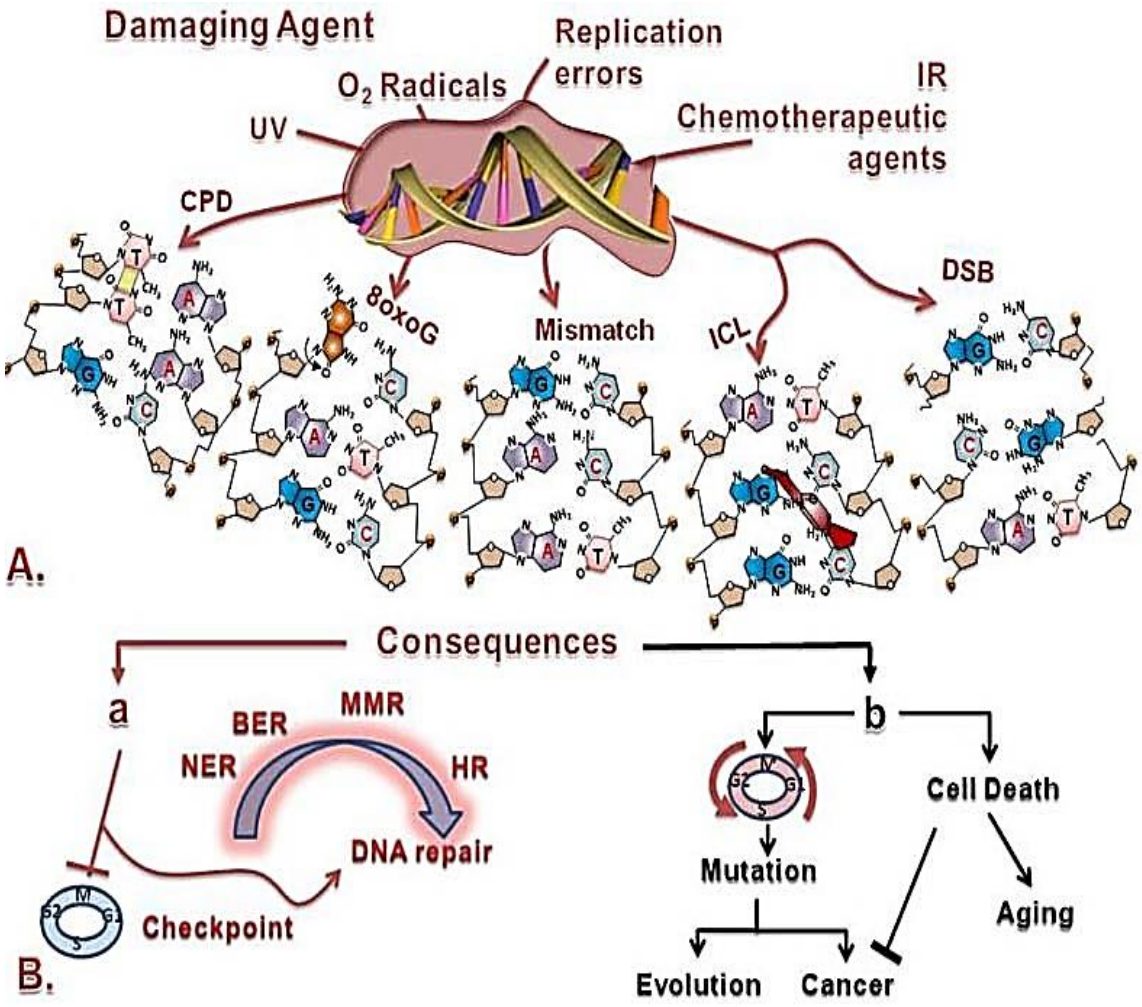

Figure 1.9 Showing (a) DNA damaging agent, and (b) Long-term consequences of DNA lesions (Moraes *et al.*, 2012).

1.6.3 Oxidative stress

Oxidative stress is defined as a disturbance of the equilibrium (imbalance) between the production of reactive oxygen species (free radicals) and antioxidant defences, which can lead to damage of tissues and cellular components in biological systems. It has thus become a topic of significant interest for environmental toxicology studies (Halliwell, 1992; Davies, 1995; Valavanidis *et al.,* 2006). Free radicals are also formed in large amounts by the products of many biochemical processes, and they may be also be generated in the body in response to electromagnetic radiation from the environment or acquired directly as oxidizing pollutants, such as nitrogen dioxide and ozone. It has been suggested that, if antioxidant defences are deficient then damage may occur in a variety of tissues (Betteridge, 2000). Oxygen and nitrogen free radicals are continuously produced in living cells and are considered fundamental in the physiological control of cell function in biological system (Halliwell and Gutteridge, 1985). Cellular metabolism in aerobic organisms involves the production of oxygen free radicals and non-radical reactive species (ROS) (Valavanidis *et al.,* 2006). ROS encompass a variety of diverse chemical species including hydrogen peroxide, hydroxyl radicals and superoxide anions, some of these are extremely unstable, for instance superoxide or hydroxyl radicals, while others, such as hydrogen peroxide, are freely diffusible and relatively long-lived (Finkel and Holbrook, 2000).

Many studies and experiments have shown how living organisms use ROS and free radicals such as the superoxide anion radical ($O_2^{\bullet -}$), nitrogen oxide ($NO^{\bullet}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($ROO^{\bullet}$) and others, for advantageous biological effects (Dröge, 2002). In the higher organisms and maintenance of ‘‘redox homeostasis, the regulated production of free radicals has been suggested are essential for the physiological health of organisms’’ (Ames *et al..,* 1993). However, during these metabolic processes, free radicals may escape from the protective shield of antioxidant mechanisms causing oxidative damage to cellular components (Valavanidis *et al.,* 2006). Other endogenous sources of ROS within cells are several oxidizing enzymes which can produce $O_2^{\bullet -}$, such as cytochrome P450 reductase, tryprophan dioxygenase and xanthine oxidase, while the enzymes that generate H_2O_2 are glucose oxidase, superoxide dismutase (SOD) and guanyl cyclase (Citron, 1992). Cytochrome P450 is of additional interest in toxicology because it is involved in the metabolism and bioactivation of xenobiotics (Mccord and Fridovich, 1978). Oxidative damage to

essential cellular biomolecules has been shown to be caused by superoxide anions and hydroxyl radicals (Sies, 1997). With a lifetime of a few nanoseconds, the hydroxyl radical ($HO^{\bullet}$) is considered a free radical of great toxicological and biological importance. Two reasons make it important: (1) its potent oxidative potential; (2) its indiscriminate reactivity with cellular components, such as DNA, enzymes and other proteins, and lipids of biological membranes (Richter, 1987; Levine and Stadtman, 2001; Jackson and Loeb, 2001).

Several toxic affects at the cellular level by environmental pollutants can be induced by reactive oxygen species (ROS) (Winston and Di Giulio, 1991; Livingstone, 2003; Regoli and Principato, 1995; Regoli, 1998). Also oxidative DNA damage may be particularly important in the presence of redox cycling pollutants, including polycyclic aromatic hydrocarbons, nitroaromatic compounds and transition metals, which have all been observed to enhance the intracellular levels of ROS (Halliwell and Arouma, 1991; Livingstone, 2003). In aquatic organisms, the oxidative stress is affected naturally by temperature (Abele *et al.*, 1998), ultra violet radiation (Stark, 1991), level of dissolved oxygen (Storey, 1996; Pannunzio and Storey, 1998), diet and reproductive status (Rojas *et al.*, 1999) (Figure 1.9). The balance between pro-oxidant challenge and antioxidant defences has been shown to be based on of the effectiveness of cellular antioxidant defences and on oxidative stress which may affect the elevation of intracellular levels of ROS (Winston and Di Giulio, 1991; Collins, 1999).

1.7 Aims and objectives of the research

The main purpose of this study was to determine the bioavailability of SWCNTs to mussels (*Mytilus edulis)* and their potential toxic effects, both separately and in combination with metals at various nominal concentrations, and to assess the potential for SWCNTs to sequester and concentrate benign concentrations of metals. The increasing commercialisation of these materials is leading to concern about the potential release to the environment and toxicity to exposed organism (Muller and Nowack, 2008), including humans (Gorman, 2002).

SWCNT toxicity studies have been predominantly concerned with the impacts on humans, and *in vivo* SWCNT toxicity studies in aquatic organisms in particular are still rare.

Before starting biomarkers assays, the characterization of SWCNTs should be fully comprehended, because there is a strong likelihood that biological activity will depend on the physicochemical properties of SWCNTs (Oberdörster, 2005; Al-Shaeri *et al.*, 2013), which will also determine the behaviour and interaction of CNTs with other substances, such as metals (Qiao and Aluru, 2003), potentially increasing the toxicity of otherwise benign concentrations of these substances. In order to fill these gaps, several specific objectives were investigated:

Characterization of SWCNTs

- To assess the characterization of SWCNTs in this study a several technique was used to understand the characteristic SWCNTs such as SEM, TEM and Raman spectroscopy;
- To assess the agglomeration size and the surface charge of SWCNTs DLS and Zeta potential was used;
- To assess synthesized SWCNTs that contain residual metal impurities (RMI) by using atomic absorption spectrometry (AAS);
- To assess the chemical interaction between SWCNTs and dissolved metals (Cd^{+2} and Zn^{+2}) by using AAS.

Potentiating toxicological interactions of the single-walled carbon nanotubes with dissolved metals

- To detect the presence of SWCNTs on the gill tissue of mussels *Mytilus edulis* by using Raman spectroscopy;
- To assess the uptake of metals (Cd and Zn) by mussel's gill filtration system using atomic absorption spectrometry;
- To assess the impact of SWCNTs and metals, as well as the handling of samples on cell viability of mussel haemocytes and gill's cells using flow cytometry;
- To study the genotoxicity of SWCNTs and metals (Cd and Zn); their impact on DNA damage of mussel gill cells and haemocytes using the Comet assay;
- To study the oxidative stress effect of SWCNTs and metals (Cd and Zn); their impact on superoxide dismutase (SOD), and lipid peroxidation in gills.

Furthermore, the effect of agglomerated SWCNTs on the cell viability, chlorophyll *a* concentration and growth rate of the marine green alga *(Tetraselmis suecica)*, were investigated with the following objectives:

Impact of agglomerated single-walled carbon nanotubes on the marine green algae, *Tetraselmis suecica*

- To assess the adherence of SWCNTs to external surface of *T. suecica* using LM and SEM;
- To detect the presence of SWCNTs on *T. suecica* cells using Raman spectroscopy;
- To assess the uptake of SWCNTs by *T.suecica* using TEM;
- To assess the effects of SWCNTs on the growth rate and cell viability of *T. suecica* using an improved Neubauer haemacytometer and flow cytometry;
- To assess the effects of SWCNTs on chlorophyll *a* concentration using chlorophyll fluorometer.

The impact of SWCNTs on the feeding behaviour of mussels on algal cells (*Tetraselmis suecica*) was of interest and the following questions addressed:

Green algae interacting with single-walled carbon nanotubes affect the feeding behaviour of mussels, mitigating nanotube toxicity

- To assess the psuedofaeces of algal cells by mussels using flow cytometry;
- To assess the genotoxicity of SWCNTs in the presence of algae *(T.suecica)* through the filter feeding of mussels using Comet assay;
- To assess the oxidative stress effect of SWCNTs in the presence of algae *(T.suecica)* on superoxide dismutase (SOD), and lipid peroxidation in gills;
- To assess the trophic transfer of SWCNTs from algae to mussels using a histological assay.

2. MATERIAL AND METHODS

2.1 characterization of stock SWCNT

This section describes the instrumental requirements and procedures for the characterization of SWCNTs used in this study. The instruments and techniques used were transmission electronic microscopy (TEM), dynamic light scattering (DLS), zeta potential, Raman spectroscopy, and atomic absorption spectrometry, (AAS).

2.1.1 Transmission and scanning electronic microscope (TEM, SEM)

Capabilities:

- High resolution evaluation of primary particles size and shape of NMs.
- Diffraction patterns of materials can be used to determine their composition and structure.

A SWCNT stock (1mg L^{-1}; Sigma–Aldrich; manufacturer's specifications: diameter 1.1 nm × length 0.5–100μm) was prepared in distilled water, using 0.02% Suwannee River Natural Organic Matter (SRNOM) as a dispersant. Prior to use, the SWCNT stock was dispersed using an ultrasonic bath (Decon FS300 Frequency Sweep) for 2 hours. SWCNT dispersion was assessed, both in the stock and under exposure conditions, using spectrophotometric analysis at absorbance 234 nm (Smith *et al.,* 2007) and visualized using a transmission electron microscope (TEM; Philips CM120-Biotwin), and prepared by evaporating a drop of stock suspension on a membrane grid (carried out by Steve Mitchel, the EM technician at Edinburgh University).

2.1.2 Dynamic light scattering (DLS) and Zeta potential

Capabilities:

- Used to evaluate the agglomeration of NMs in a variety of solutions.
- Used to determine hydrodynamic size of NMs in a variety of solutions
- Measures the zeta potential (from which the surface charge can be inferred) of NMs in solution.

In this study, the surface charge of single walled carbon nanotubes (SWCNTs) was inferred by measuring the zeta potential at 8.4 pH; suspensions were prepared with nominal concentrations of SWCNTs of 5μg L^{-1}, 10μg L^{-1}, 50μg L^{-1}, 100μg L^{-1}, and 500μg L^{-1} in seawater and in distilled water, respectively. After two hours of ultrasonic

dispersion, the SWCNTs were further characterised under exposure conditions by zeta potential and DLS measurements. A red laser with a wavelength of 633nm was used for both measurements by using Malvern Nano-ZS Zetasizer, Reference. No 2011143.

2.1.3 Atomic Absorbance spectrometry (AAS)

Capabilities:

- Analyse NMs stock to detect any residual metals impurities (RMI) that may contribute to cellular responses.
- Determine the concentration of NMs remaining in the media.

Single-walled carbon nanotube powder, as received from the manufacturer, was examined for RMIs prior to stock suspension preparation by digesting (70% HNO3) 20 mg of SWCNT powder and made up to 25mL of deionized water, and analysed using flame atomic absorption spectrometry (PerkinElmer Precisely). The RMI in SWCNTs stock were calculated using the following equation:

$$\frac{The\ concentration\ of\ metals \times 25ml\ water}{20\mu g.L^{-1}\ SWCNTs} = final\ concentration\ of\ metals\ in\ SWCNTs \qquad \textbf{Eq. 2.1}$$

2.1.4 Raman spectroscopy

Capabilities:

- Localization of single carbon nanotubes
- Diameter and chirality of carbon nanotubes
- Electronic behaviour of carbon nanotube (semiconducting/metallic)
- Number of layers in graphene materials;
- Stress/strain characterization in graphene structures.

A sample of single-walled carbon nanotube stock was placed on a quartz sample holder consisting of a vinyl spacer sandwiched between 2 quartz-covered glasses. Raman microspectroscopy was performed on the samples using an inVia Raman spectroscopy with an integrated confocal microscope (Renishaw) using a 785-nm laser operating at 5mW output power. A camera was used to capture transmitted light optical images of the stock SWCNT with a x20 magnification, 0.4 NA Leica N-plan microscope objective, and Raman spectra of the same region of SWCNTs were acquired using a x50, 0.75 numerical aperture Leica N-plan objective to focus the excitation beam into the SWCNTs and collect the scattered light. High-resolution confocal measurements were made, and Raman scattered light was collected over an extended range of Raman

shifts (100–3200 cm^{-1}) to detect the characteristic peaks (radial breathing mode, D band, G band, and G′ bands) of SWCNTs.

2.2 Chemical exposure and preparation

Cadmium was chosen as a known genotoxic agent, while zinc was selected both for its toxic potential and for its role as an essential metal involved in DNA repair (Pruski and Dixon, 2002). For the exposure experiments, all metallic compounds (cadmium chloride, Sigma-Aldrich, catalogue C5081; zinc sulphate, BDH, catalogue 306215J) were prepared in aqueous solution.

2.2.1 Atomic Absorbance spectrometer (AAS)

2.2.1.1 The interaction between dissolved metals and SWCNTs

Chemical analysis was used to understand the interaction of the dissolved metals (Cd^{+2} and Zn^{+2}) with SWCNTs. Water was spiked with metals and SWCNTs, and then samples were centrifuged using a MSE Mistral 1000 with speed of 3000 rpm for 10 minutes, as shown in Figure 2.1. The SWCNTs were spun down and the metals were analyzed separately in the pellet and in the supernatant by flame atomic absorption spectroscopy.

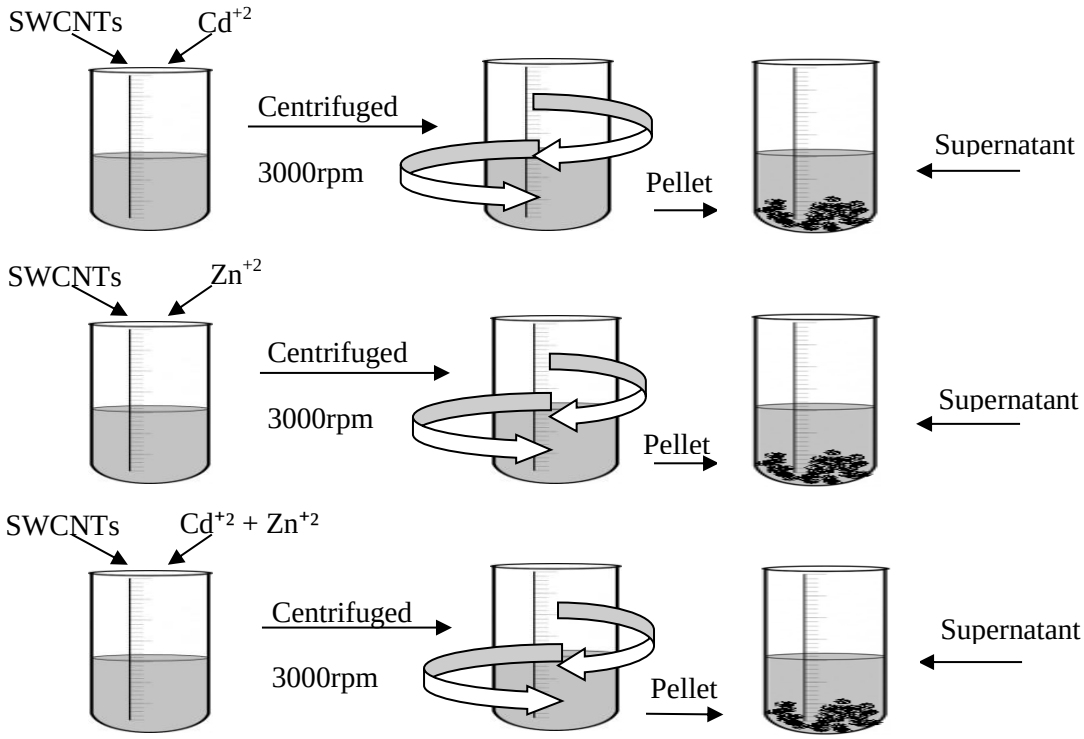

Figure 2.1 The determination of metal (Cd^{+2}, Zn^{+2}) partitioning behaviour in an aqueous SWCNTs suspension.

2.3 Potentiating toxicological interactions of the single-walled carbon nanotubes with dissolved metals

The aim of this study was to determine the bioavailability of SWCNTs to mussels (*Mytilus edulis)* and their potential genotoxic effects, both separately and in combination with metals (cadmium and zinc) at various nominal concentrations. Secondly it aimed to assess the potential for SWCNTs to sequester and concentrate benign concentrations of metals.

This chapter describes how mussels were selected and collected, how the experimental tanks were prepared, the chemical concentrations used, and how the mussels were dissected and haemocytes and gills extracted. The preparation stages of chemical solutions and buffers used are also described, as well as protocols for the assays applied; the cell viability (flow cytometry), chemical analysis, gel electrophoresis, superoxide dismutase (SOD) and thiobarbituric acid reactive substances (TBARS).

2.3.1 Mussel collection

Mussels, *Mytilus edulis* L (1757), Mytilidea were used as biomarker organisms in this study, because firstly, they are biologically and physiologically well understood, secondly, their low mobility helps to determine the area of pollution, and once the planktonic larvae have settled (Table 2.1), they are stationary. Mussels are particularly suited to this study because of their high filter-feeding efficiency and their ability to remove small particulate material from the water column. They are also known to accumulate a large number chemical substances through their gill filtration system (Rank *et al*, 2005). Additionally, mussels are easy to obtain and maintain, and they have significant economic importance (Pimentel *et al.*, 2005).

Table 2.1 Stages, size, age and characteristics of the mussel's life (Newell, 1989)

Stage	Size (length)	Age and characteristics
Fertilized egg	86-70 μm	0-5h Non motile.
Trochophore	70-110 μm	5-24h Ciliated and motile.
Veliger		Up to 35 days; Feeds end. Swims with ciliated velum.
a) Prodissoconch I	110-	Umbo on shell.
b) Prodissoconch II	-260 μm	Development of pigmented 'eye spot'.
c) Eyed larvae	220-260 μm	Development of foot.
d) Prediveliger	260 μm	Up to 6 months; temporarily attached
Plantigrade	0.26-1.5 mm	to filamentous substrates.
Juvenile		Up to 2 years; sexually immature.
Adult	Up to 100 mm	Up to 20 years; sexually mature.

2.3.2 Geographic range

The range of a species is the geographical area within which that species can be found, and understanding the forms that the geographic range limits of species take, their causes and their consequences are key issues in ecology and evolutionary biology (Gaston, 2009). Specimens of the blue mussel *M. edulis* of similar length (5±0.5 cm) were collected from the walkway to Cramond Island at the mouth of the River Almond, on the northwest outskirts of Edinburgh, UK, a well-characterized area showing good water quality after recovering from historical discharges (Scottish Environmental Protection Agency, 2006) and providing animals with a known exposure history (Boisson *et al.,* 1998). The geographical coordinates are 55° 58' 04" North, 3° 18' 46" West (Figure 2.2). The mussels were hand-collected at low tide.

Figure 2.2 Location of Cramond beach in Edinburgh

2.3.3 Aquarium seawater preparation

The collected mussels were transported straight to the laboratory, then divided evenly into two large holding tanks and left to acclimatize in the Aquarium Unit containing aerated filtered seawater (32‰/15 °C) for at least 72hrs before use (Hartl, 2002) as illustrated in Figure 2.3). From time to time the tanks were checked to make sure no mussels were dead, the aquarium air pumps were still working, and the seawater was changed regularly (twice a week).

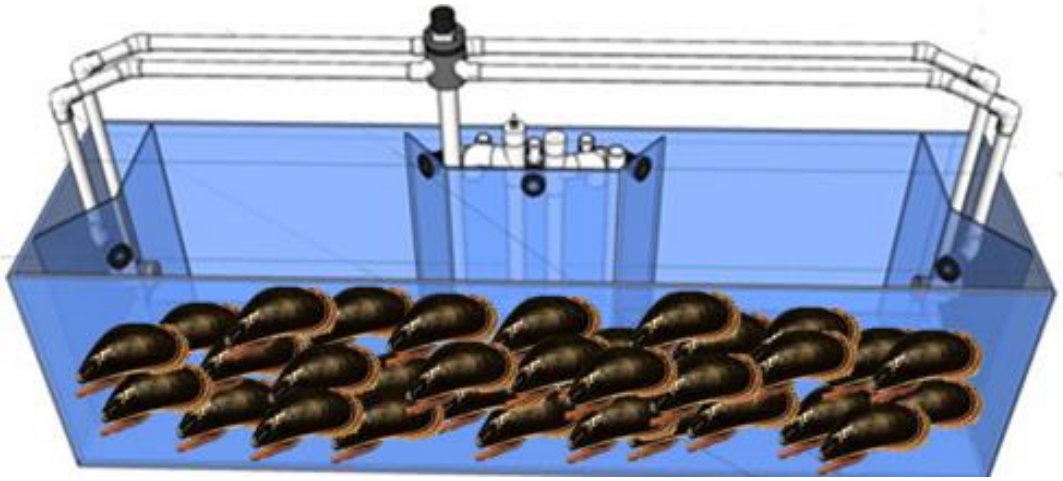

Figure 2.3 The large holding tank used in the aquarium unit.

2.3.4 SWCNT-gill interaction

The interaction of SWCNT with the surface of the gills was observed by exposing mussels to 100µg L^{-1} SWCNTs in a 1L beaker glass. After 15 min, the mussels were removed, opened by severing the abductor mussels, and placed under a Leica MZ7s dissecting microscope (x50 magnification) equipped with a Leica DC300 digital camera connected to a personal computer for image capture.

2.3.5 Raman spectroscopy

The aim of this experiment was to detect and identify the presence of SWCNTs in mussel gills using Raman spectroscopy.

2.3.5.1 Treatment

In order to detect adsorbance of SWCNT onto mussel gill epithelia, two beakers were prepared with 1 litre of seawater added to each. The first tank was used as treatment and 5 mussels were put into it. The second tank was used as a control and also contained 5

mussels. In the treatment tank, mussels were exposed to three replicates SWCNTs 100µg L^{-1} for 24 hours (Figure 2.4).

After 24hrs, mussels from both treatment and control tanks were dissected and their gills extracted and placed in 2.5mL Hanks balanced salt solution at 4°C. Gill tissue of both control and treatment groups was sectioned and placed on a quartz sample holder consisting of a vinyl spacer sandwiched between 2 quartz-covered glasses and the retained material subject to Raman microspectrometrical analysis.

Raman microspectroscopy was performed on both control and treatment samples using an inVia Raman spectrometer with an integrated microscope (Renishaw) using a 785-nm laser operating at 5mW output power. A camera was used to capture transmitted light optical images of the tissue with a x20 magnification, 0.4 NA Leica N-plan microscope objective. Raman spectra of the same region of tissue were also acquired, using a x50, 0.75 numerical aperture Leica N-plan objective to focus the excitation beam into the tissue and collect the scattered light. High-resolution confocal measurements were made, and Raman scattered light was collected over an extended range of Raman shifts (100–3200 cm^{-1}) to detect the characteristic peaks (radial breathing mode, D band, G band, and G′ bands) of SWCNTs.

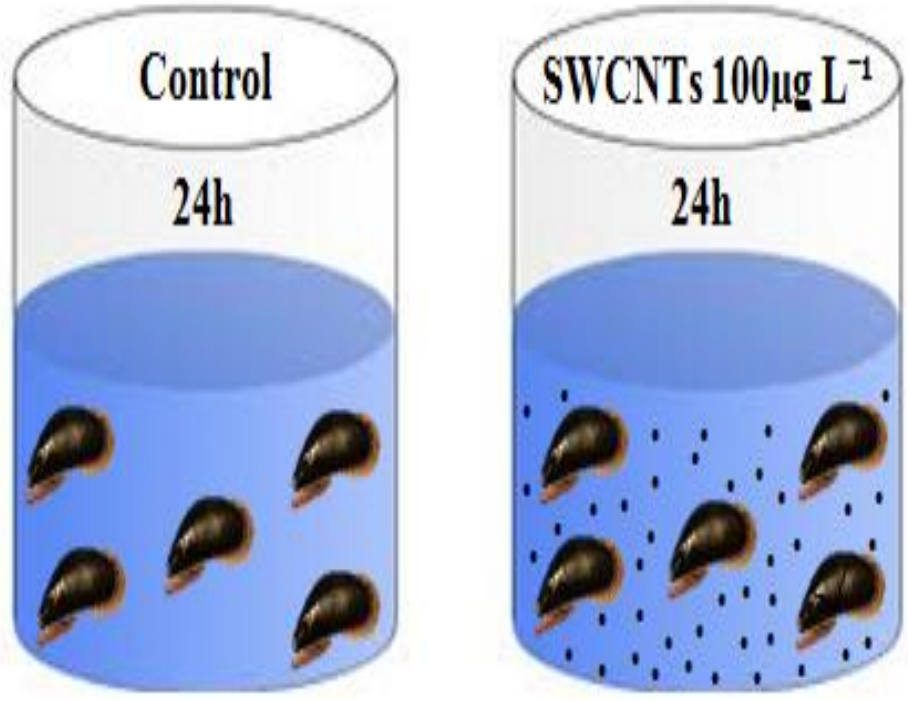

Figure 2.4 Control tank and treatment tank spiked *in vivo.*

2.3.6 Chemical analysis

The aim of this experiment was to assess the uptake of heavy metals by mussels, using atomic absorption spectrometry.

2.3.6.1 Treatment

Glass tanks were filled with 5 L of constantly aerated, filtered seawater (salinity range 33±1 ppt, 15 °C) and the aquarium air pumps were fitted. Five mussels of similar length (5 ±0.5 cm) were placed in each glass tank. The mussels were exposed to three replicates of Cd 0.001µM, Cd 0.001µM + SWCNTs 5µg L^{-1}, Zn 1.0µM, Zn 1.0µM + SWCNTs 5µg L^{-1}. All the spiking described was *in vivo* for 72 hours.

2.3.6.2 Determination of dissolved metals (Cd, Zn) in seawater by AAS

After 72 hours 15ml water samples were taken from each tank and kept at 4C°. 70% nitric acid (S.G.1.42 (70%); Batch (0755171) from Fisher Scientific was added to each sample for 24 hours at 25ºC to extract Cd and Zn from the sediments. Samples were centrifuged using a MSE Mistral 1000 with speed of 3000 rpm for 10 minutes. Pellets were gently removed using tweezers and were kept in 5ml distilled water. The concentrations of cadmium standard solution (1000ppm), Reference: 20/21/22-36/38 and zinc standard solution (1000ppm), Reference: 36/38 from Spectrosol were measured by an atomic absorption spectrometer (AAnalyst 200; Reference 2005526. from Perkin Elmer. Then all samples were analysed in the AAS to detect the spiked amount of Cd and Zn in seawater tanks.

2.3.6.3 Determination of spiked metals (Cd, Zn) in mussel gills by ASS

Protocol described by Liu and Kueh (2005).

Following 72 hours of exposure, two gills per mussel were extracted separately and frozen. All empty glass beakers (10ml) were weighed, then the thawed wet gills placed in the beakers, oven dried at 60°C for at least 24 hours and then weighed (Table 2.2). In order to extract Cd and Zn from gill tissue, 70% nitric acid (NHO3) was added to each gill sample for 24 hours, then 5ml distilled water was added in order to dilute the nitric acid. All the samples were left for 24h in a 60°C aquatic bath (SUB36, Reference; 2008254, from Grant). Each gill sample was filtered and all beakers were filled with distilled water up to 50ml (Figure 2.5). Samples were then analysed using flame atomic absorption spectrometry.

Table 2.2 Empty beaker weight and dry weight of mussel gills

Chemical exposure	Empty beaker weight	Dry weight gill	Net dry weight
Control sample 1	20.635g	21.363g	0.728g
Control sample 2	18.368g	18.144g	0.776g
Control sample 3	22.484g	23.231g	0.747g
Control sample 4	19.865g	20.342g	0.477g
Control sample 5	20.424g	21.143g	0.719g
Cd sample 1	22.633g	23.114g	0.481g
Cd sample 2	18.658g	19.257g	0.599g
Cd sample 3	18.421g	19.110g	0.689g
Cd sample 4	19.678g	20.232g	0.554g
Cd sample-5	22.982g	23.347g	0.365g
Zn sample 1	23.328g	24.132g	0.804g
Zn sample 2	22.551g	23.222g	0.671g
Zn sample 3	19.224g	20.150g	0.926g
Zn sample 4	18.333g	19.201g	0.868g
Zn sample 5	19.658g	20.261g	0.603g
Cd + SWCNT sample 1	20.661g	21.425g	0.764g
Cd + SWCNT sample 2	20.417g	21.113g	0.696g
Cd + SWCNT sample 3	18.201g	19.012g	0.811g
Cd + SWCNT sample 4	21.821g	22.333g	0.512g
Cd + SWCNT sample 5	19.664g	20.197g	0.533g
Cd + Zn + SWCNT sample 1	18.518g	19.177g	0.659g
Cd + Zn + SWCNT sample 2	22.325g	23.150g	0.825g
Cd + Zn + SWCNT sample 3	21.958g	22.447g	0.489g
Cd + Zn + SWCNT sample 4	22.894g	23.514g	0.620g
Cd + Zn + SWCNT sample 5	19.654g	20.339g	0.685g

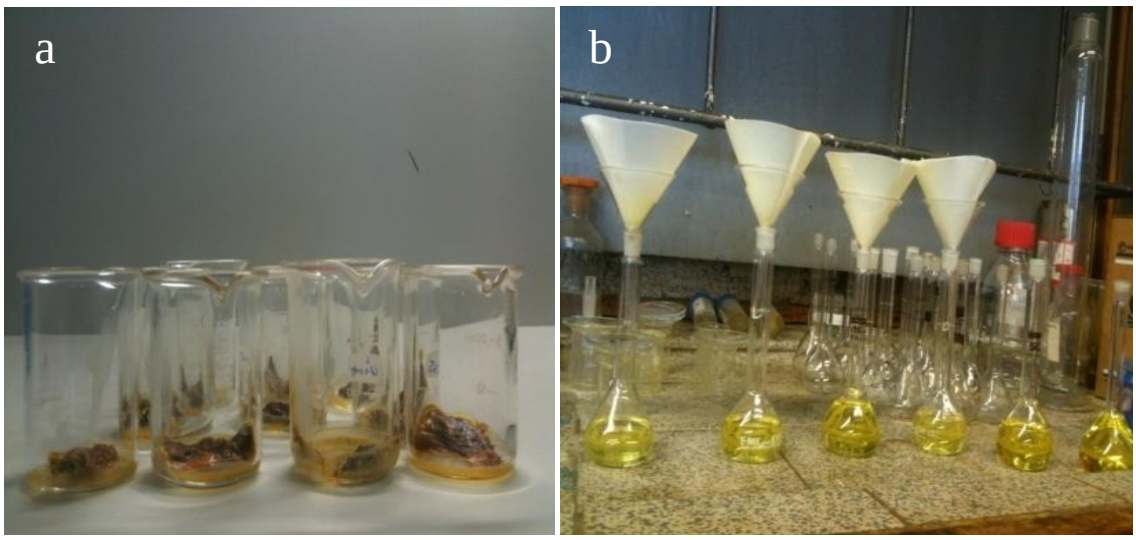

Figure 2.5 (a) Dried mussel gill tissue and (b) filtered extract for AAS analysis.

2.3.7 Biomarker analysis

The exact mechanisms whereby the interaction of single-walled carbon nanotubes (SWCNTs) with dissolved metals (cadmium and zinc) induce effects on mussels *Mytilus edulis L* remain unknown. In this study, the potential genotoxicity of manufactured SWCNTs and metals, both separately and in combination, was assessed in terms of their effect on cell viability, oxidative stress and DNA damage to mussels. The endpoints of toxicity treatments, as shown in Figure 2.6 were flow cytometry (Fc), superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS) and single cell gel electrophoresis or comet assay.

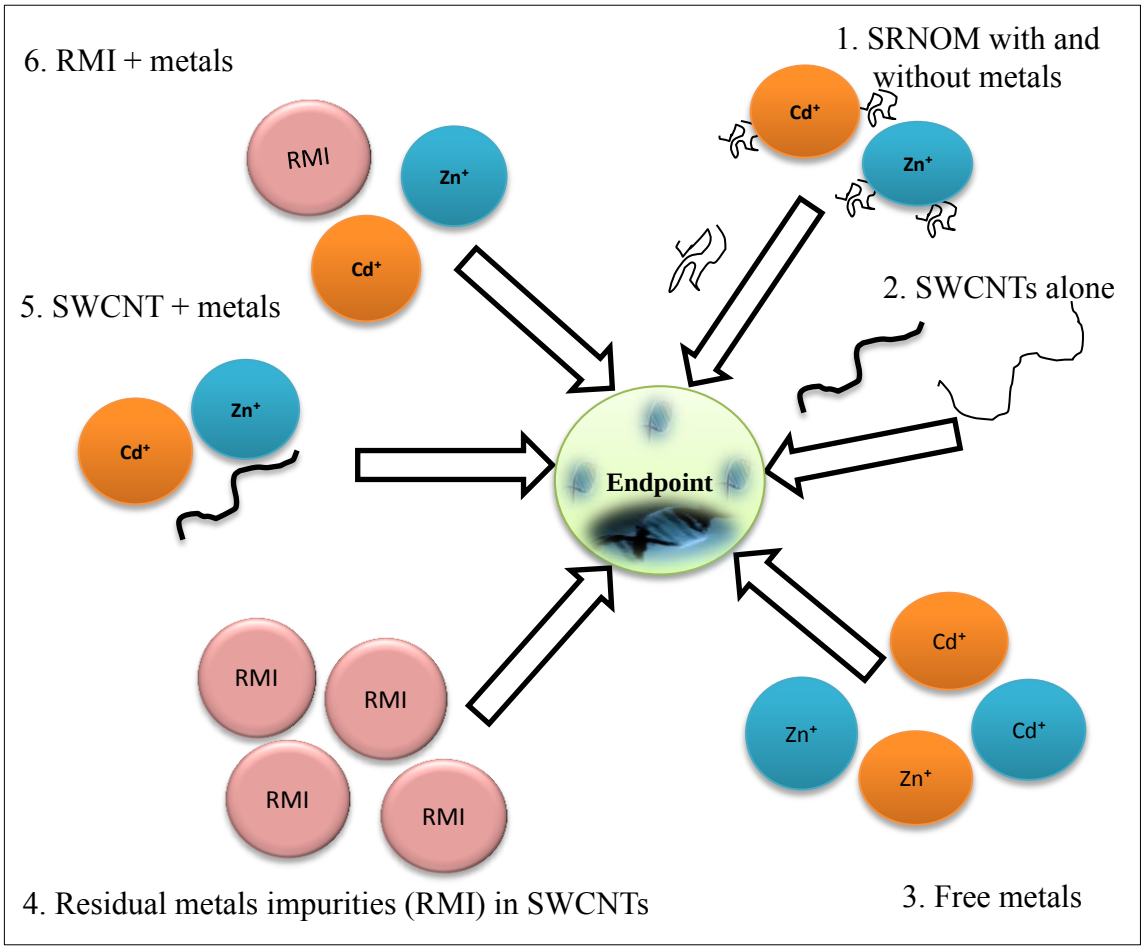

Figure 2.6 Treatments for toxicity endpoint

2.3.7.1 *In vivo* studies

M. edulis was used as an *in vivo* model to evaluate the toxicity of SWCNTs and metals. Mussels were exposed in an aquarium unit under simulated environmental conditions.

2.3.7.2 Cell isolation

Haemocytes and gill cell suspensions were prepared according to the procedure for the clam *Tapes semidecusatus* (Coughlan *et al.,* 2002) and adapted for mussels, as previously described by Singh and Hartl (2012).

In the laboratory the mussels were dissected to isolate the haemocytes and gill cell suspensions. Instruments used for dissection of the mussels were scissors, scalpel blade, needle, tweezers and 1mL Plastic Luer syringe Reference No. (300013) (21G1 ½ needles; Sigma-Aldrich) and petri dish.

2.3.7.2.1 Extraction of haemocyte samples

Hank's Balanced Salt Solution (HBSS) purchased from Invitrogen (Reference. No.14175-053 500mL; Ca and Mg-free) was used as a tissue culture medium. Mussel haemocytes and gill cells have an osmolarity of 990 mOsmol L^{-1}; therefore, the HBSS was adjusted by adding 22.2g L^{-1} of sodium chloride (NaCl), which was purchased from Sigma Aldrich. This protocol was as reported by Hartl *et al.* (2010).

For the dissection position, all mussels were laid on their beak (umbo). The valves were forced partly open with scissors and excess seawater drained from the interior. Before taking the blood sample, the syringe was primed with 0.1ml HBSS. Then 0.1ml of the haemocytes was gently extracted by inserting the needle into the posterior adductor muscle. The needle was gently removed from the syringe, which contained a mixture of 0.2ml (0.1ml HBSS + 0.1ml haemocytes), and the mixture transferred to an Eppendorf tube (1.5ml: purchased from Greinerbio-one) and placed on ice (Figure 2.7).

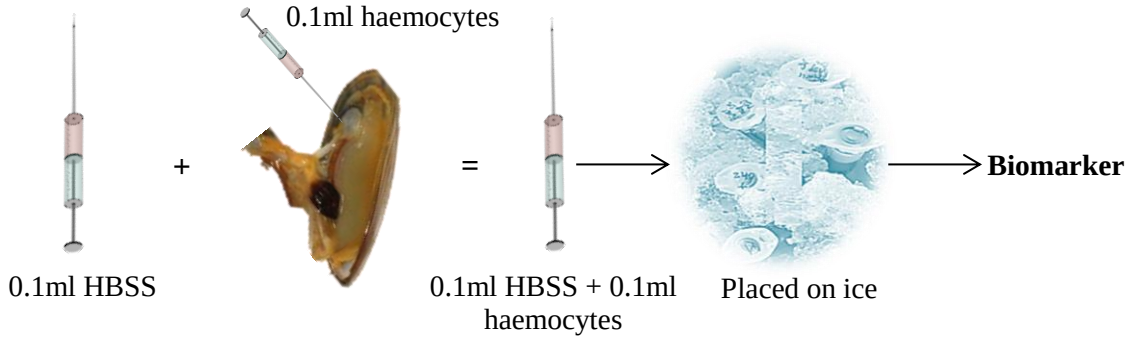

Figure 2.7 Shows the procedure of haemocyte isolation

2.3.7.2.2 Extraction of Gill samples

The procedure for gill extraction was adapted from Coughlan et al. (2002) and is summarized in Figure 2.8. Both muscles were severed using a scalpel and the mussels opened up completely. Extraction of the gills was done using fresh scalpel blades. During the isolation of the gills, Petri dishes were filled with 2.5mL of HBSS, osmotically adjusted with 11.1g L^{-1} of NaCl. The gills were placed on the Petri dish and chopped 20 times using fresh scapel blades in a scissor-like movement. In the next steps, the chopped gills with the salt buffer (HBSS) were transferred to tubes (PP-Test tubes, 15mL) using wide-mouthed Pasteur pipettes. A 2.5% trypsin solution (trypsin solution 10x from porcine pancreas was purchased from SAFC and was kept at -20°C) was diluted with HBSS to achieve a 1% working trypsin concentration. 2.5 mL were added to the chopped gill tissue to achieve a final concentration of 0.05%. Using a gyro-rocker (SSL3), the tubes were gently rocked for 10 minutes at room temperature. After finishing a rocking session, all the tubes were topped up with HBSS to 10ml final volume, filtered through a 40 µm cell strainer and centrifuged using a MSE Mistral 1000) with speed of 3000 rpm for 10 minutes. The pellets were gently removed and re-suspended in 0.5ml fresh trypsin-free HBSS.

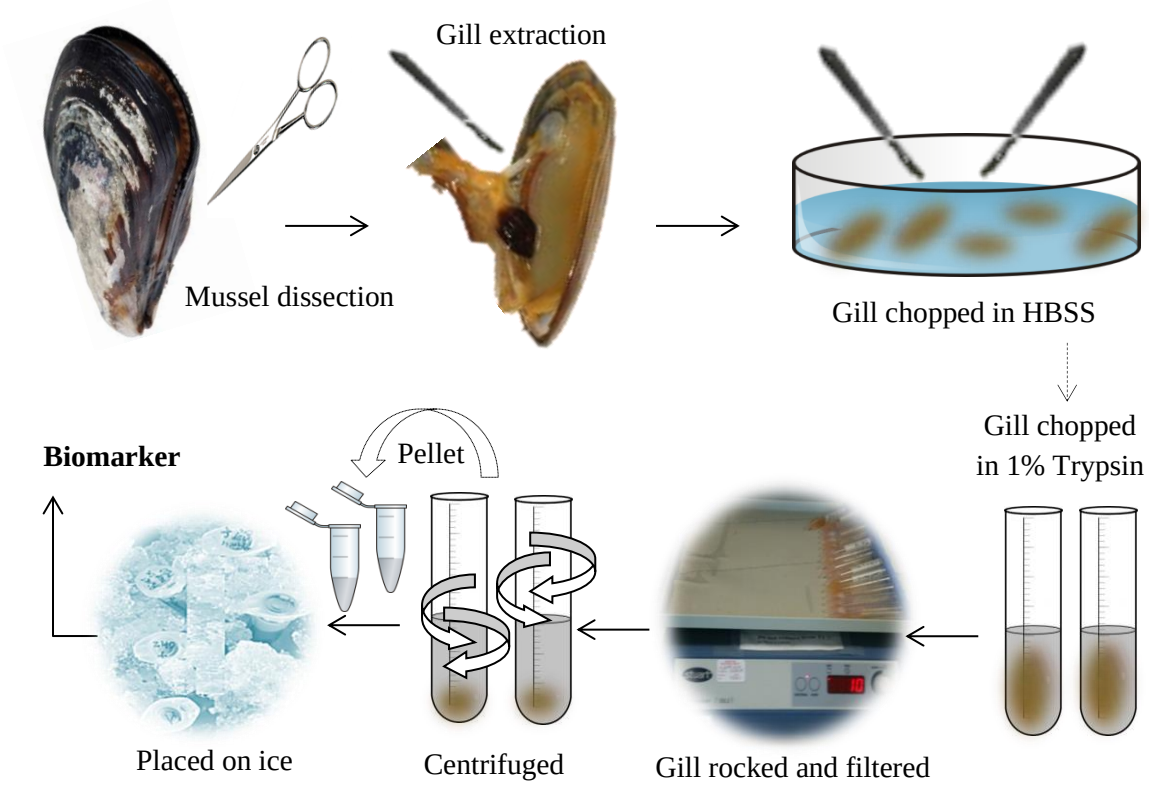

Figure 2.8 Shows the procedure of gill isolation.

2.3.7.3 Cell viability

Cell viability was examined to study the impact of SWCNTs and metals, as well as the handling of samples on mussel haemocytes and gill cells. Traditional methods rely on exclusion dies such as trypan blue (Absolom, 1986). The purpose of this part of the study was to develop a more rapid and reliable technique using flow cytometry.

2.3.7.3.1 Treatment

Glass tanks were filled with 1 L of constantly aerated, filtered seawater (salinity range 33±1 ppt, 15 °C) and the aquarium air pumps were fitted. 3 mussels of similar length (5 ±0.5 cm) were placed in each glass tank. The mussels were exposed to three replicates of SWCNTs 100µg L^{-1}, Cd 0.001µM, Cd 0.001µM + SWCNTs 100µg L^{-1}, Zn 1.0µM, Zn 1.0µM + SWCNTs 100µg L^{-1} and Cd 0.001µM + Zn 1.0µM + SWCNTs 100µg L^{-1}. All this spiking was carried out *in vivo* for 72 hours.

After 72 hours of exposure, all mussels were taken out from the tanks and were dissected to extract their haemocytes and gill cells. The haemocytes and gill cells were extracted and put in Eppendorf tubes and were kept on ice.

Cell viability was assessed by flow cytometry (using Partec CyFlow reference 2005442-1711) following treatment with propidium iodide (Pi) (Sigma, catalogue P4170). Cell suspensions (200 µL) were then mixed with 800 µM Hanks balanced salt solution in flow cytometry tubes (Röhren; 3.5 mL, 55 x 12 mm, PS reference 55.484). All the tubes were kept on ice, and the propidium iodide was kept in a dark tube. Cells were stained with propidium iodide (Pi) (final concentration 2 µg mL^{-1}) immediately before analysis. The results of CyFlow were automatically saved as graphs on the computer (Figure 2.9)

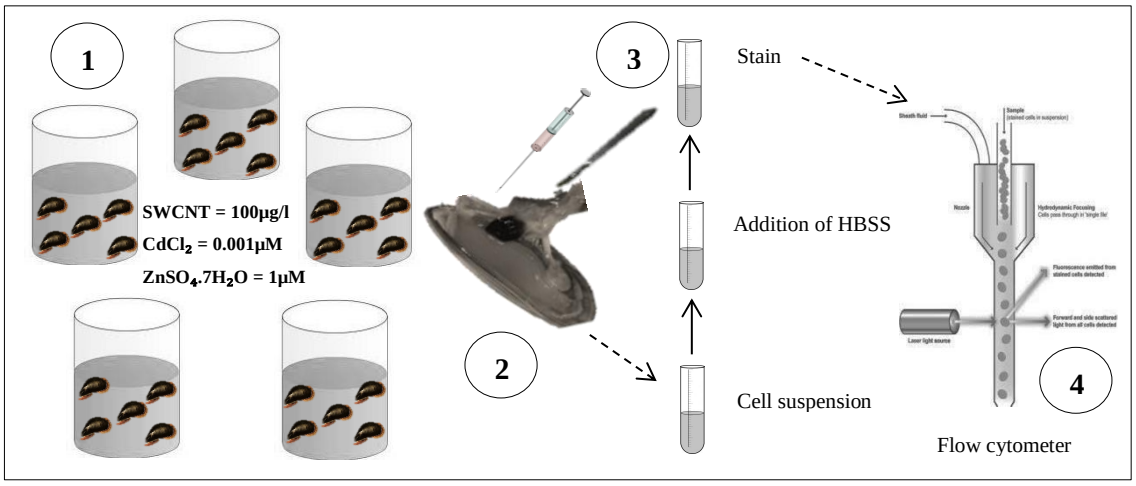

Figure 2.9 Summary of flow cytometry procedure.

2.3.7.4 Comet assay

Genotoxicity of SWCNTs and heavy metals (cadmium and zinc) and their impact on DNA damage of mussel gill cells and haemocytes was assessed using the Comet assay, carried out according to the procedure developed for the clam *T. semidecusatus* (Coughlan *et al.*, 2002) and adapted for mussels, as previously described by Hartl et al. (2010). A minor change here was the replacement of ethidium bromide with GelRed (Biotum, catalogue BT41003; solution 2 µL in 10 mL) as a fluorescent DNA stain.

2.3.7.4.1 Treatment

Glass tanks were filled with 5 L of constantly aerated, filtered seawater (salinity range 33±1 ppt, 15 °C) and the aquarium air pumps were fitted. 5 mussels were placed in each glass tank with a similar length of mussels (5 ±0.5 cm). Mussels were exposed in quintuplicate to:

Group 1. SWCNTs 5µg L^{-1}, Cd 0.001µM, Cd 0.001µM + SWCNTs 5µg L^{-1}, Zn 1.0µM, Zn 1.0µM + SWCNTs 5µg L^{-1}, Cd 0.001µM + Zn 1.0µM and Cd 0.001µM + Zn 1.0µM + SWCNTs 5µg L^{-1}. The spiking was carried out *in vivo* for 72 hours.

Group 2. SWCNTs 10µg L^{-1}, Cd 0.001µM, Cd 0.001µM + SWCNTs 10µg L^{-1}, Zn 1.0µM, Zn 1.0µM + SWCNTs 10µg L^{-1} and Cd 0.001µM + Zn 1.0µM + SWCNTs 10µg L^{-1}. The spiking was carried out *in vivo* for 72 hours.

Group 3. SWCNTs 50µg L^{-1}, Cd 0.001µM, Cd 0.001µM + SWCNTs 50µg L^{-1}, Zn 1.0µM, Zn 1.0µM + SWCNTs 50µg L^{-1} and Cd 0.001µM + Zn 1.0µM + SWCNTs 50µg L^{-1}. The spiking was carried out *in vivo* for 72 hours.

Group 4. SWCNTs 100µg L^{-1}, Cd 0.001µM, Cd 0.001µM + SWCNTs 100µg L^{-1}, Zn 1.0µM, Zn 1.0µM + SWCNTs 100µg L^{-1} and Cd 0.001µM + Zn 1.0µM + SWCNTs 100µg L^{-1} . The spiking was carried out *in vivo* for 72 hours.

Group 5. SWCNTs 500µg L^{-1}, Cd 0.001µM, Cd 0.001µM + SWCNTs 500µg L^{-1}, Zn 1.0µM, Zn 1.0µM + SWCNTs 500µg L^{-1} and Cd 0.001µM + Zn 1.0µM + SWCNTs 500µg L^{-1}. The spiking was carried out *in vivo* for 72 hours.

Group 6. Suwannee River Natural Organic Matter (SRNOM), Cd 0.001µM, Cd 0.001µM + SRNOM, Zn 1.0µM, Zn 1.0µM + SRNOM and Cd 0.001µM + Zn 1.0µM + SNORM. The spiking was carried out *in vivo* for 72 hours.

Group 7. Residual metal impurities (RMI): (Fe [0.013 µM] + Mn [0.009 µM] + Ni [0.006 µM]) alone, Cd (0.001 µM) + RMI, Zn (1 µM) + RMI, and Cd (0.001 µM) + Zn (1 µM) + RMI. The spiking was carried out *in vivo* for 72 hours.

- **Chemicals, reagents and stains used in the experiment**

All the following solutions were prepared according to the protocol reported by Hartl *et al*. (2007).

Phosphate buffered saline (PBS)

1 tablet of PBS Reference (096K8217) from Sigma-Aldrich was dissolved in 200ml distilled water. The PBS solution was stored at laboratory temperature.

Normal Gel Agarose (NGA)

1% of Normal Gel Agarose NGA) (Agarose, Type V; Reference (08K1093) from Sigma-Aldrich) (was dissolved in a 100ml conical flask of PBS which was already prepared. The resulting solution was kept at 4 °C.

Low melting Agarose (LMP)

1% of low melting point Agarose (Agarose, Type I-B; Reference (075K0077) from Sigma- Aldrich) LMP was dissolved in a 100ml conical flask of PBS which was already prepared. The resulting solution was kept at 4 °C.

Sodium hydroxide (NaOH)

10N NaOH (M.M 40, Reference 28248.367; from Sigma-Aldrich) was dissolved in 1000ml of distilled water.

Ethylenediaminetetra-acetic acid (EDTA)

23.37g EDTA (ACS reagent, 99.4-100.06%; Reference (028k00581) from Sigma-Aldrich) was first put in 200ml water, then NaOH was added, to obtain the correct pH of 7. Finally, the second 200 ml water was added to give the total volume of 400ml.

Tris hydrochloride (Tris)

31.52g of Tris ($C_4H_{11}NO_3$ CIH = 157.6/mol: Reference (441514A) from VWR International Ltd) was dissolved in 500ml distilled water.

Lysing solution stock

Lysing solution was prepared using several chemical compounds. In 1500mL distilled water, 2.5M NaCl and 100mM EDTA were added, followed by 10mM Tris; then NaOH was added to bring the pH up to 10.

Lysis working solution

Lysis solution was prepared in a small black tank by adding 1% (1.5ml) Triton X-100 (Reference 22-41-51/53) and 15ml Dimethyl sulphoxide (DMSO) purchased from Sigma-Aldrich (Reference BCBB4025) to 135mL of the prepared Lysis solution. The solution was stored in the black tank at 4 °C.

Electrophoresis solution

60ml of 10N NaOH and 10ml of 200mM EDTA were dissolved in 2 litres of chilled distilled water. The final concentrations were 300mM NaOH and 1mM EDTA, which were kept at 4 °C

GelRed stain

GelRed (Biotum, catalogue BT41003; solution 2 µL in 10 mL), as a fluorescent DNA stain.

- **Comet assay procedure**

Preparation of microscope slides and tips:

- Microscope slides (0.1-1.2mm purchased from Fisherbrand, (FB58628).
- Microscope cover slips (22mm X 22mm purchased from Thermo Scientific.
- Pipette tip 200µl, yellow (Ref No. 70.760.002 from Sarstedt).

Several microscope slides were first marked with a pencil; 1% normal Gel Agarose (NGA) was melted in and the slides frosted by pipetting 100µl of NGA onto each slide and smearing it evenly across the slides, which were allowed to dry for 24 hours.

First Layer

The marked and frosted microscope slides were warmed on a hot plate, which was fixed at 40°C, to help spread the NGA and to eliminate any bubbles. 100µl NGA, which had been prepared in advance (see above), was drawn with a pipette and directly added to

the slide and gently covered by a microscope cover slip (22mm X 22mm), and the slides stored on ice or at 4°C for 15-20 mins to accelerate the setting of the gel.

Second Layer

Next the cover slips were carefully removed. The next step after the adhesion of NGA on each slide was the second layer, which consisted of a mixture of haemocytes or gill cell suspension + Low melting point (LMP) agarose gel. LMP was melted and the hot plate temperature was as used with first layer. However, for this layer, 3 different volumes of pipettes (30µl, 70µl and 100µl) were prepared. The first pipette was used to gently draw 30µl of blood cells or gill cell suspension, which had been already prepared and then add it to Eppendorf tubes (see above). The second pipette drew 70µl of melted LMP and this was then added to 30µl blood cell or gill cell suspension; the third pipette was used to draw100µl of the mixture of both the cells and LMP from the tube and deposit them on the slide, where they were gently covered by a microscope cover slip (22mm X 22mm) and left to set at 4 C° for 15-20 minutes to form the second layer.

Third Layer (last layer)

The next step was similar to the first layer. However, in this case LMP was used instead of NGA and just 100µl of LMP was drawn and added to each slide and gently covered by a microscope covers slip (22mm X 22mm) and then kept at 4 C° to be a third layer.

Removal of cell membranes

Alkaline lysis solution was used to remove the cell membrane in order to detect single strand breaks. A chilled lysis solution had been previously prepared in tank by adding 1% (v/v) 1.5ml triton X-100 and 10 % (v/v) 15mL dimethyl sulphoxide (DMSO) to 135mL Lysis solution.

The cover slips were gently removed and the slides were placed next to each other in chilled lysis solution in the dark, cold room at 4°C, to avoid any DNA damage. DNA can be damaged by ultraviolet (UV) light forming pyrimidine dimers. This is because the pyrimidine dimers are incised by an endonuclease which causes single strand (DSS) DNA fragments that would mask any affects of the treatment of interest (Kantor and Barnhart 1973).

Electrophoresis buffer and unwinding

At this stage, the alkaline solution (300mM L^{-1} NaOH and 1mM L^{-1} EDTA, pH > 13) was dissolved in 2 litres of chilled distilled water and then left at 4°C for exactly 30 minutes. This solution allowed the DNA to denature and unwind. Single DNA strand breaks (SDS) were detected by placing slides horizontally on the electrophoresis plate (Rated 0-250V, 0-100mA, Model A3-1, Serial No.233712 from Owl Separation Systems, Inc). The slides were spaced randomly, but in the same orientation (left to right), with the face of the slides marked in pencil upwards. The next step was to pour alkaline solution on the electrophoresis plate to cover all the slides. In this case, the current and power were off for exactly 30 minutes, after which the power and current were turned on and adjusted to 300mA and 250V, respectively for exactly 25 minutes in a dark, cold room at 4°C.

Neutralization and staining

After electrophoresis, the slides were removed from the electrophoresis plate and the gels neutralized repeated 3 times with 5 drops of 0.4 M Tris (pH 7.5), rinsed with 5 drops of chilled distilled water and, following removal of excess fluid, stained with 5 drops of GelRed stain. The slides were gently covered with cover slips and kept in humid boxes at 4°C for processing.

Observation of single strand DNA breaks under a microscope

The Zeiss Axiophot microscope (Reference No. 58) was used to detect single strand DNA breaks. The slides covered by cover slips were placed between stage clips on the microscope's stage. The magnification of the objective was set at ph_2-plan-Neofluar 40x/o.75 (∞/0.17) and the ocular lens was set at Pl 10x/25.

In the second step, 50 cells per slide were analysed to detect the % DNA tail, using live video scoring with Comet Assay IV (from Perceptive Instruments). After the number of cells from the slides was scored, the final results of % tail intensity (=% DNA in the tail) were saved automatically in Microsoft Excel format (Figure 2.10).

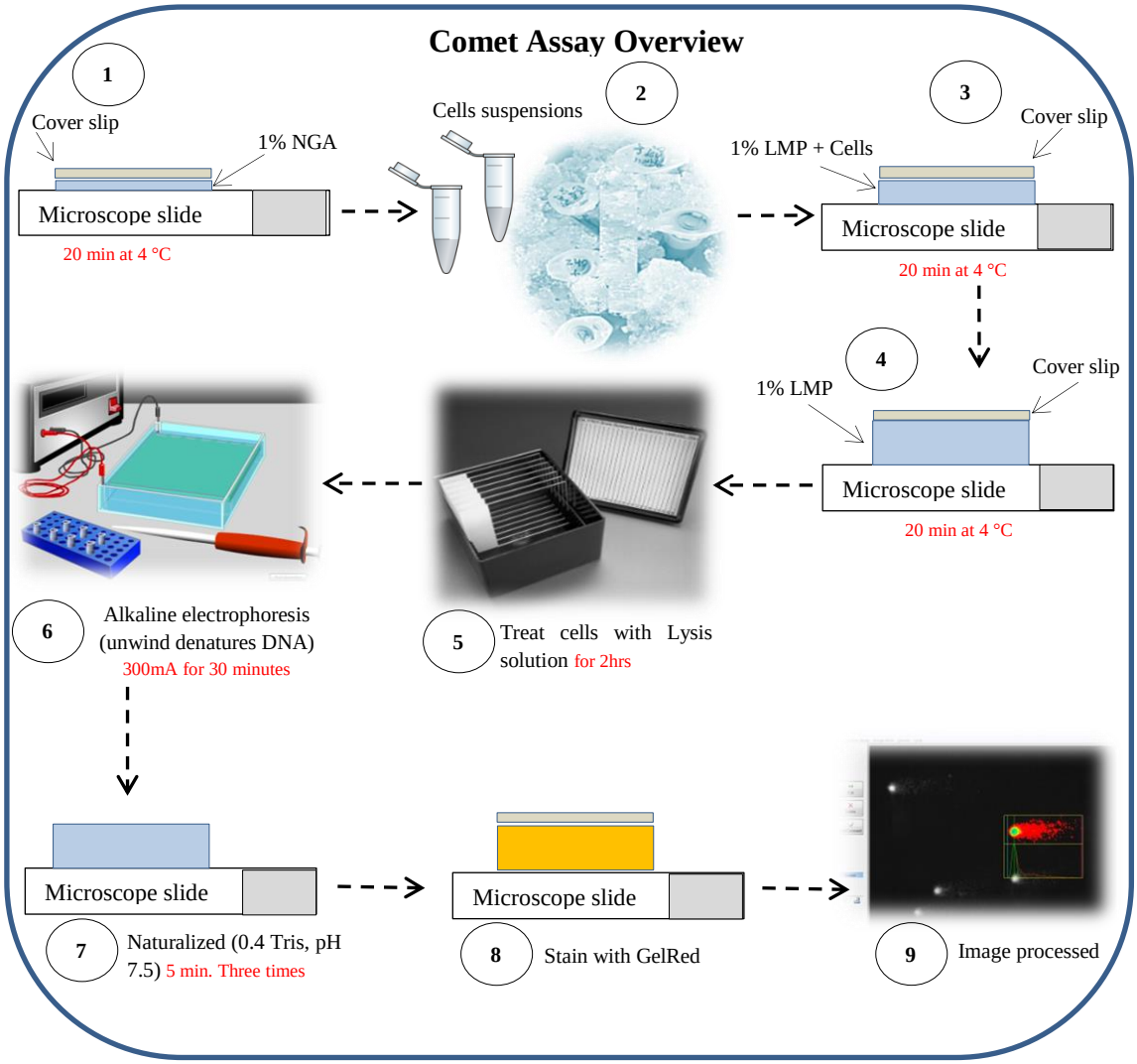

Figure 2.10 The comet assay procedure summary

2.3.7.5 Oxidative stress

To prepare tissues for oxidative stress assays, part of the gills excised during gill cell isolation for the comet assay were diverted, immediately immersed in liquid nitrogen, and stored at (–80 °C) until further use. The tissues were then homogenized on ice with approximately buffer (1:5 volumes of Tris-HCl 50 mM, 0.15M KCl, pH 7.4) and centrifuged at 10,000g for 20 min at 4 °C. The pellet was retained for thiobarbituric acid reactive substance (TBARS) analysis, while the supernatant fraction was transferred to a 5ml Beckmann ultracentrifuge tube and centrifuged at 40,000 g for 60 min at 4 °C to obtain the cytosolic fraction, which was then used to analyse the superoxide dismutase activity (SOD) (Figure 2.11).

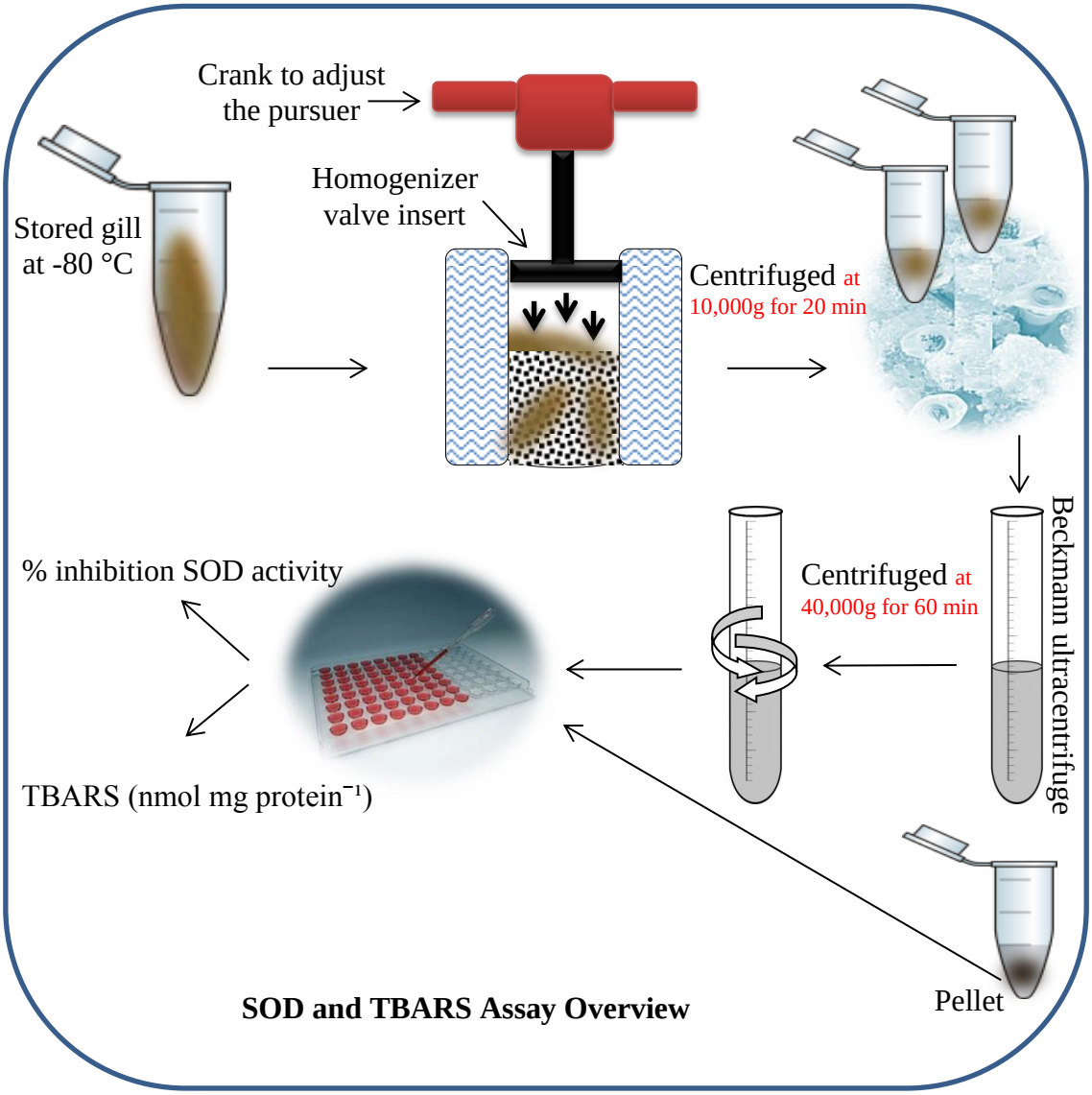

Figure 2.11 Summary procedure of SOD and TBARS assay.

2.3.7.5.1 Superoxide dismutase (SOD) Assay kit-working solution (WST)

Superoxide dismutase (SOD) was determined using a kit (Sigma 19160 superoxide dismutase determination kit). The activity of xanthine oxide (XO) is linearly related to the rate of reduction with O_2, which is inhibited by SOD, and therefore, to the IC_{50} (which measures how much of SOD activity or a particular substance/molecule is needed to inhibit some biological process by 50%). The SOD kit contains 5ml working solution (WST), 50ml of dilution buffer, and 100ml of buffer solution and 100µl of enzyme solution.

2.3.7.5.1.1 Working solution (WST) preparation

1- **Working solution:** 1ml of WST was diluted with 19ml of buffer solution

2- **Enzyme solution:** the enzyme solution was centrifuged for 5 sec, and then 15µl was diluted with 2.5ml of dilution buffer.

Each sample contains 20µl of the supernatant (cytosolic fraction), which was drawn with a pipette and gently added to the 96-well plate, and then the reagents added. Table 2.3 shows the amount of solution in each well of a 96-well microtitre plate and the SOD standard and each solution sample, blanks 1, 2 and 3.

The plates were then incubated at 50°C for 20 minutes and read at 450 nm using a spectrophotometer (spectra Max M5). Subsequently, the activity of SOD was expressed as % inhibition, and was then calculated using the following equation:

$$\%\ inhibition\ of\ SOD\ activity = \frac{[(Abs\ blank1 - Abs\ blank3) - (Abs\ sample\ x - Abs\ blank2)]}{(Abs\ blank1 - Abs\ blank3)}\ x\ 100 \qquad \textbf{Eq. 2.2}$$

Table 2.3 Amount of each solution for sample and blanks 1, 2 and 3.

Solution	**Sample**	**Blank 1**	**Blank 2**	**Blank 3**
Sample solution	20 µl	----------	20 µl	----------
ddH2O	----------	20 µl	----------	20 µl
WST	200 µl	200 µl	200 µl	200 µl
Enzyme working solution	20 µl	20 µl	----------	----------
Dilution buffer	----------	----------	20 µl	20 µl

2.3.7.5.1.2 Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) determination was adapted from the procedure described by Smith *et al*. (2007) for trout. Briefly, to each well of a 96-well microtitre plate, 40µL of gill homogenate, 10µL of butylated hydroxytoluene (BHT), 140 µL of phosphate-buffered saline (PBS), 50µL of trichloroacetic acid (TCA), and 75 µL of thiobarbituric acid (TBA) were added. The plates were then incubated for 60 min at 50 °C, read at 530 nm and 630 nm (correction for turbidity 530–630 nm) using a spectrophotometer (spectra Max M5) against a tetraethoxypropane (TEP) standard series (0.5 nmol, 2.5 nmol, 5 nmol, 15 nmol, and 25 nmol), and expressed as nmol mg^{-1} protein. Total homogenate protein was determined according to Bradford (1976) and TBARS concentration calculated using the following equation:

$$Abs = A_{530nm} - A_{630\text{nm}} \quad \textbf{Eq .2.3}$$

$$TBARS = \frac{Abs}{protein}\ nmol\ mg\ protein^{-1} \quad \textbf{Eq. 2.4}$$

2.4 Impact of agglomerated single-walled carbon nanotubes on the marine green algae, *Tetraselmis suecica*

The marine algae *Tetraselmis suecica*, a widely used food source in aquaculture (Muller-Feuga *et al.*, 2003; Tredici *et al.*, 2009). In this study, algae were used as a bioindicator to determine the influence of algal interaction on the toxicity of SWCNTs.

2.4.1 *In vivo*

Examination of the SWCNTs on the algae *Tetraselmis suecica* was performed using a light microscope (LM), Raman mircospectroscopy, a scanning electron microscopy (SEM) and a transmission electron microscopy (TEM). These techniques also helped to illustrate the agglomeration and the presence of SWCNTs inside and outside the algal cells. Toxicity was assessed using the biomarker assays described above.

The aims of this study were;

- To assess the adherence of SWCNTs to the external surface of *T. suecica* using a LM and a SEM;
- To detect the presence of SWCNTs on *Tetraselmis suecica* cells, using Raman spectroscopy;
- To assess the uptake of SWCNTs by *T.suecica* using aTEM;
- To assess the effects of SWCNTs on the growth rate and cell viability of *T. suecica,* using an improved Neubauer haemacytometer and flow cytometry;
- To assess the effects of SWCNTs on algal chlorophyll *a* content using fluorometry.

2.4.2 Observation of algae-SWCNTs agglomerate using a light microscope

Tetraselmis suecica strain PLY305 were obtained from Plymouth Algal Culture Collection; The Marine Biological Association, Citadel Hill, Plymouth. The algal cells were cultured in autoclaved 250mL flasks containing 100 mL seawater to which 1mL/L Guillard's f/2 medium + vitamin mix 100µl/L were added. The flasks were kept in a shaking incubator (Infors HT, Ref: 2011225) where the shaking speed was 225rpm and

the temperature 23 ± 0.5 °C, with illumination by white incandescent lights (115 ± 15 $\mu Em^{-2}\ s^{-1}$) (Long *et al.*, 2012). All of the flasks in the experiments were shaken for 8 days.

2.4.2.1 Treatment

In order to detect the agglomeration of SWCNTs in the algal culture medium, 9 autoclaved 250 mL flasks were prepared. Algae *Tetraselmis suecica* were exposed to SWCNTs at nominal concentrations (100µg L^{-1} and 500µg L^{-1}); three replicates per treatment and control were incubated for 8 days respectively.

A light microscope was used to observe the agglomeration of SWCNTs with algae *Tetraselmis suecica.* 5µl of *Tetraselmis suecica* culture were drawn with a pipette, placed on slide and covered by a cover slip, and viewed with a Zeiss Axiophot microscope (Ref No. 5822/018030; magnificantion: ph_3-plan-Neofluar 100x/1.30 oil (∞/0.17) and the ocular lens was set at Pl 10x/25).

2.4.3 Scanning electronic microscope (SEM)

The aim of using the SEM was to assess the adherence and agglomeration of SWCNTs to the algal cell wall.

In order to detect the agglomeration of SWCNT in the algal culture medium, six autoclaved 250 mL flasks were prepared. The algae were exposed to three replicates SWCNTs 500µg L^{-1} and control. The flasks in the experiment were incubated for 8 days. Conventional methods for SEM analysis of phototrophic biota involve dehydration, using either critical point drying (CPD) or chemical compounds (e.g. osmium tetraoxide) (Kersey and Wessells, 1976). The basic steps involved in SEM sample preparation included stabilizing the sample with a fixative, rinsing, dehydrating, drying, and coating the sample with a layer of a material that is electrically conductive, gold, in this case (Bozzola and Russell, 1999). Because each of these steps is crucial and affect the outcome of the study (Stadtländer, 2007), they are all described individually in more detail below. The SEM protocol is adopted from Stadtländer (2007) and Guiselin *et al.* (2009).

In order to eliminate undesirable salt crystals, all samples were filtered through a pore size 0.45 µm filtration unit (Whatman membrane filters, cellulose nitrate; Cat No 7184 002), and then the filters were rinsed with ultrapure water. CPD was performed as

follows: algal samples were slightly pre-fixed by immersing in a solution comprising 50:50 of acid Lugil's iodine and 1.5% glutaraldehyde solution (sold as Grade I, 25% in H_2O, specially purified for use as an electron microscopy fixative, from Sigma-Aldrich) and prepared in 0.1 cacodylic acid buffer (pH 7.3) and incubated at 4°C overnight. The fixative solutions were prepared under a standard fume hood using proper protection (plastic gloves, mask and glasses).

All samples were carefully dehydrated performed with a graded absolute ethanol series: 30% ethanol for 5 min, 70% ethanol for 10 min, 80% ethanol for 10 min, 90% ethanol for 15 min and 100% ethanol for 20 min, at 4 °C. This dehydration process was used to allow the water in the biological samples to be slowly exchanged through liquids with lower surface tension (Stadtländer, 2007).

The SEM operates under a vacuum. Thus, the specimens were essentially required to be dry: unless the samples are dry, they will be destroyed in the SEM chamber. Critical point drying (CPD) is considered as the gold standard for SEM specimen drying (Hoppert and Holzenburg, 1998). Algal cells were critical point dried in liquid CO_2 using a CPD Mod. 8/4 from Peak Scientific LTD (carried out by James B Buchanan, the EM technician Heriot-Watt University). Before SEM observations, all dried filters were mounted onto SEM stubs and coated with gold-palladium. The SEM was equipped for imaging with a secondary electron detector (Guiselin *et al.* 2009)

2.4.4 Raman microspectroscopy

The aim of this experiment was to confirm the presence of SWCNTs in the algae *Tetraselmis suecica* using Raman spectroscopy.

2.4.4.1 Treatment

In order to detect absorbance of SWCNTs in the algal culture medium, six autoclaved 250 mL flasks were prepared. The algae were exposed to three replicates SWCNTs 500µg L^{-1} and control for 24 hours (Figure 2.12). Following exposure, 5µl of algae were placed on a quartz sample holder consisting of a vinyl spacer sandwiched between 2 quartz-covered glasses. Raman microspectroscopy was performed on both control and treatment samples using an in Via Raman spectrometer with an integrated microscope (Renishaw) using a 785-nm laser operating at 5mW output power. A camera was used to capture transmitted light optical images of the algal cells with a x20 magnification,

0.4 NA Leica N-plan microscope objective and Raman spectra of the same region of algal cells were acquired using a x50, 0.75 numerical aperture Leica N-plan objective to focus the excitation beam into the algal cells and collect the scattered light. High-resolution confocal measurements were made, and Raman scattered light was collected over an extended range of Raman shifts (100–3200 cm^{-1}), to detect the characteristic peaks (radial breathing mode, D band, G band, and G′ bands) of SWCNTs.

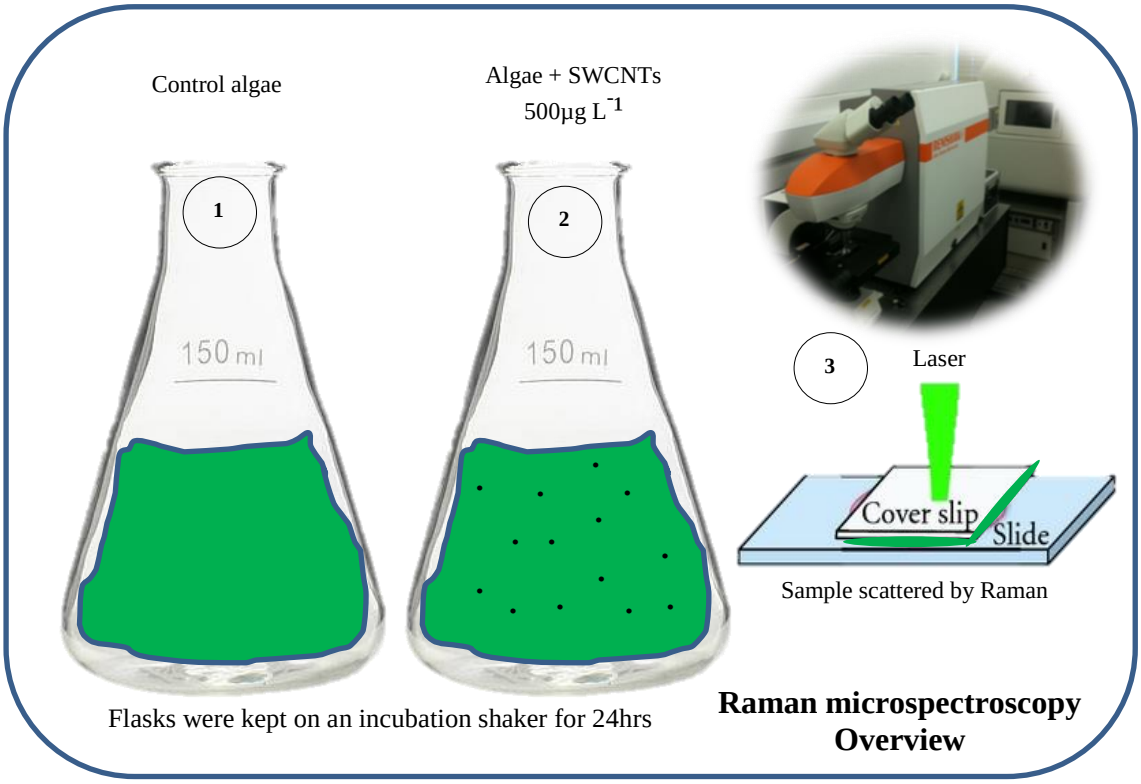

Figure 2.12 Control algae flask and treatment flask spiked *in vivo*.

2.4.5 Transmission electronic microscope (TEM)

For determination of the uptake of 500µg L^{-1} SWCNTs by the algae *Tetraselmis suecica* under the transmission electron microscope (TEM), algal samples were prepared using methods adopted by Azma *et al.* (2010) and Youn *et al.* (2011). Algal culture samples were centrifuged at 474g for 10 min. The centrifuged supernatants were discarded, while pellets were primarily fixed in 1 mL 4% glutaraldhyde, and stored at 4°C overnight to improve fixation. The samples were washed 3 times in 0.1 M sodium cacodylate buffer (pH 7.24) for 10 min. The cells were post-fixed with 1% buffered osmium tetroxide and stored at 4°C for 2 hours. Before cell dehydration, the algal cells were washed again 3 times in 0.1 M sodium cacodylate buffer (pH 7.24) for 10 min each time. The fixed cells were dehydrated through a gradient series of acetone 35%, 50%, 75% and 95% for 15 min per each concentration, followed by 100% acetone.

The dehydrated cells were infiltrated by adding a mixed graded acetone and Spurr's epoxy resin (agar 100 resin 10mL; dodecenyl succinic anhydride 5.5mL, methyl nadic anhydride 6mL and benzyl dimethyl amine 0.5mL). The infiltration was processed by first adding 1 mL of acetone with a ratio of 1:1 of a mixed resin for 1 hour, then adding 1mL of acetone with a ratio of 1:3 for 2 hours. Finally, 1mL (100% resin) was added to the samples for 2 hours, and then the cells were stored at room temperature overnight, followed by 100% resin for 2 hours. The cells were placed into beam capsules and then were filled up with resin. The cells were cured at 60°C for 24 hours to become polymerized cells.

Using a glass knife and a cyro-ultramicrotome the cells were cut into ultra thin sections and placed on a grid, and then the contrast of sections was improved by adding 2% aqueous uranyl acetate stain and Reynold's lead citrate for 10 min and they were washed by 50% filtered alcohol. The sections were then examined with a transmission electron microscope (TEM). TEM samples were prepared by Steve Mitchel at the University of Edinburgh EM Unit.

2.4.6 Cell counting using a Haemocytometer (Growth rate)

The aim of this experiment was to assess the effects of SWCNTs on the algal cell growth rate by using an improved Neubauer haemocytometer. The haemocytometer procedure was adapted from Lobban *et al*. (1988).

Eighteen flasks were sterilized in an autoclave (Astell, Swiftlock Secure-Touch Touchscreen: Reference 2008129), and then filled with 100ml of cultured *Tetraselmis suecica*. The three replicated treatments were control, SRNOM and nominal SWCNT concentrations 5µg L^{-1}, 10µg L^{-1}, 50µg L^{-1}, 100µg L^{-1}, and 500µg L^{-1}, respectively. Sterilized tips were used for spiking throughout this experiment. *T.suecica* were exposed for 8 days under environmentally-relevant conditions. The flasks were shaken to make sure no SWCNT could settle, and the *Tetraselmis* cells in each flask were checked each day to make sure the shaker was working and the light was on. The initial concentration of algal cells was 54 cells/ µL. Live algal cells were collected daily and growth rates determined using an improved Neubauer haemacytometer, and the then growth rate calculated using the following equation:

$$\text{Mean } x = \frac{\sum x_i}{n} \qquad \textbf{Eq. 2.5}$$

Where x_i = total no; $\sum x_i$ = sum of all totals and n = no. of counts.

2.4.7 Cell viability

The aim of this experiment was to study the impact of SWCNTs, and also the effect of sample handling of samples on algal cell viability.

2.4.7.1 Treatments

Tetraselmis suecica were exposed to three replicates SWCNTs 500µg L^{-1} and control and incubated for 8 days.

On day 8, both the control and the treated algae were filtered through a 100µm mesh, stained with Dihexyloxacarbocyanine iodide (DiOC6), and then the algal cell viability was determined using flow cytometry. Chlorophyll *a* was slightly fluorescent when exited in the blue laser, and although stain was added to algal cells, however, the chlorophyll was observed clear without stain rather than with stain.

2.4.8 Chlorophyll measurement

The aim of measuring the chlorophyll *a* concentration of green algae *Tetraselmis suecica* is to estimate the abundance of phytoplankton in the algal culture medium. The algae were exposed to three replicates of 500µg L^{-1} SWCNTs and controls for 8 days. From t_0 to t_3, 100µl gum + 1ml algal sample + 4ml acetone was added, and then all samples were kept in the dark at laboratory temperature in order to extract the chlorophyll.

The chlorophyll *a* samples (the controls and those treated) were analysed regularly (t_0, t_1, t_2, and t_3), using a chlorophyll fluorometer (Turner Design Instrument; model: #7200-00) which calibrates the low and high standard and f2/medium. The blank chlorophyll was measured and finally, concentrations of chlorophylls *a* were measured in µg L^{-1}.

2.5 Effect of the interaction of Green algae with single-walled carbon nanotubes on the feeding behaviour of mussels

The aim of this study was to detect the effect of algae-SWCNT interactions on mussel health, by determining the rate of pseudofaeces production, as well as DNA damage and oxidative stress.

2.5.1 Pseudofaeces

The procedure for determining pseudofaeces production using flow cytometry was performed according to Cucci *et al*. (1985).

Five mussels have not been fed, they were placed in the main test tank, and then they were fed a known final concentration of 1) algal cells (*Tetraselmis suecica*): 2) SWCNT 500µg L^{-1} alone and 3) *Tetraselmis suecica* + SWCNTs 500µg L^{-1} combined were applied to the tanks and the mussels left to feed for 10 minutes in order to observe any sedimented pseudofaeces, after which they were transferred to clean seawater and left to depurate for 24hrs. The control tank contained algal cultures *(Tetraselmis suecica)* only, without mussels, to correct for algal cell division during the course of the experiment (Cucci *et al.*, 1985) (Figure 2.13). All the experiments were replicated tree times.

At the end of the exposure feeding period, the rejected food (algae) and SWCNTs from the mussels were subsequently observed to be visibly sedimented in the tanks thought thier filtration system. The pseudofaeces of algal cell from mussels were analyzed by using flow cytometry.

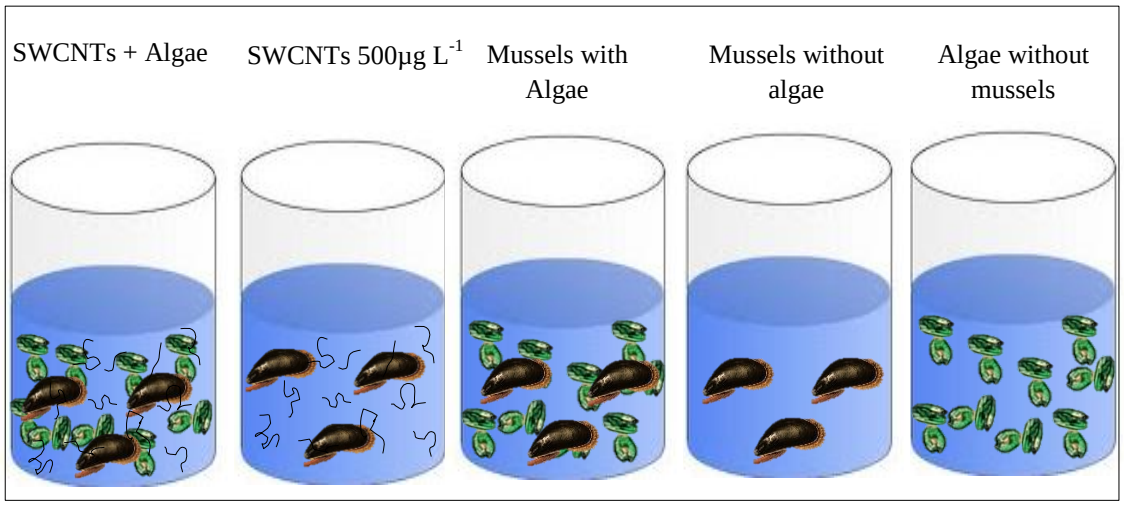

Figure 2.13 Combinations of algae and SWCNT and controls for the psuedofaeces test.

2.5.2 Comet assay and oxidative stress

Mussels were fed algae (*Tetraselmis suecica*), SWCNT 500µg L^{-1} alone and *Tetraselmis suecica* + SWCNTs 500µg L^{-1} for 24 hours. All treatments were replicated three times. The procedures of comet assay and oxidative stress were assessed in the same manner as previously described from p15 to p23.

2.6 Trophic transfer of SWCNTs from algae to mussels

The aim of this study was to assess the previously treated algal cells (*Tetraselmis suecica*), SWCNT 500µg L^{-1} alone and *Tetraselmis suecica* - SWCNTs 500µg L^{-1} to mussels through their filtration system. In order to assess the ingestion period of algae or SWCNTs, algae fed SWCNT 500µg L^{-1} for 7 days, and then three replications of mussels fed algae and divided in two groups; the first group of mussels were left to feed for 10 minutes, and the second group were left to feed for 24hrs. After the exposure period, the mussels were dissected to extract their guts to study them histologically and assess whether either the algae or the SWCNTs can be ingested into the mussels' guts. Before studying the trophic transfer histologically, parts of the gills were placed on a microscope slide to assess the presence of algae or SWCNTs on the gill epithelium, using a light microscope. For the histological study of the potential ingestion of algal cells or SWCNT into the mussels' guts, below the paraffin processing of tissue:

The mussels were placed whole in a wide-mouth jar filled with Davidson's fixative (20ml Formalin (40%), 10ml glycerol, 10ml glacial acetic acid, 30ml ethanol (100%) and 30ml seawater. The specimens were left in fixative for at least a week to ensure preservation of all tissues. After this time, the fixative was decanted and 70% ethanol added, for storage until processing (Kim *et al*., 2006). In the next step, all tissues were placed in labelled plastic cassettes, marked using a lead pencil which survives the processing fluid/solvent. The processing machine automatically transfer the cassettes using an automatic tissue processor (Shandon Duplex Tissue Processor), in a basket through the following; samples are carefully dehydrated with a graded absolute ethanol series: 70% ethanol for 1h (1x), 90% ethanol for 2h, and 100% ethanol for 1h (2x), and Histo-clear for 3hrs (1x), and finally, added molten wax for 2hrs (2x) at 60-66ºC. This dehydration process was used to allow the water in the biological samples to be slowly exchanged through liquids with lower surface tension (Stadtländer, 2007). The next step is embedding the tissues in paraffin blocks. In order to create smooth wax blocks, a small amount of molten paraffin was placed in the mould, a warm forceps was used to transfer the tissue into the mould, then, the mould was transferred to a cold plate, and gently, the tissue was pressed flat. Hot paraffin was then added to the mould from the paraffin dispenser. The paraffin was solidified for 30 min, until the tissue block was ready for sectioning. The tissue block was sectioned using a rotary microtome (LKB, Bromma, 2218 Historange microtome, Ref. No. 577). A fresh blade was placed on the microtome, the blade was angled at 5°, subsequently, and the block was inserted into the

microtome, the block was cut and the ribbon was floated on the surface of 37°C water bath. Sections were floated onto the surface of clean glass slides and allowed to dry in a warm. Once the sections are dried onto slide (about 1hour) they can be stained. All tissues are routinely stained with haematoxylin and eosin method. Stained slides were mounted under a glass cover slip using a synthetic mounting medium (Histomount). Finally, stained slides were seen clearly under the light microscope.

2.7 Data analysis

Statistical analysis was carried out using Sigmastat 2.03 (Systat Software). All graphs were produced using Sigmaplot 2001 (Systat Software). Superoxide dismutase data were arcsin transformed and analyzed with a one-way analysis of variance (ANOVA) followed by a Tukey all pairwise multiple comparisons procedure (Sparks, 2000). The thiobarbituric acid reactive substances data did not require transformation and were subjected directly to an ANOVA/Tukey analysis, as described above. The DNA damage was expressed as a percentage of tail DNA (Lovell and Omori, 2008) and determined using the image analysis software package Comet Assay IV (Perceptive Instruments). Following arcsin transformation, the data were statistically analyzed as described above. Algal cell growth, chlorophyll rates and the produced pseudofeaces of algal cells by mussels were analyzed with a one-way variance (ANOVA), followed by a Tukey all pairwise multiple comparison procedure.

3. RESULTS

3.1 Synthesis and characterization of stock SWCNT

3.1.1 Transmission Electronic Microscopy (TEM)

Single-walled carbon nanotubes were characterized in the stock suspension and, as far as possible, in environmental media under exposure conditions. The dispersion of SWCNT aggregates in 0.02% Suwannee River natural organic matter (SRNOM) for the stock preparation (1 g L^{-1}) and the effects of sonication are shown in Figure 3.1A and B, and following the transfer to seawater at 100µg L^{-1} and 500µg L^{-1} in Figure 3.1C and D; (spectrophotometric peaks at concentrations <100µg L^{-1} were not resolvable). SWCNT dispersion was assessed, both in the stock and under exposure conditions using spectrophotomteric analysis at abs 234nm (Buzea *et al.*, 2007) (Figure 3.1) and visualised using a TEM (Philips CM120-Biotwin) in stock suspensions (Figure 3.2).

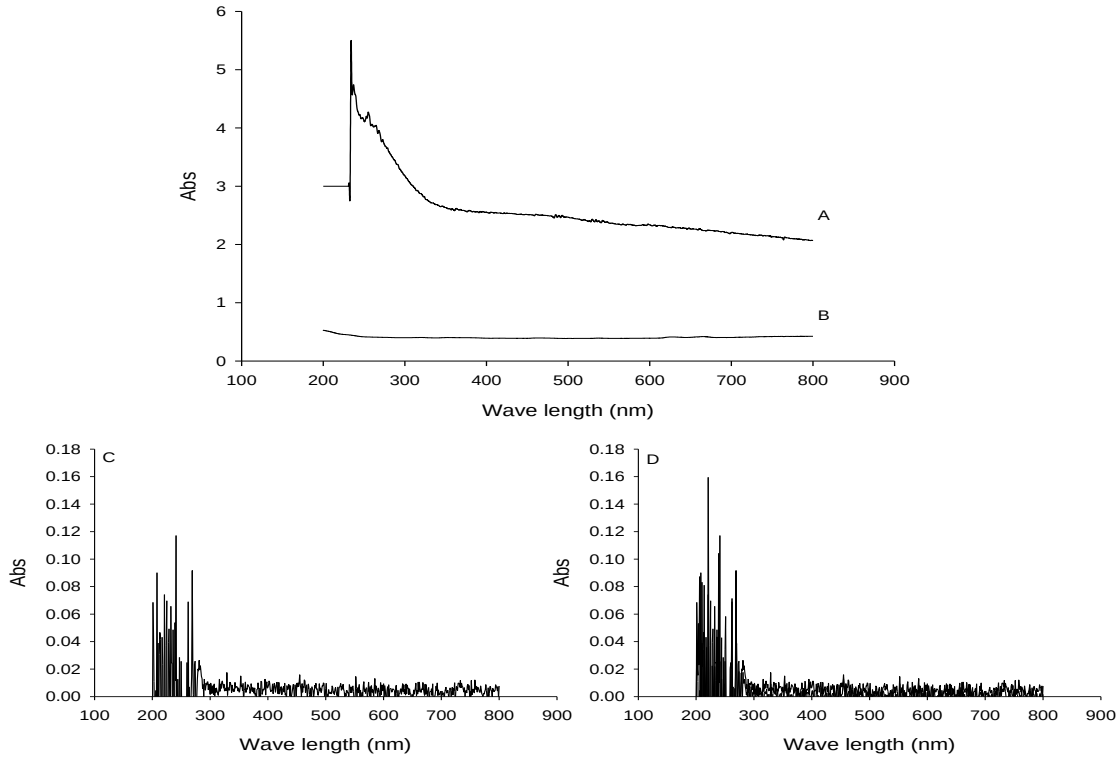

Figure 3.1 Spectrophotometric characterization of single-walled carbon nanotube (SWCNT) dispersion: spectra for SWCNT prepared in 0.02% Suwannee River natural organic matter solution with sonication (A) and without (B); spectra of SWCNT stock spiked in seawater at the final exposure concentrations of (C) 100mg L^{-1} and (D) 500mg L^{-1}. Abs = absorbance.

A visual examination of dispersion using TEM was only possible in the stock preparations (Figure 3.2).

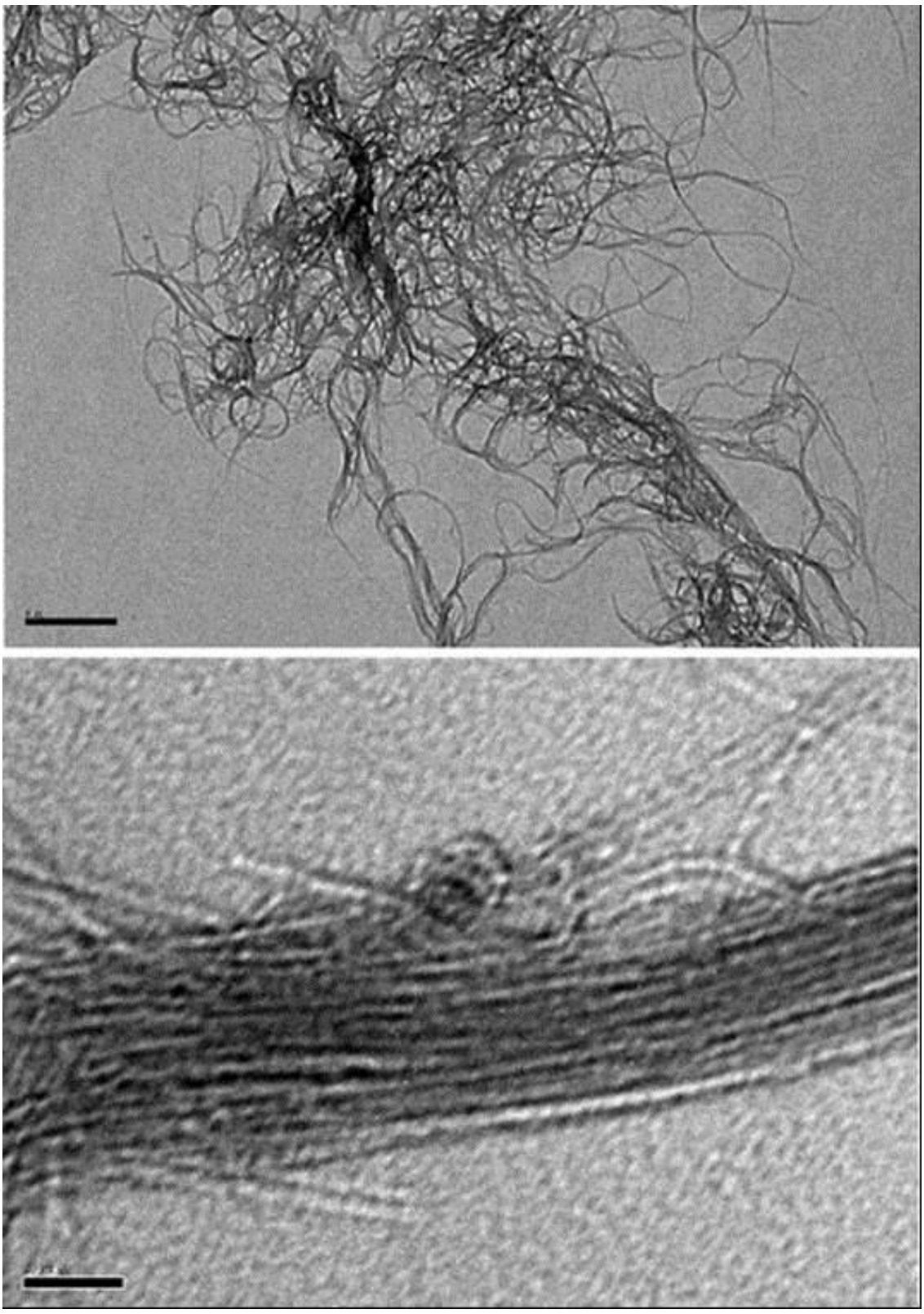

Figure 3.2 Transmission electron micrographs of single-walled carbon nanotube (SWCNT) stock preparations (1mg L^{-1} in 0.02% Suwannee River natural organic matter). Scale bars = 1µm (top) and 20 nm (bottom).

3.1.2 Scanning Electronic Microscope (SEM)

SWCNTs were characterized in the stock suspension and, as far as possible, in environmental media under exposure conditions. SWCNT dispersion in SRNOM was assessed using SEM is shown in Figure 3.3.

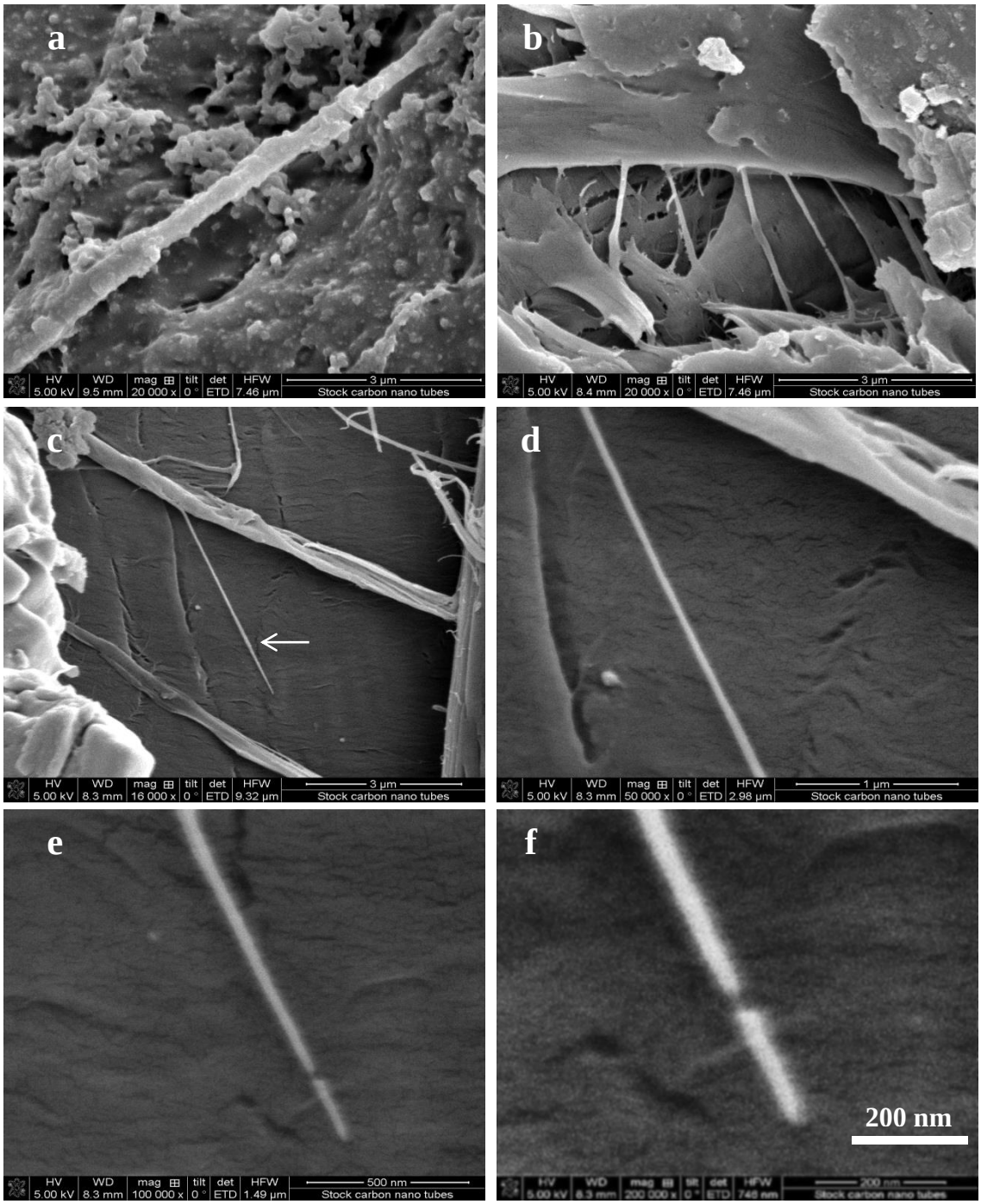

Figure 3.3 Scanning electron microscope images of SWCNT. (a) Thickness of SWCNT layers coated with 0.02% SRNOM, (b) Crystallized SWCNT-SRNOM films (c-f) SEM images of an individual SWCNT.

3.1.3 Dynamic light scattering (DLS) and Zeta potential

The surface of SWCNT aggregates dispersed in SRNOM were negatively charged under exposure conditions (Table 3.1), and their respective size approximations increased in a concentration-dependent manner (Figure 3.4).

Table 3.1 Single-walled carbon nanotube (SWCNT) characterization: Zeta potential

SWCNT (μgL^{-1})	Zeta potential (water)	Zeta potential[a] (seawater)	DLS (nm)	Zeta potential (%0.02 SRNOM)
5	–2.95	–8.84	475	-12.24
10	–4.49	–10.83	1384	
50	–7.75	–10.13	1740	
100	–5.25	–15.93	4982	
500	–6.86	–13.73	6206	

[a]pH 8.4, salinity 32 (±1) ‰.
DLS = dynamic light scattering

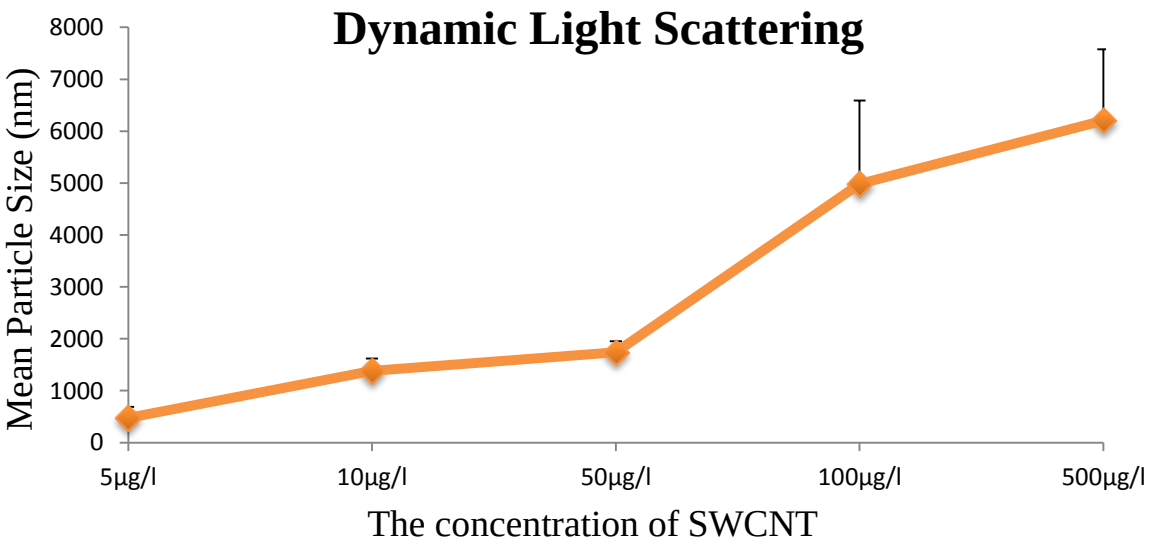

Figure 3.4 Dynamic light scattering (DLS) was used to measure SWCNTs size at nominal concentrations in seawater. Based on SWCNTs concentration, it was shown that gradual increases in SWCNTs concentration increase the mean of their agglomerate size (nm); (n=3).

3.1.4 Raman spectroscopy

The intensity of the Raman spectrum of SWCNT stock was observed at high frequency tangential (D-band) at 1590 cm^{-1}, and at low frequency, the RBM was observed at 268 cm^{-1}. Figure 3.5 shows a typical Raman spectrum acquired from SWCNT stock. Spectra were collected using a x50, 0.75 numerical aperture Leica N-plan objective to focus the excitation beam onto the SWCNTs and collect the scattered light.

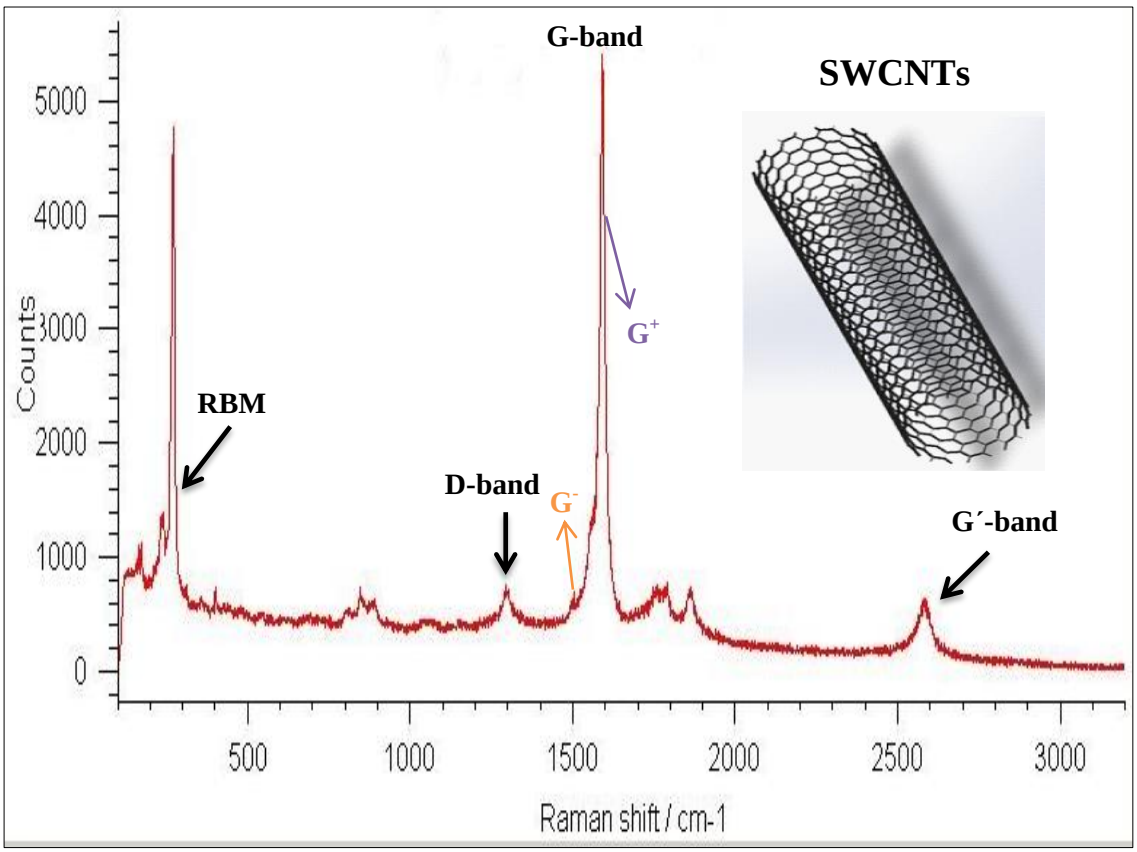

Figure 3.5 Spectrum from SWCNTs stock clearly shows the characteristic peaks of SWCNTs: radial breathing mode (RBM) at 268 cm^{-1}, D band at 1290 cm^{-1}, G band at 1590 cm^{-1}, and G´ band at 2585 cm^{-1}.

3.1.5 Atomic Absorbance spectrometer (AAS)

Chemical analysis was used to examine the SWCNT powder used for the presence of residual metal impurities (RMI) associated with the SWCNTs showed traces of Iron (Fe), Manganese (Mn) and Nickel (Ni) (Tables 3.2), and Figures 3.6-3.8 observed calibration of Fe, Mn and Ni standard curves between absorbance and concentration.

Table 3.2 Chemical analysis of residual metals in stock carbon nanotubes and metal–carbon nanotube association following experimental exposure

	Actual[a]	Single-walled carbon nanotube alone[a]	Metals remaining in supernatant	Metals associated with single walled carbon nanotubes[b]
Cd	6.95	ND	1.18	4.26
Zn	5.44	ND	1.73	3.43
Fe	___	0.7	ND	ND
Mn	___	0.05	ND	ND
Ni	___	0.38	ND	ND

[a]Concentrations in parts per million without Cd/Zn spike
[b]Concentrations in parts per million following metal spike.
ND = not detected.

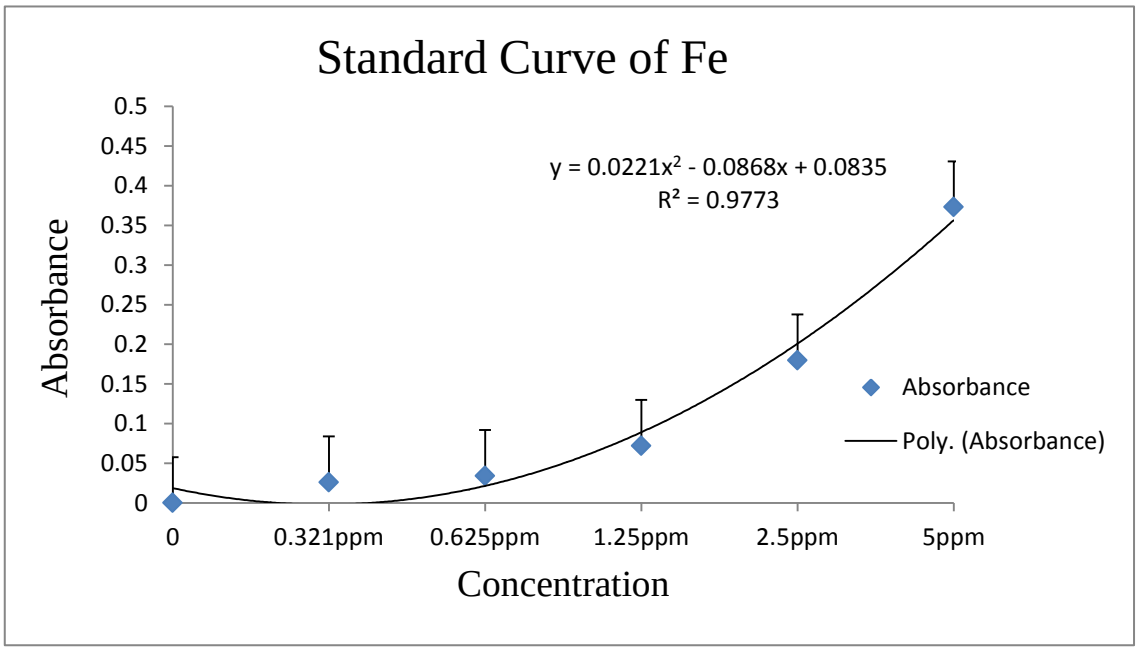

Figure 3.6 Calibration of Fe standard curve between absorbance and concentration.

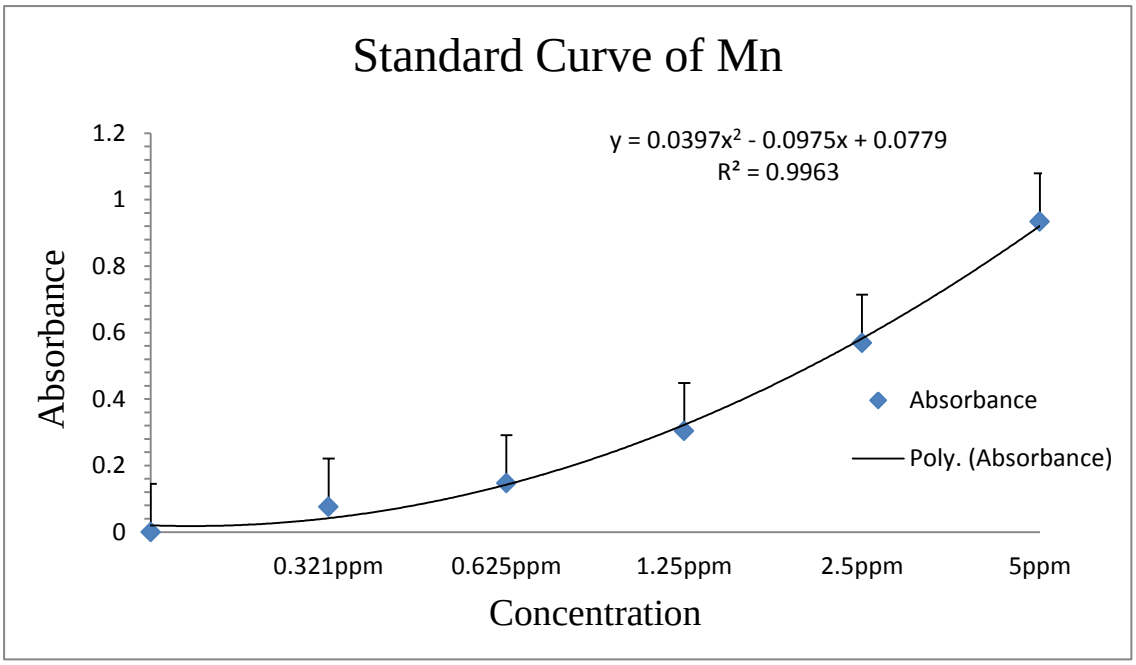

Figure 3.7 Calibration of Mn standard curve between absorbance and concentration.

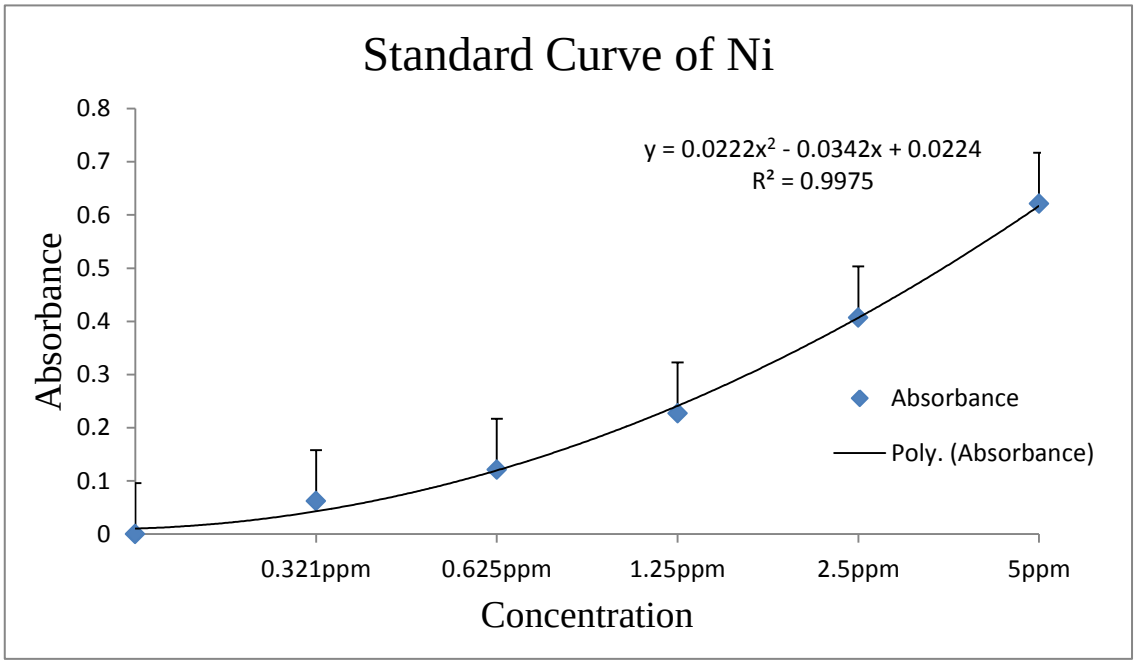

Figure 3.8 Calibration of Ni standard curve between absorbance and concentration.

3.1.6 The interaction between dissolved metals and SWCNTs

Figures 3.9-3.10 observed metal scavenging behaviour of SWCNTs for Cd^{+2} and Zn^{+2} under exposure conditions as shown in Tables 3.3-3.10.

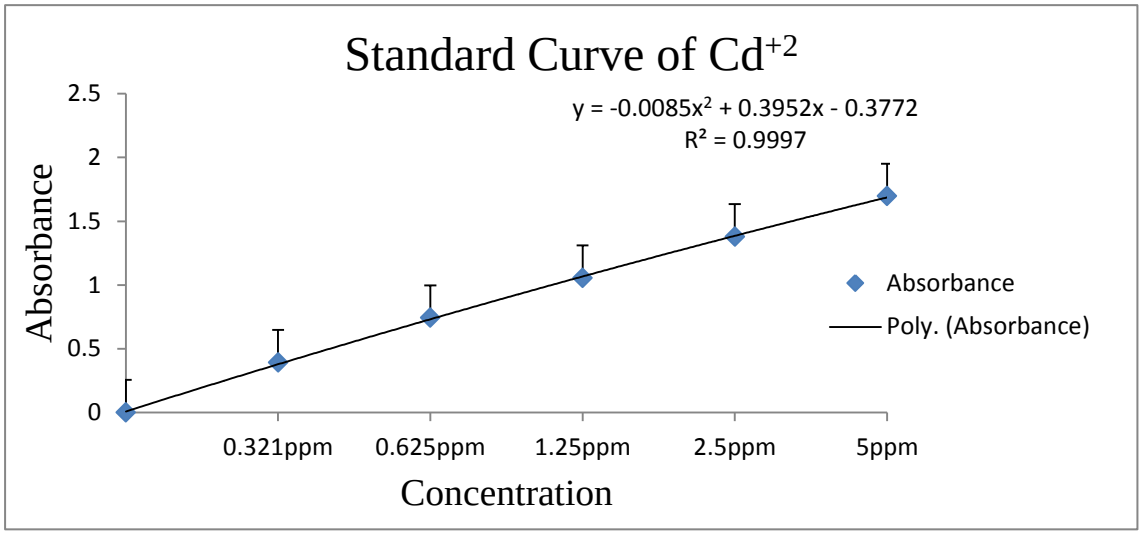

Figure 3.9 Calibration of Cd^{+2} standard curve between absorbance and concentration.

Table 3.3 Average of absorbance and recovery of chemical analysis for the cadmium concentration with CNT in supernatant**.**

Cadmium nominal	Cadmium measured (n=2)	Recovery %
6.954ppm	1.176ppm	16.91%

Table 3.4 The average of absorbance and recovery of chemical analysis for the cadmium concentration with CNT in pellet**.**

Cadmium nominal	Cadmium measured (n=2)	Recovery %
6.954ppm	4.260ppm	61.26%

Table 3.5 Average of absorbance and recovery of chemical analysis for the cadmium + zinc concentration with CNT in supernatant.

Cadmium nominal	Cadmium measured (n=2)	Recovery %
6.954ppm	1.653ppm	23.77%

Table 3.6 Average of absorbance and recovery of chemical analysis for the cadmium + zinc concentration with CNT in pellet.

Cadmium nominal	Cadmium measured (n=2)	Recovery %
6.954ppm	4.157ppm	59.79%

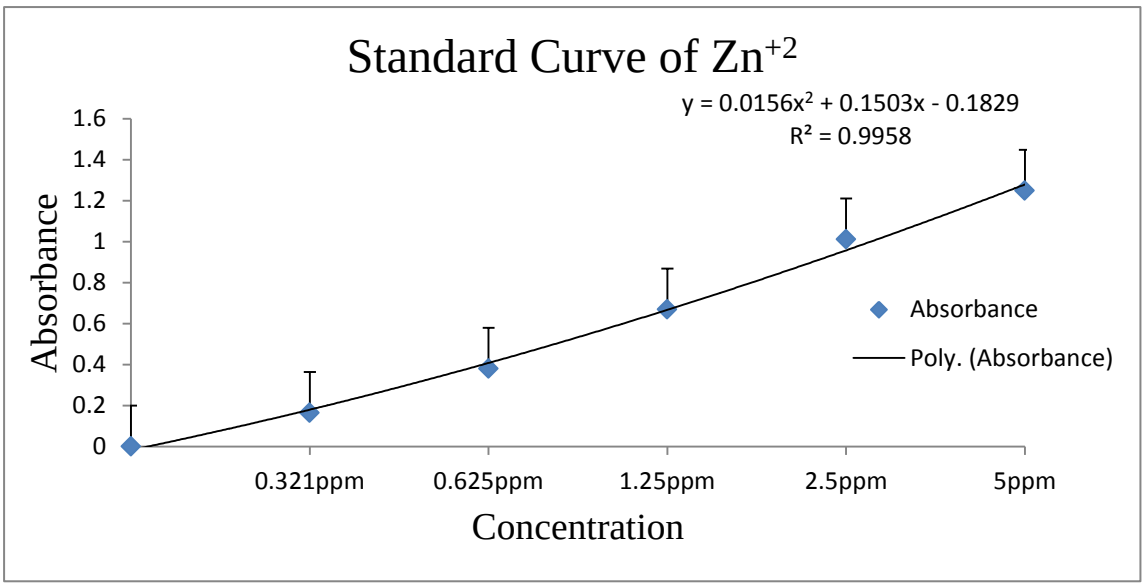

Figure 3.10 Calibration of Zn^{+2} standard curve between absorbance and concentration.

Table 3.7 Average of absorbance and recovery of chemical analysis for the zinc concentration with CNT in supernatant.

Zinc nominal	Zinc measured (n=2)	Recovery%
5.4ppm	1.734ppm	32.1%

Table 3.8 Average of absorbance and recovery of chemical analysis for the zinc concentration with CNT in pellet.

Zinc nominal	Zinc measured (n=2)	Recovery%
5.4ppm	3.432ppm	63.55%

Table 3.9 Average of absorbance and recovery of chemical analysis for the cadmium + zinc concentration with CNT in supernatant.

Zinc nominal	Zinc measured (n=2)	Recovery%
5.4ppm	1.673ppm	30.98%

Table 3.10 Average of absorbance and recovery of chemical analysis for the cadmium + zinc concentration with CNT in pellet.

Zinc nominal	Zinc measured (n=2)	Recovery%
5.4ppm	3.427ppm	63.46%

3.2 Potentiating toxicological interactions of single-walled carbon nanotubes with dissolved metals

3.2.1 SWCNT-gill interaction

The SWCNT aggregates were very effectively removed from the water column by filter-feeding *M. edulis*. On contact with the gills, the SWCNTs were coated in mucus, concentrated, transported across the gill surface to the nearest food groove, rolled into a food string, transported to the labial palps where they were visibly sorted out to bypass the mouth, and excreted as pseudofaeces (Figure 3.11). It is unclear whether any were ingested. Some, however, remained attached to the gill membranes for at least 24hrs following exposure. The black residues retained within the gills were confirmed as SWCNTs using confocal Raman spectroscopy (Figure 3.12).

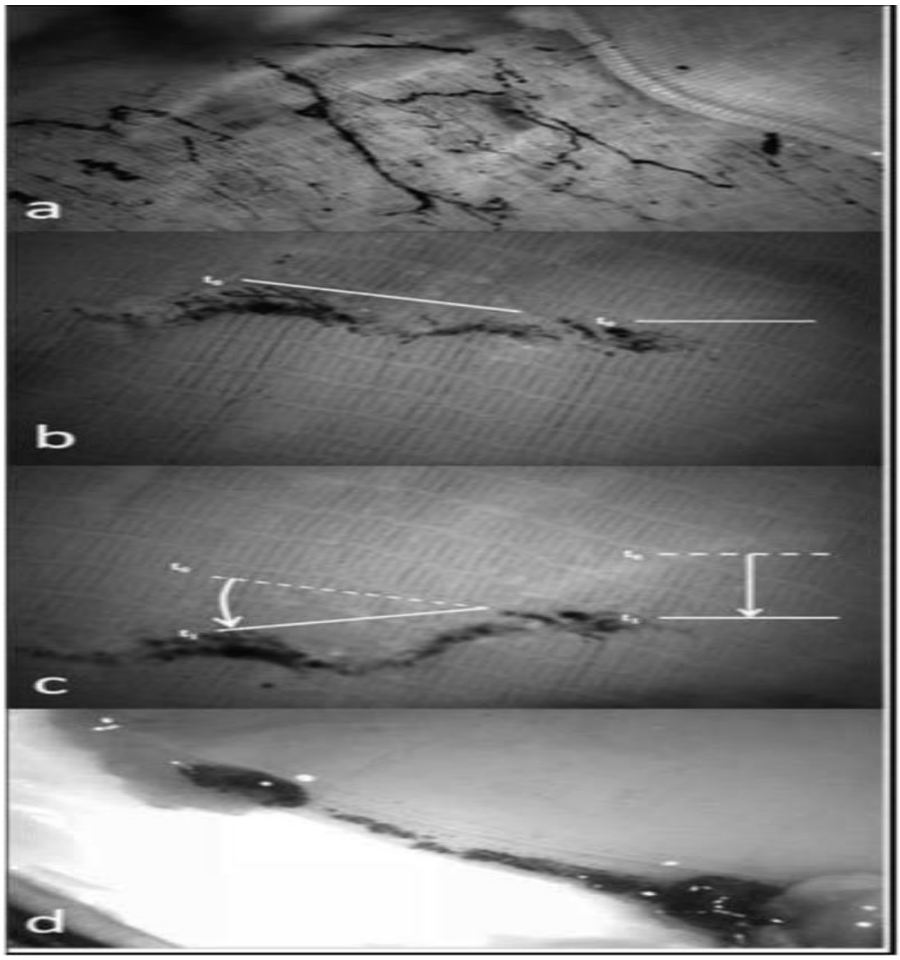

Figure 3.11 Light micrographs of single-walled carbon nanotubes (SWCNTs) interacting with mussel gills. (a) SWCNTs being collected on the inner demibranch; (b) a concentrated black front is formed; (c) the front moves rapidly toward the dorsal food groove $t_0 \rightarrow t_1$ approximately 2 min); and (d) food groove containing mucous-bound SWCNTs.

3.2.2 Raman microspectroscopy

The presence of SWCNTS in mussel gills was confirmed using Raman spectroscopy (Figure 3.12.)

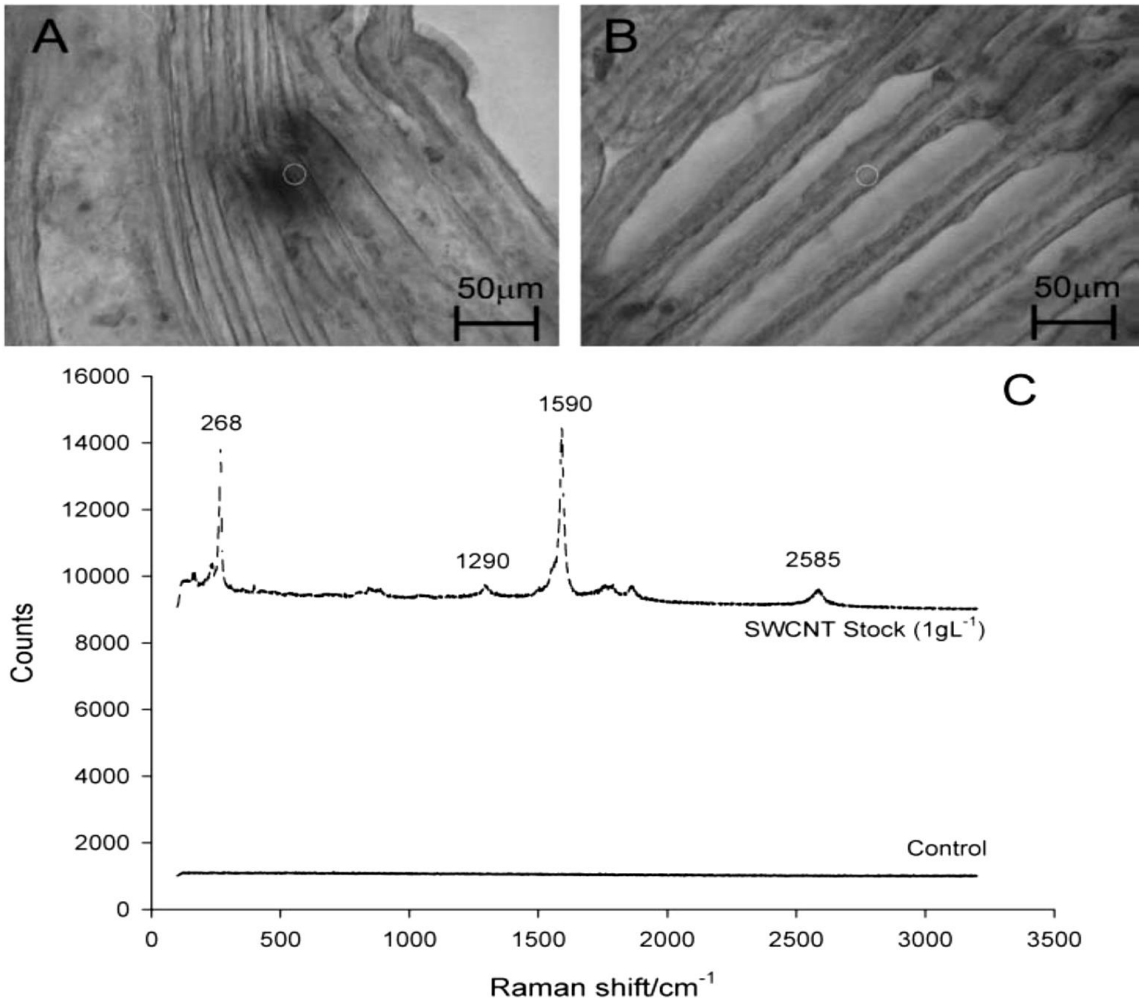

Figure 3.12 Raman spectroscopy were obtained using excitation at 785nm laser line, and the laser power was maintained at 5mW output power to avoid sample heating, because it is near infrared, so does not heat water or biological tissue therefore does not damage the samples. (A) Magnification (x 20) of gill tissue from mussels exposed to 100µg L^{-1} single-walled carbon nanotubes (SWCNTs) for 24hrs. (B) Magnification (x 20) of gill tissue from mussels not exposed to SWCNTs. The white circle in the centre of (A) and (B) marks the position of the Raman excitation beam spot, which was focused to a diameter of 1.7µm. (C) Representative Raman spectra acquired from SWCNT stock (top spectrum), mussel sample spiked with 100µg L^{-1} SWCNT for 24hrs (middle spectrum), and control gill tissue (bottom spectrum). Both spectra were collected using a 50x, 0.75 numerical aperture microscope objective lens. Spectrum from spiked sample.

3.2.3 Chemical analysis of cadmium and zinc in seawater and mussel's gills

M. edulis can take up and accumulate metals (Cd^{+2} and Zn^{+2}) through their filtration system; the results also showed that the uptake amount of metals by mussels was significant when metals interacted with SWCNTs (Table 3.11). Figure 3.13 shows the AAS standard calibration curve for Cd^{+2} and Zn^{+2}.

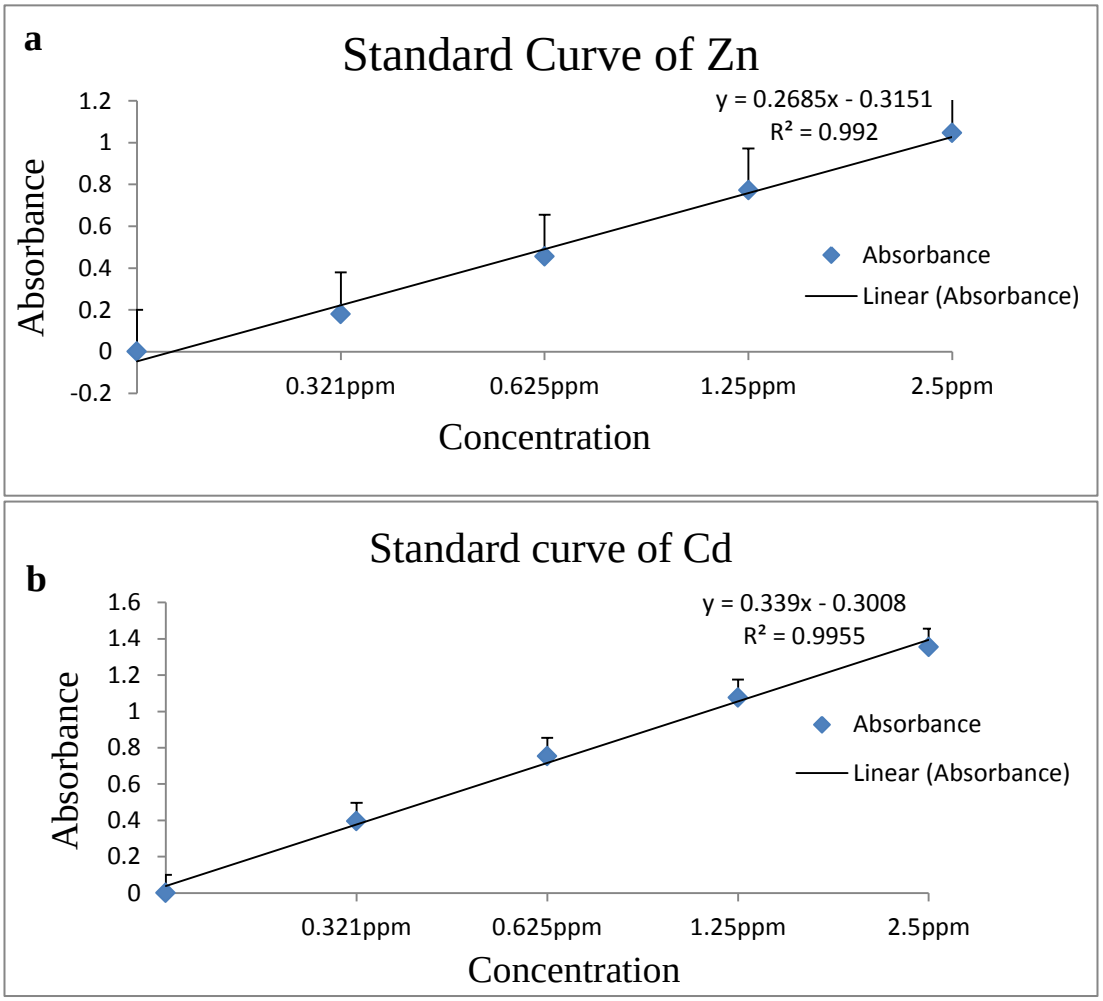

Figure 3.13 Calibration of (a) Cd^{+2} and (b) Zn^{+2} standard curve between absorbance and concentration.

Table 3.11 Chemical analysis of metals in gills and water following cadmium or zinc spike, with and without single-walled carbon nanotubes (SWCNTs)[a]

	Nominal Water	Gills (Metal only)	Recovery in water	Gills (Metal + SWCNT)	Recovery in water
Cadmium	6.954	1.16	2.01	1.79	1.97
Zinc	5.44	1.20	1.91	1.18	2.11

[a]Calibration in parts per million.

3.2.4 Biomarker analysis

3.2.4.1 Cell viability

Gill and haemocyte viability in *M. edulis* after the 72hrs exposure to SWCNTs 100µg L^{-1}, Cd 0.001µM, Cd 0.001µM + SWCNTs 100µg L^{-1}, Zn 1.0µM, Zn 1.0µM + SWCNTs 100µg L^{-1} and Cd 0.001µM + Zn 1.0µM + SWCNTs 100µg L^{-1} remained above 85% and 86%, respectively; n=3 Tables 3.12-3.13. The following example graph represents the cell populations of the mussel haemocyte or gill (Figure 3.14).

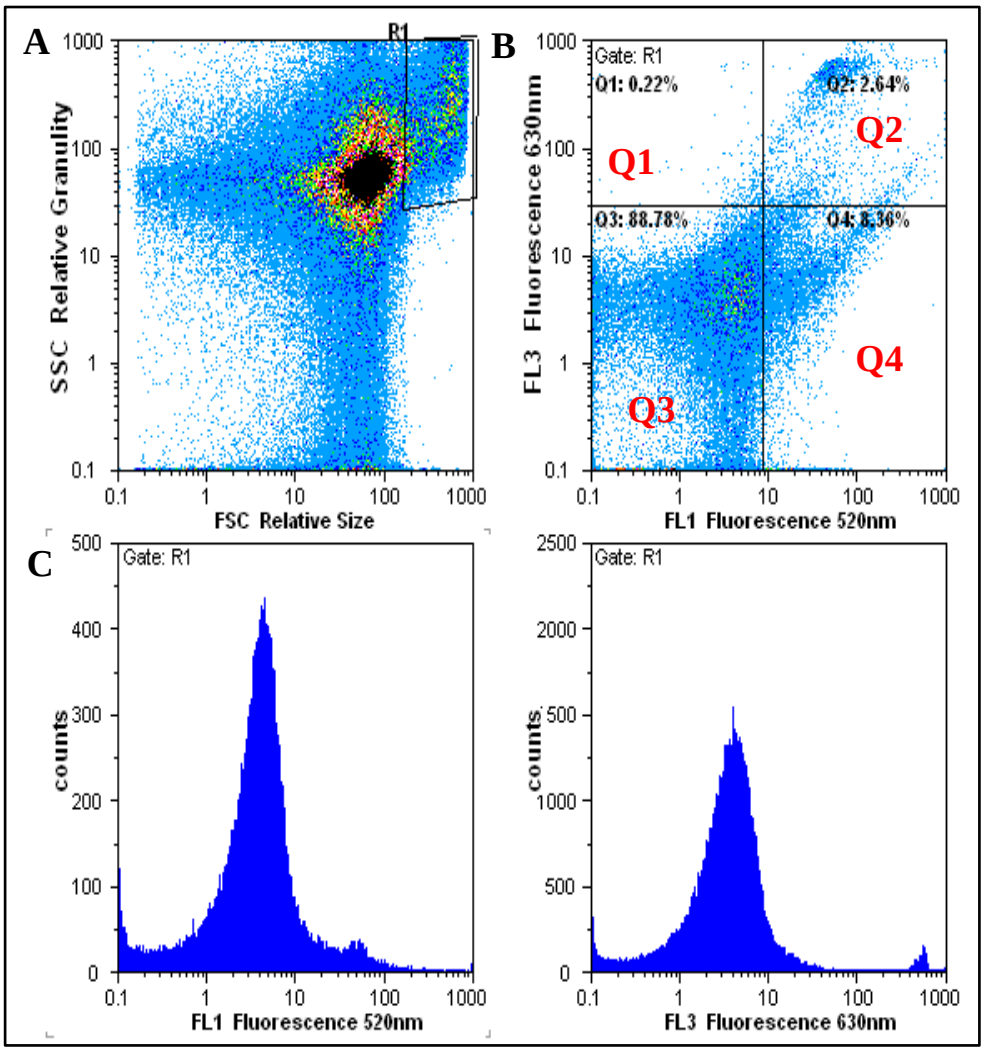

Figure 3.14 (A) R1 is the defined cell population from which other graphs are extrapolated. (B) R1 represents the cell population as a histograms. (C) Shows the ratio of live and dead cells. Live cells appear as one large fluorescence peak, whereas dead or necrotic cells are represented as one small fluorescence peak. Q1: background counts; Q2 apoptotic cells; Q3 live cells; Q4 necrotic cells. FL1 and FL3 are the lasers which excite the Pi and the fluorescence is recorded as counts on the histograms.

Table 3.12 Cell viability expressed as percentage of Q1 background counts; Q2 apoptotic cells; Q3 live cells; and Q4 necrotic cells in the mussel's haemocytes (n=3).

Number of mussels	Length of mussels	Spiked	Q1 (%)	Q2 (%)	Q3 (%)	Q4 (%)
3	5 ±0.5 cm	control	0.67	0.13	98.64	0.56
3	5 ±0.5 cm	Cd	1.86	3.53	95.73	3.88
3	5 ±0.5 cm	Zn	0.13	0.01	99.72	0.14
3	5 ±0.5 cm	SWCNTs	1.34	1.89	94.78	1.99
3	5 ±0.5 cm	Cd + SWCNTs	2.47	5.87	86.67	4.99
3	5 ±0.5 cm	Zn + SWCNTs	1.58	3.28	92.42	2.72
3	5 ±0.5 cm	Cd + Zn + SWCNTs	0.22	2.64	88.78	8.36

Table 3.13 Cell viability expressed as percentage of Q1: background counts; Q2 apoptotic cells; Q3 live cells and Q4 necrotic cells in the mussel's gills (n=3).

Number of mussels	Length of mussels	Spiked	Q1 (%)	Q2 (%)	Q3 (%)	Q4 (%)
3	5 ±0.5 cm	control	0.71	0.13	99.13	0.03
3	5 ±0.5 cm	Cd	16.33	0.00	92.27	0.00
3	5 ±0.5 cm	Zn	2.74	0.00	97.26	0.00
3	5 ±0.5 cm	SWCNTs	10.49	0.00	87.15	0.00
3	5 ±0.5 cm	Cd + SWCNTs	17.42	0.75	85.46	0.37
3	5 ±0.5 cm	Zn + SWCNTs	7.73	0.00	87.67	0.00
3	5 ±0.5 cm	Cd + Zn + SWCNTs	12.85	0.00	89.51	0.00

3.2.4.2 Comet assay and Oxidative stress

In the present study, *M. edulis* exposed to 0.001µM $CdCl_2$ for 72hrs did not incur DNA damage in haemocytes or gill cells significantly above that of the control group (p = 0.996). Unlike cadmium, zinc is less genotoxic, and in the present study even concentrations as high as 1µM failed to induce DNA damage in both haemocytes (p = 0.984) and gill cells (p = 0.999) higher than the level recorded in the controls. Furthermore, metals in combination (0.001µM $CdCl_2$ + 1µM Zn) did not incur DNA damage in haemocytes (p = 0.999) or gill cells (p = 0.834) significantly above that of the control group (Figures 3.15, 17, 19, 21, 23A and B). A SRNOM control and SRNOM control with either 0.001µM $CdCl_2$ or 1µM Zn and both metals combined had also no significant genotoxic effect in either gill cells or haemocytes (p = 0.997; p = 0.865; Figures 3.25A and B). No significantly increased DNA damage was measured in gill cells (Figures 3.15, 17, 19A; p = 0.992) and haemocytes (Figures 3.15, 17, 18B; p = 0.006) of mussels exposed *in vivo* to 5µg L^{-1}, 10µg L^{-1}, or 50µg L^{-1} SWCNTs alone. However, exposure of mussels to 100µg L^{-1} and 500µg L^{-1} SWCNTs alone did show significantly increased DNA strand breaks in both gill cells (Figures 3.21, 23A; $p<0.05$) and haemocytes3.21, 23B; $p<0.05$). The combined effect of in vivo exposure of mussels to SWCNTs (5–500µg L^{-1}) and subthreshold concentrations of metals (0.001µM $CdCl_2$ or 1µM ZnSO4) on the degree of DNA damage in both gill cells and haemocytes was significantly elevated above that following exposure to metals or SWCNTs alone (Figures 3.15, 17, 19, 21, 23A and B; $p<0.05$).

Superoxide dismutase activity (expressed as percentage of inhibition) in gill tissue from mussels exposed to 0.001µM $CdCl_2$ alone, 1µM Zn alone, 0.001µM $CdCl_2$ + 1µM Zn and SWCNTs alone at concentrations of up to 50µg L^{-1} was not significantly increased above the control (Figures 3.16, 18, 20, 22, 24A and B). A Suwannee River natural organic matter control and Suwannee River natural organic matter with either 0.001µM $CdCl_2$ or 1µM Zn and both metals combined also no showed significantly increased oxidative stress, expressed as superoxide dismutase (SOD) activity ($P<0.001$) and lipid peroxidation in gills (P=0.032; Figures 3.26A and B). However, concentrations of 100µg L^{-1} and above did induce significantly increased superoxide dismutase activities (Figures 3.22, 23A). Lipid peroxidation (expressed as TBARS nMol mg $protein^{-1}$) in gill cells from mussels exposed to SWCNTs alone at concentrations of up to 50µg L^{-1} did not increase above the control (Figures 3.16, 18, 20B). However, concentrations of 100µg L^{-1} and above did induce significantly increased incidences of lipid peroxidation

(Figures 3.22, 24B). The combined *in vivo* exposure of mussels to SWCNTs (5–500µg L^{-1}) and subthreshold concentrations of metals (0.001µM $CdCl_2$ or 1µM ZnSO4) did induce significantly increased superoxide dismutase (SOD) activity and lipid peroxidation in gills ($p<0.05$). Exposure of mussels to appropriately upscaled concentrations of RMI found associated in trace amounts with the carbon nanotubes showed no effect on any of the endpoints measured (Figure 3.27, 28A and B).

Group 1. *Mytilus edulis* exposed *in vivo* to 0.001 µM $CdCl_2$, 1 µM ZnSO4, 0.001 µM $CdCl_2$ + 1 µM ZnSO4, 5µgL^{-1} SWCNTs, Cd 0.001µM + SWCNTs 5µgL^{-1}, Zn 1.0µM + SWCNTs 5µgL^{-1} and Cd 0.001µM + Zn 1.0µM and Cd 0.001µM + Zn 1.0µM + SWCNTs 5µgL^{-1} for 72 hours.

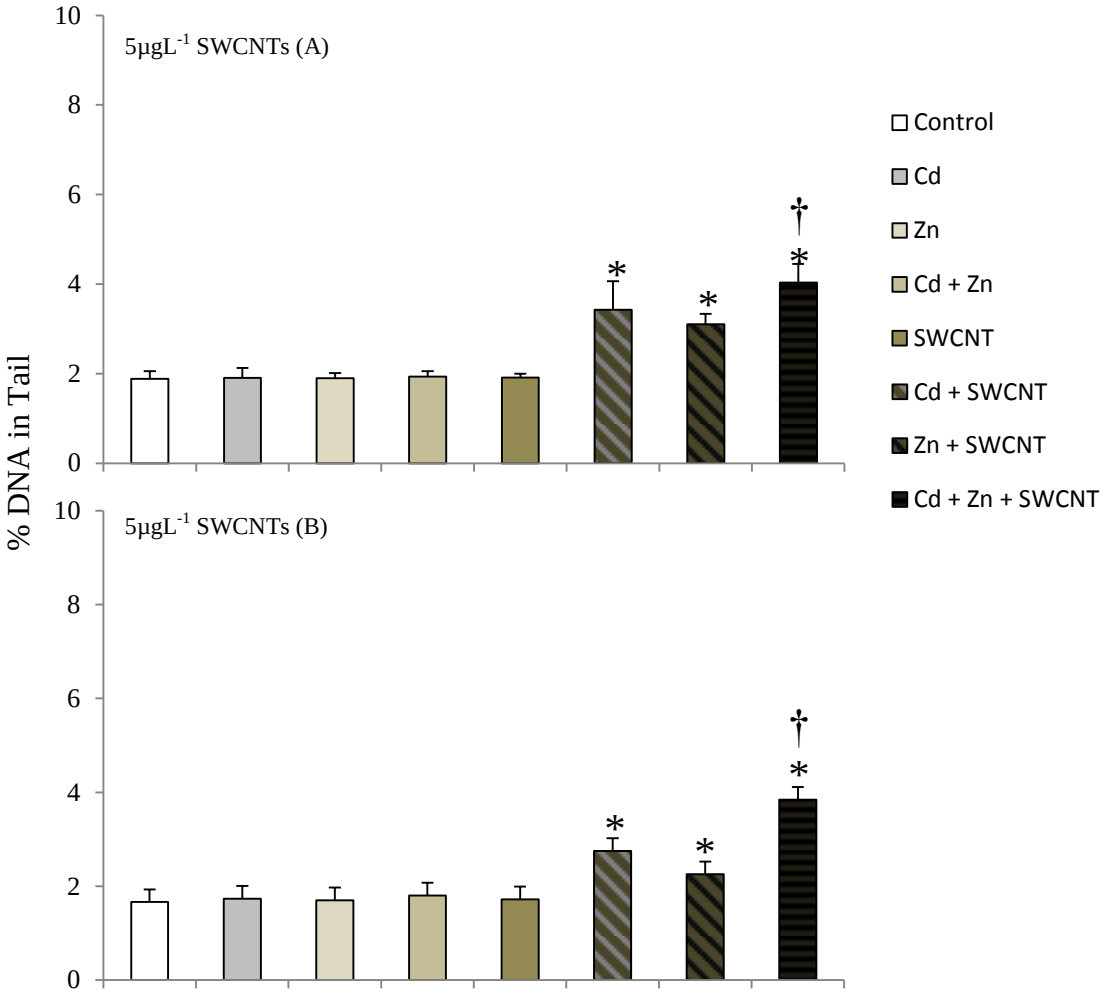

Figure 3.15 DNA damage, expressed as percentage of DNA in the tail, in (A) gill cellsand (B) haemocytes of *M. edulis*. * significantly different from control, Cd, Zn, Cd + Zn and SWCNTs alone; † significantly different from SWCNTs + Cd or Zn ($p<0.05$; means ± standard deviation, n=5).

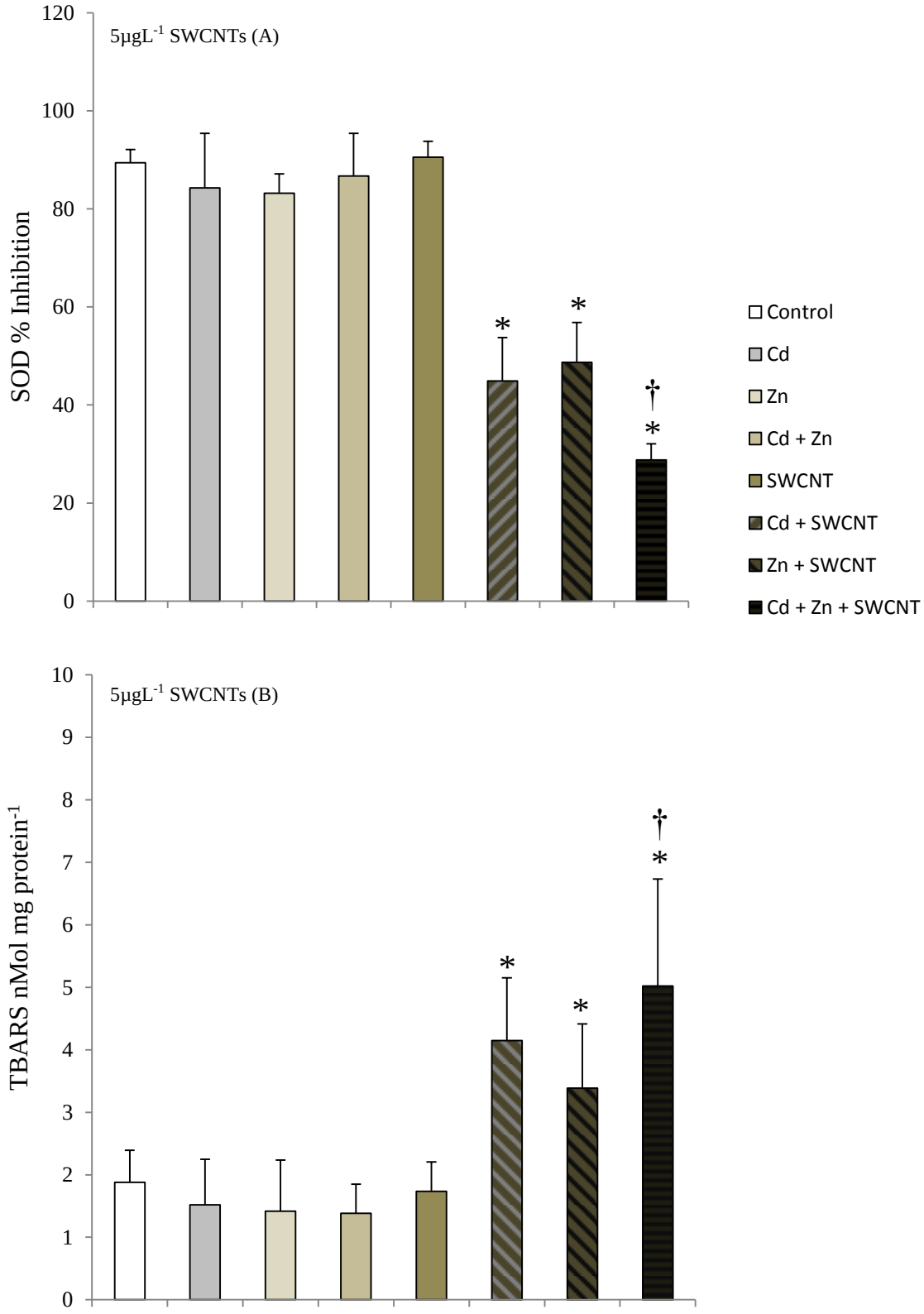

Figure 3.16 Superoxide dismutase activity expressed as percentage of inhibition (A) and (B) thiobarbituric acid reactive substances expressed as nanograms per milligram protein. * significantly different from control, Cd, Zn, Cd + Zn and SWCNTs alone; † significantly different from SWCNTs + Cd or Zn ($p<0.05$; means ± standard deviation, n=5).

Group 2. *Mytilus edulis* exposed *in vivo* to 0.001 µM $CdCl_2$, 1 µM ZnSO4, 0.001 µM $CdCl_2$ + 1 µM ZnSO4, 10µgL^{-1} SWCNTs, Cd 0.001µM + SWCNTs 10µgL^{-1}, Zn 1.0µM + SWCNTs 10µgL^{-1} and Cd 0.001µM + Zn 1.0µM and Cd 0.001µM + Zn 1.0µM + SWCNTs 10µgL^{-1} for 72 hours.

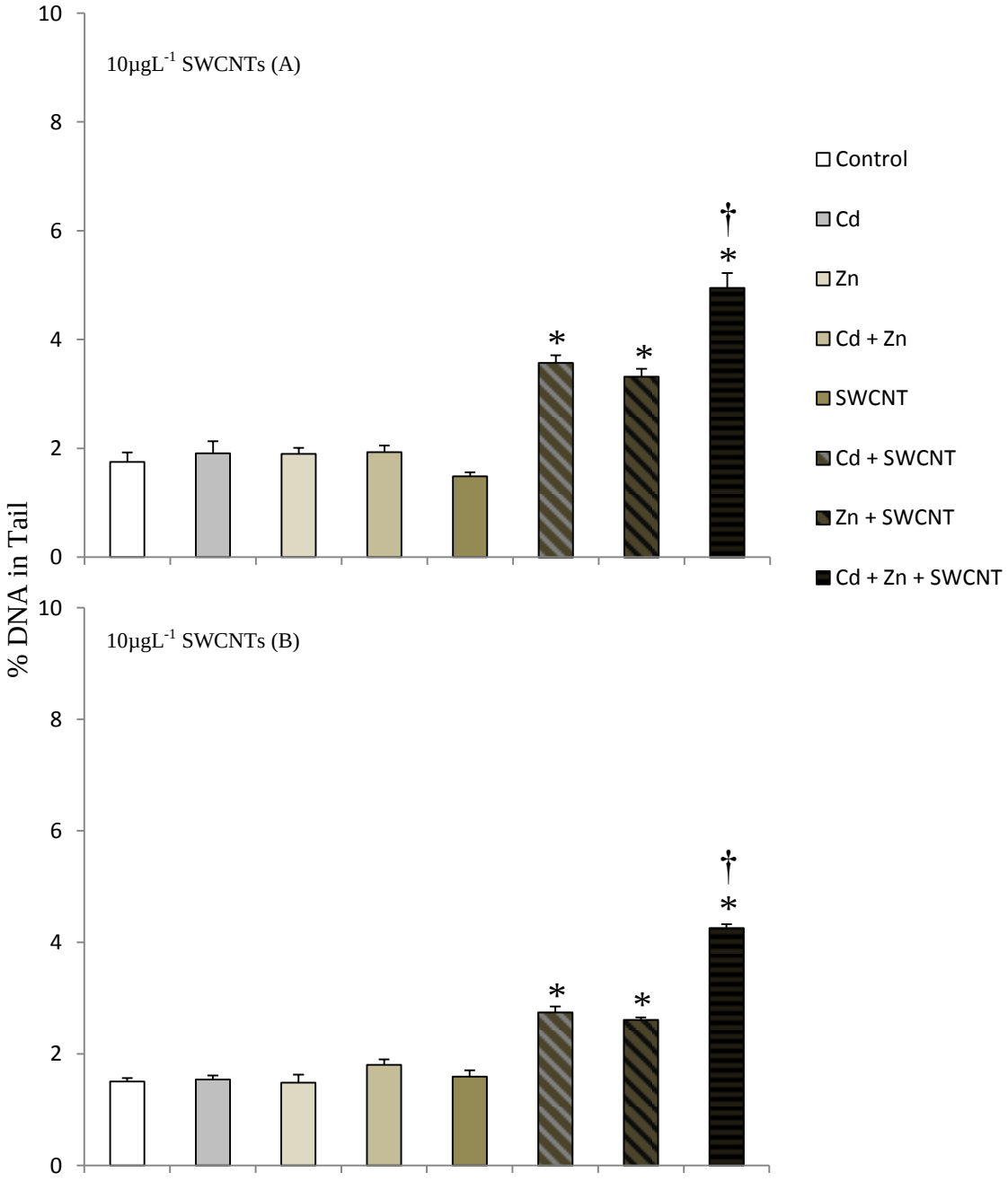

Figure 3.17 DNA damage, expressed as percentage of DNA in the tail, in (A) gill cells and (B) haemocytesof *M. edulis*. Statistically, there is significantly increased DNA damage was measured in gill and hemocytes. * significantly different from control, Cd, Zn, Cd + Zn and SWCNTs alone; † significantly different from SWCNTs + Cd or Zn ($p<0.05$; means ± standard deviation, n=5).

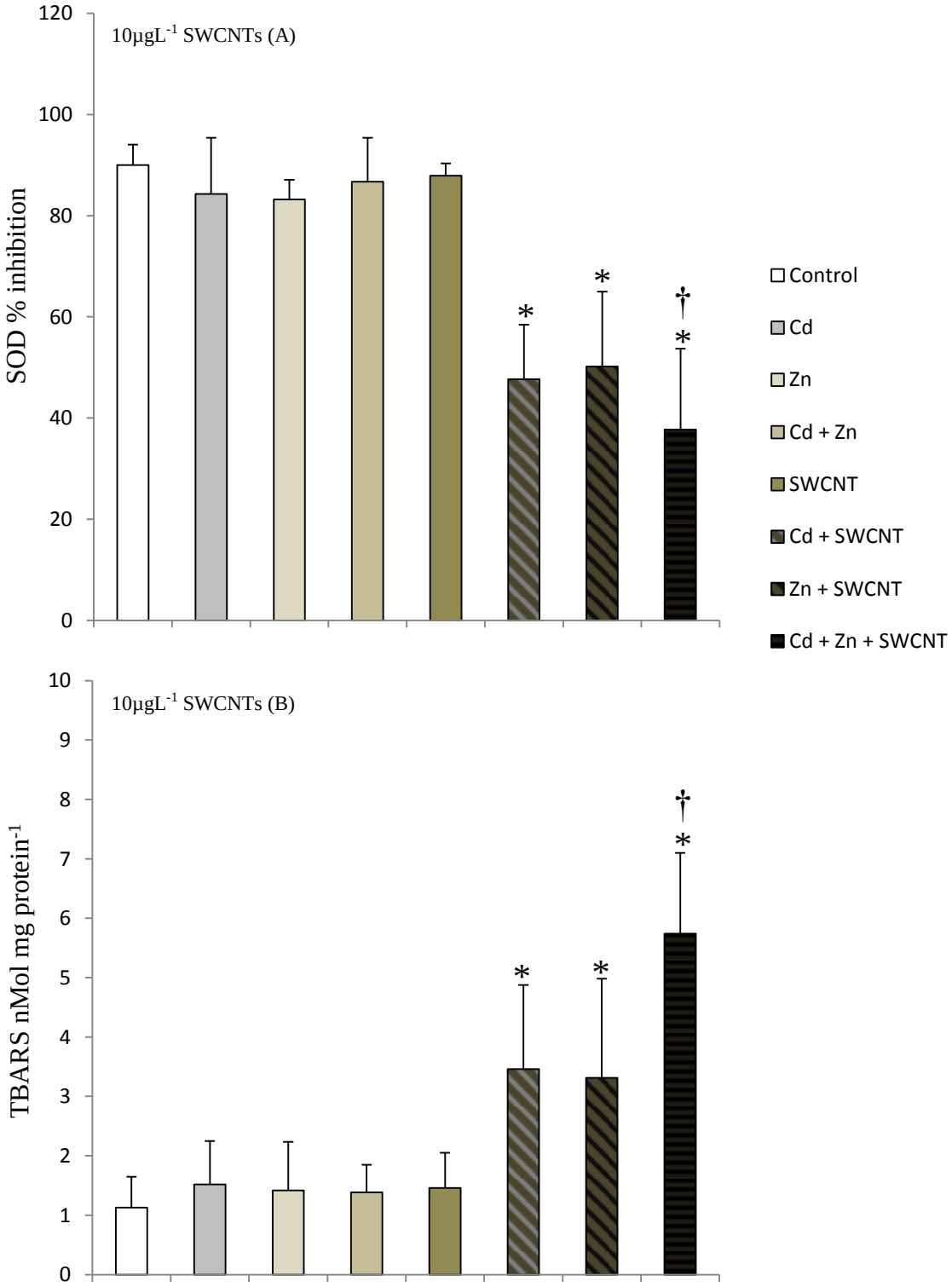

Figure 3.18 Superoxide dismutase activity expressed as percentage of inhibition (A) and (B) thiobarbituric acid reactive substances expressed as nanograms per milligram protein. * significantly different from control, Cd, Zn, Cd + Zn and SWCNTs alone; † significantly different from SWCNTs + Cd or Zn ($p<0.05$; means ± standard deviation, n=5).

Group 3. *Mytilus edulis* exposed *in vivo* to 0.001 µM $CdCl_2$, 1 µM ZnSO4, 0.001 µM $CdCl_2$ + 1 µM ZnSO4, 50µgL^{-1} SWCNTs, Cd 0.001µM + SWCNTs 50µgL^{-1}, Zn 1.0µM + SWCNTs 50µgL^{-1} and Cd 0.001µM + Zn 1.0µM and Cd 0.001µM + Zn 1.0µM + SWCNTs 50µgL^{-1} for 72 hours.

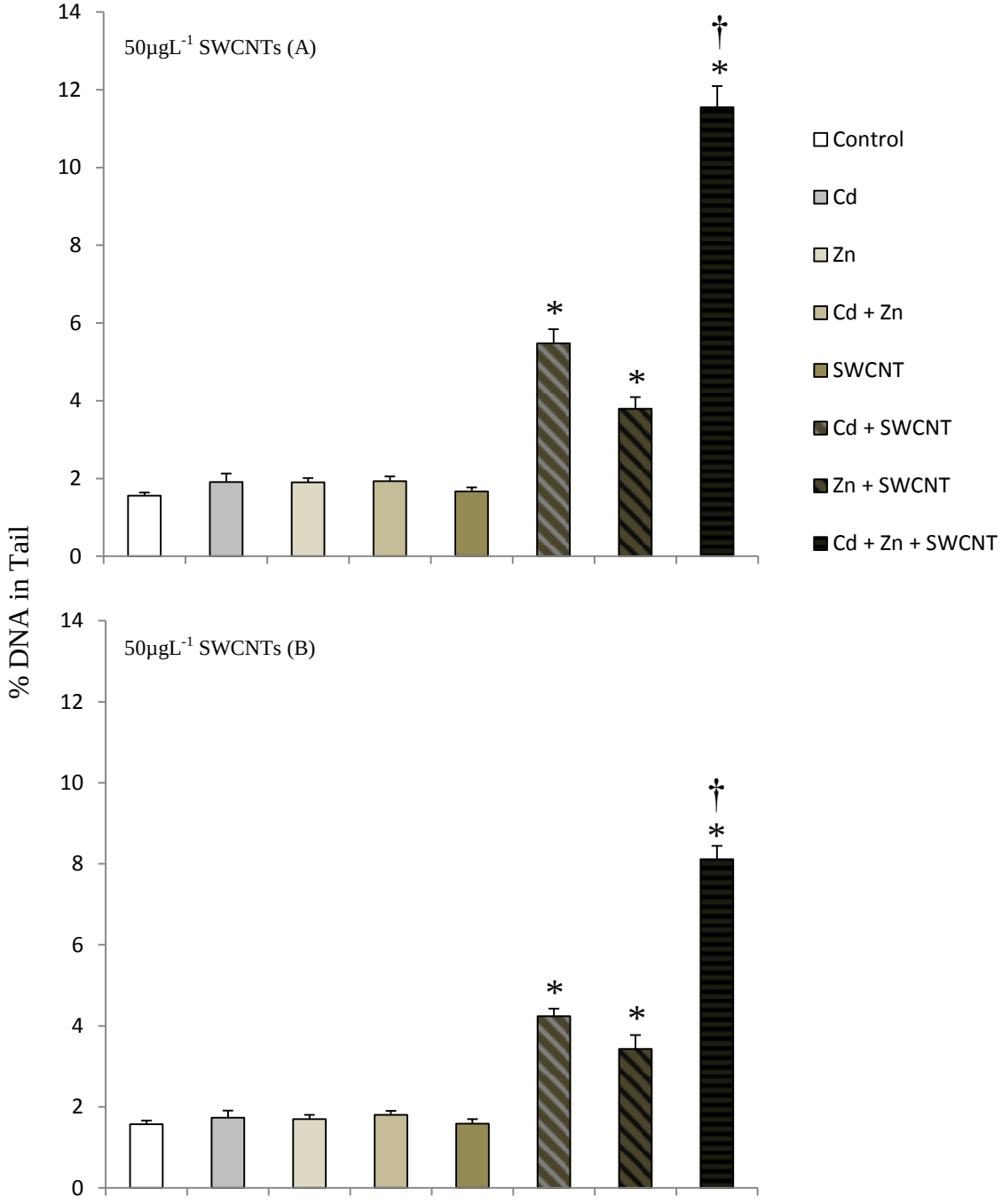

Figure 3.19 DNA damage, expressed as percentage of DNA in the tail, in (A) gill cells and (B) haemocytes of *M. edulis*. Statistically, there is significantly increased DNA damage was measured in gill and hemocytes. * significantly different from control, Cd, Zn, Cd + Zn and SWCNTs alone; † significantly different from SWCNTs + Cd or Zn ($p<0.05$; means ± standard deviation, n=5).

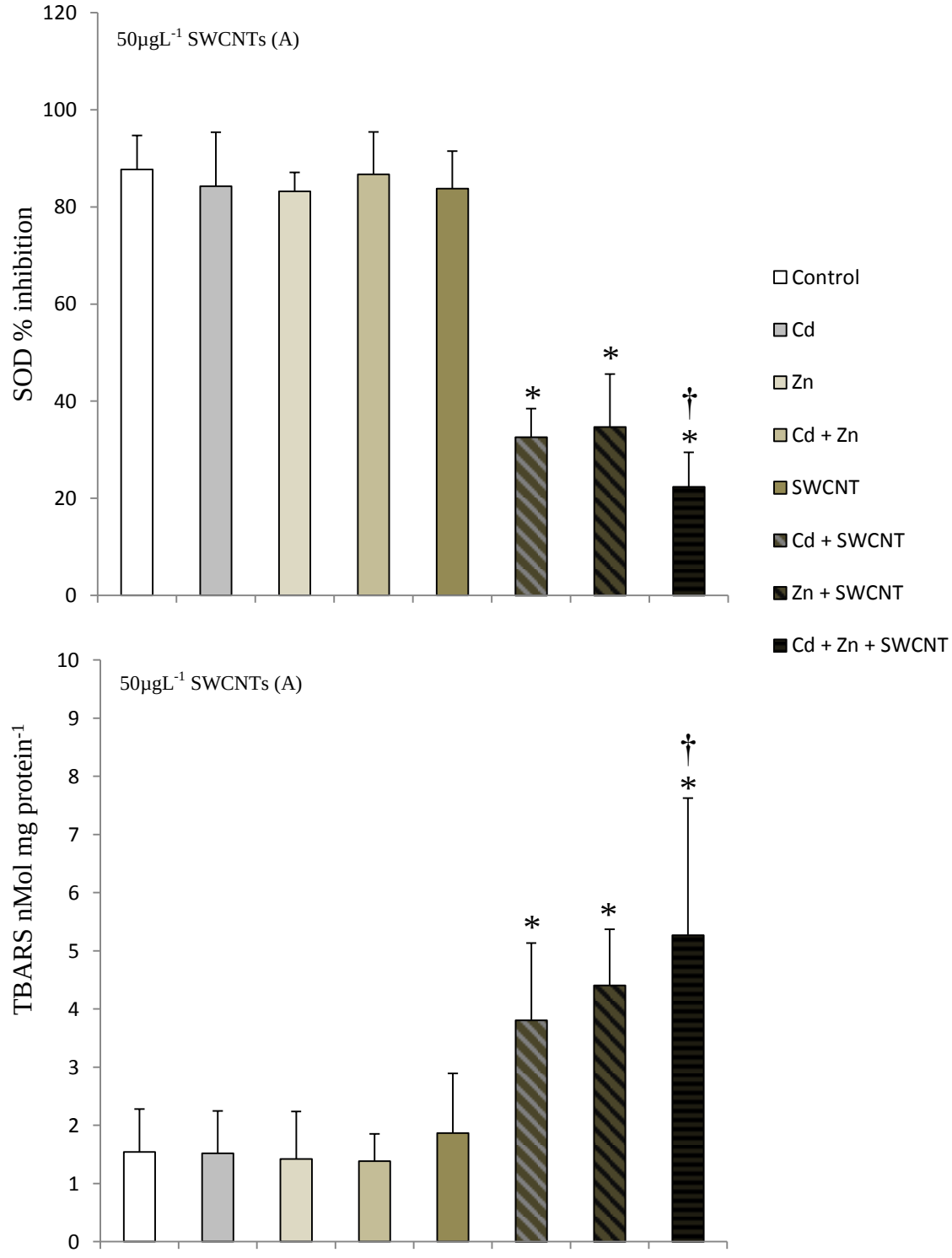

Figure 3.20 Superoxide dismutase activity expressed as percentage of inhibition (A) and (B) thiobarbituric acid reactive substances expressed as nanograms per milligram protein. * significantly different from control, Cd, Zn, Cd + Zn and SWCNTs alone; † significantly different from SWCNTs + Cd or Zn ($p<0.05$; means ± standard deviation, n=5).

Group 4. *Mytilus edulis* exposed *in vivo* to 0.001 µM $CdCl_2$, 1 µM ZnSO4, 0.001 µM $CdCl_2$ + 1 µM ZnSO4, 100µgL^{-1} SWCNTs, Cd 0.001µM + SWCNTs 100µgL^{-1}, Zn 1.0µM + SWCNTs 100µgL^{-1} and Cd 0.001µM + Zn 1.0µM and Cd 0.001µM + Zn 1.0µM + SWCNTs 100µgL^{-1} for 72 hours.

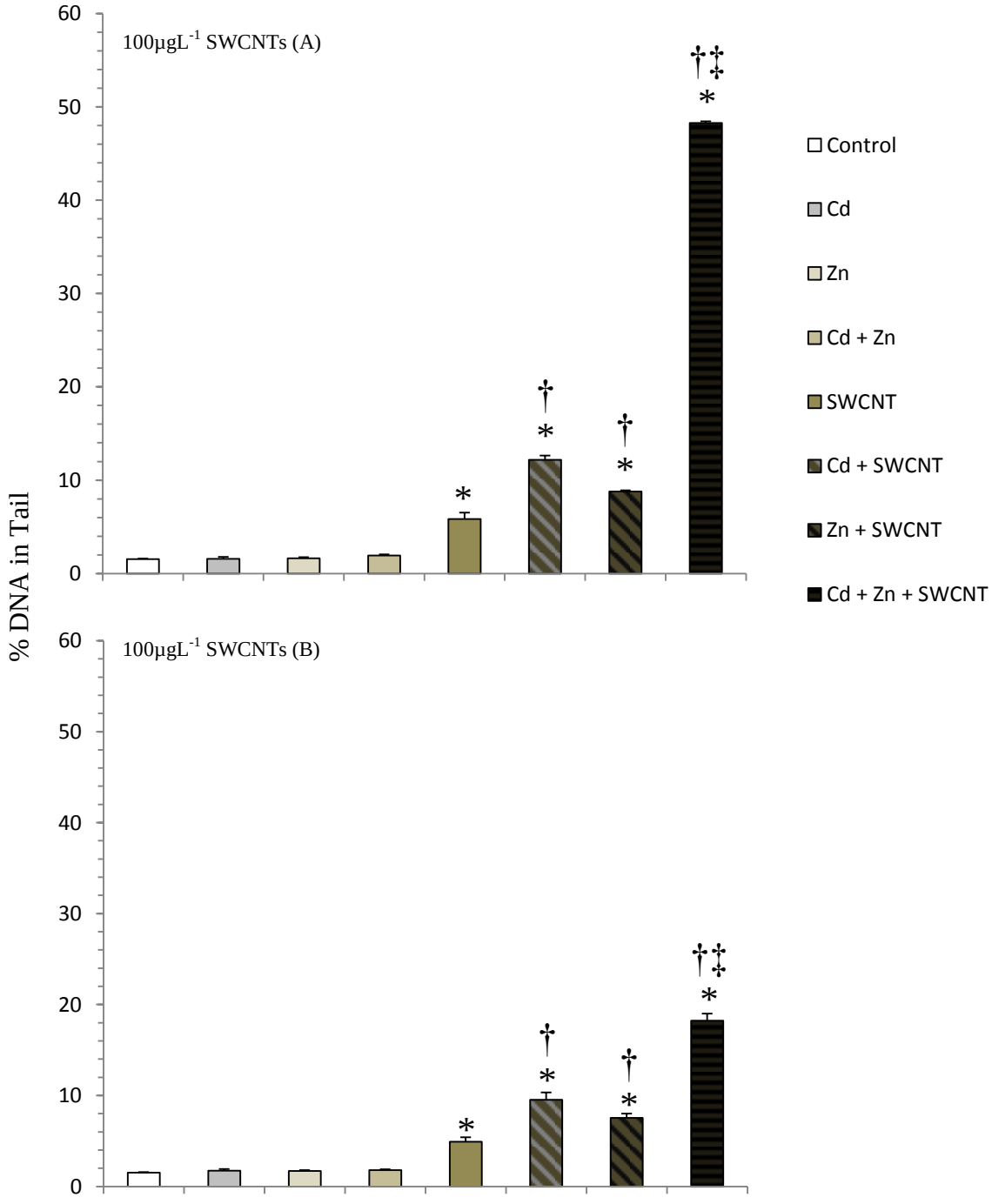

Figure 3.21 DNA damage, expressed as percentage of DNA in the tail, in (A) gill cells and (B) haemocytesof *M. edulis*. Statistically, there is significantly increased DNA damage was measured in gill and haemocytes. * significantly different from control, Cd, Z and Cd + Zn; † significantly different from SWCNTs alone; ‡ significantly different from SWCNTs + Cd or Zn ($p<0.05$; means ± standard deviation, n=5).

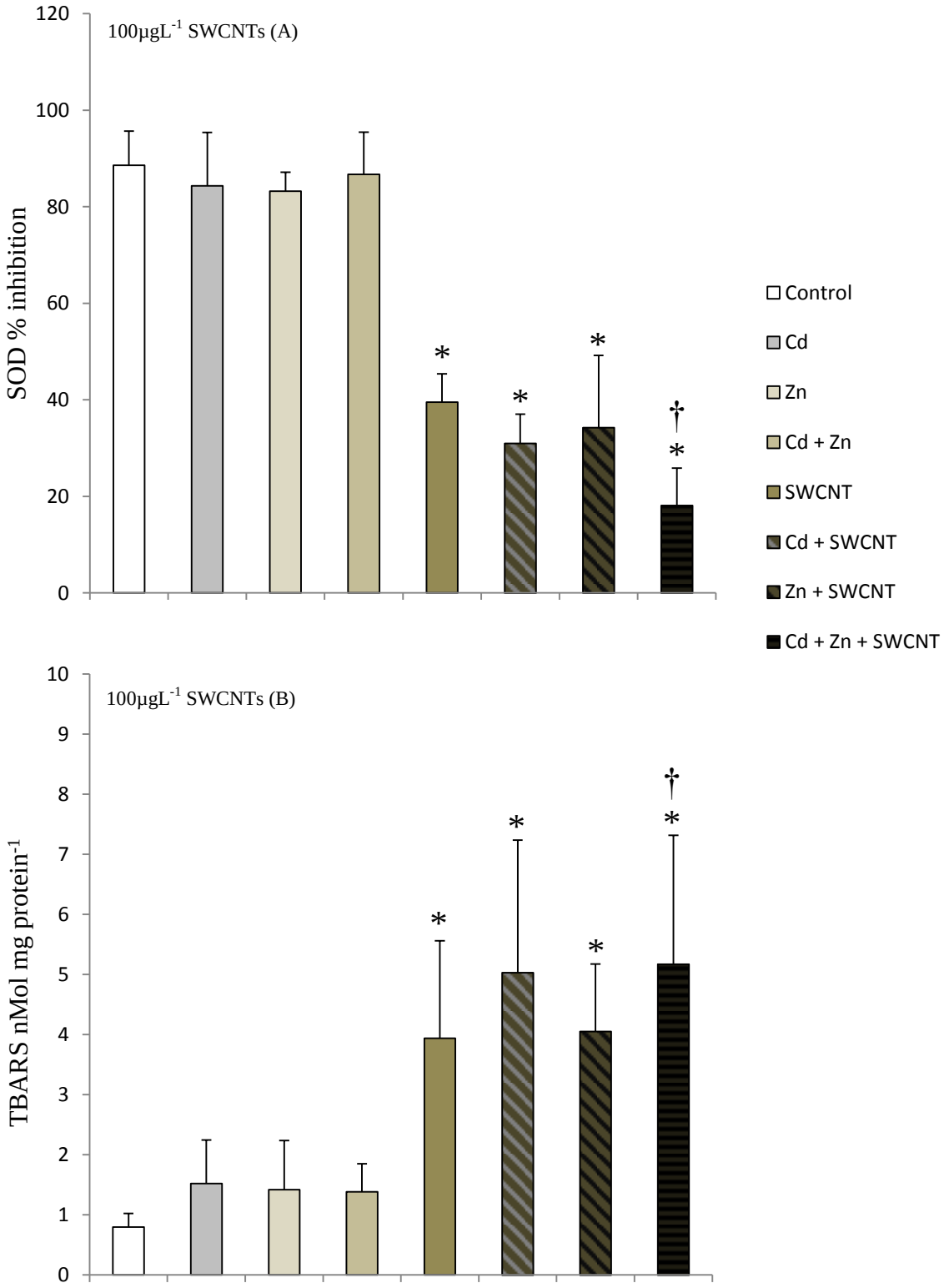

Figure 3.22 Superoxide dismutase activity expressed as percentage of inhibition (A) and (B) thiobarbituric acid reactive substances expressed as nanograms per milligram protein. * significantly different from control, Cd, Zn and Cd + Zn; † significantly different from SWCNTs alone and SWCNTs + Cd or Zn ($p<0.05$; means ± standard deviation, n=5).

Group 5. *Mytilus edulis* exposed *in vivo* to 0.001 µM $CdCl_2$, 1 µM ZnSO4, 0.001 µM $CdCl_2$ + 1 µM ZnSO4, 500µgL^{-1} SWCNTs, Cd 0.001µM + SWCNTs 500µgL^{-1}, Zn 1.0µM + SWCNTs 500µgL^{-1} and Cd 0.001µM + Zn 1.0µM and Cd 0.001µM + Zn 1.0µM + SWCNTs 500µgL^{-1} for 72 hours.

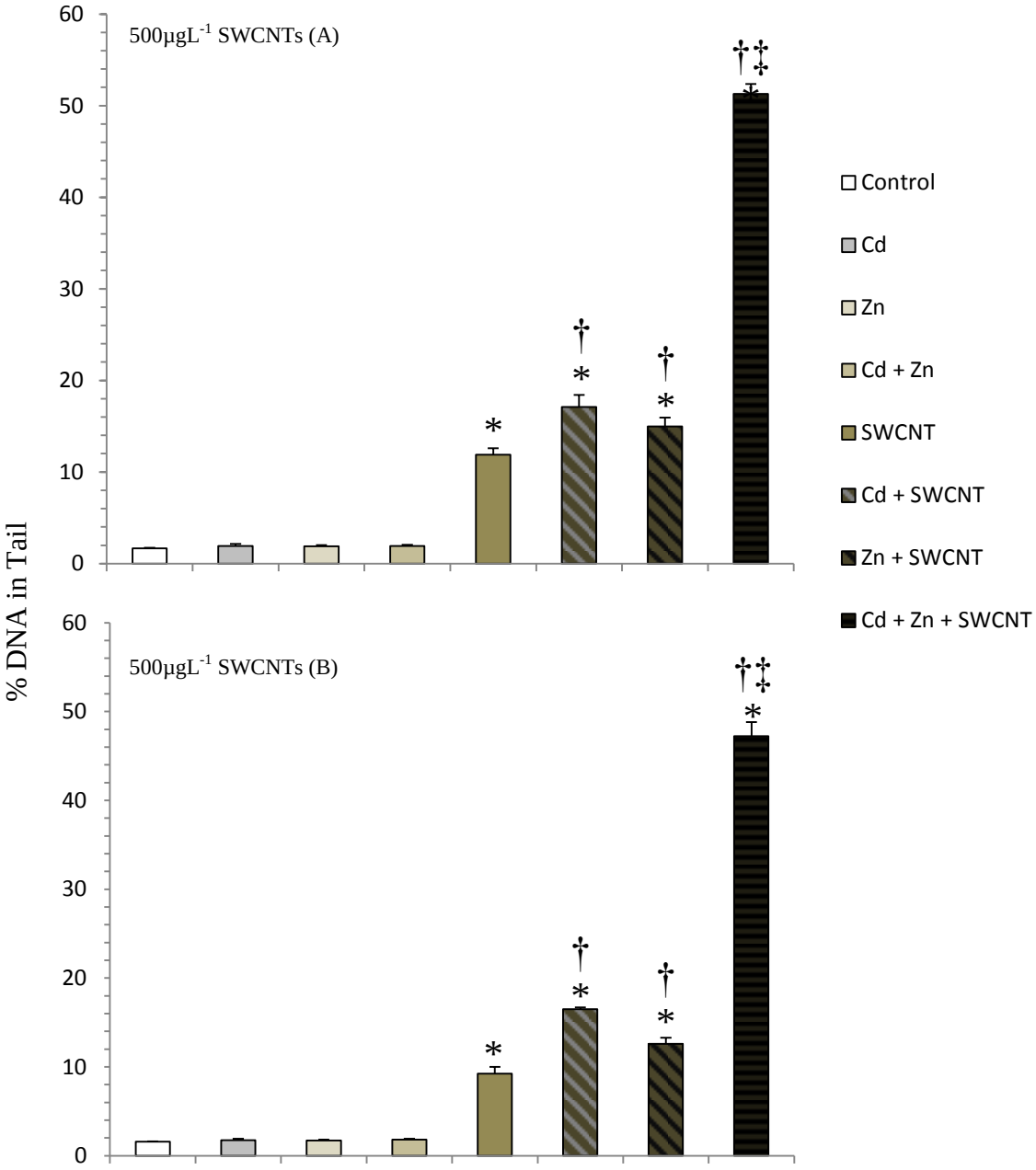

Figure 3.23 DNA damage, expressed as percentage of DNA in the tail, in (A) gill cells and (B) haemocytesof *M. edulis*. Statistically, there is significantly increased DNA damage was measured in gill and hemocytes. * significantly different from control, Cd, Zn and Cd + Zn; † significantly different from SWCNTs alone; ‡ significantly different from SWCNTs + Cd or Zn ($p<0.05$; means ± standard deviation, n=5).

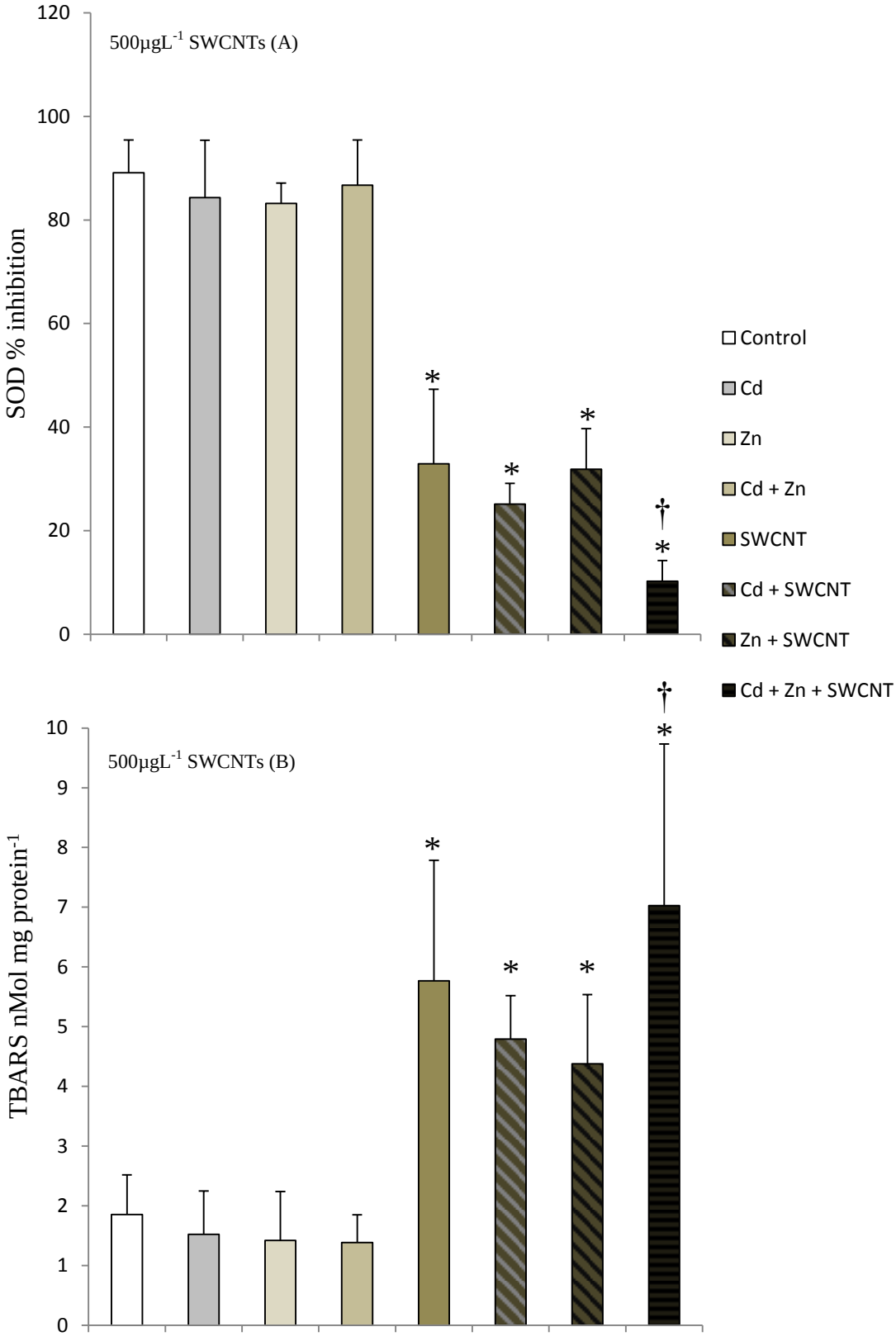

Figure 3.24 Superoxide dismutase activity expressed as percentage of inhibition (A) and (B) thiobarbituric acid reactive substances expressed as nanograms per milligram protein. * significantly different from control, Cd, Zn and Cd + Zn; † significantly different from SWCNTs alone and SWCNTs + Cd or Zn ($p<0.05$; means ± standard deviation, n=5).

Group 6. *Mytilus edulis* exposed *in vivo* to Suwannee River Natural Organic Matter (SRNOM), Cd 0.001µM, Cd 0.001µM + SRNOM, Zn 1.0µM, Zn 1.0µM + SRNOM and Cd 0.001µM + Zn 1.0µM + SNORM. The spiking was carried out *in vivo* for 72 hours.

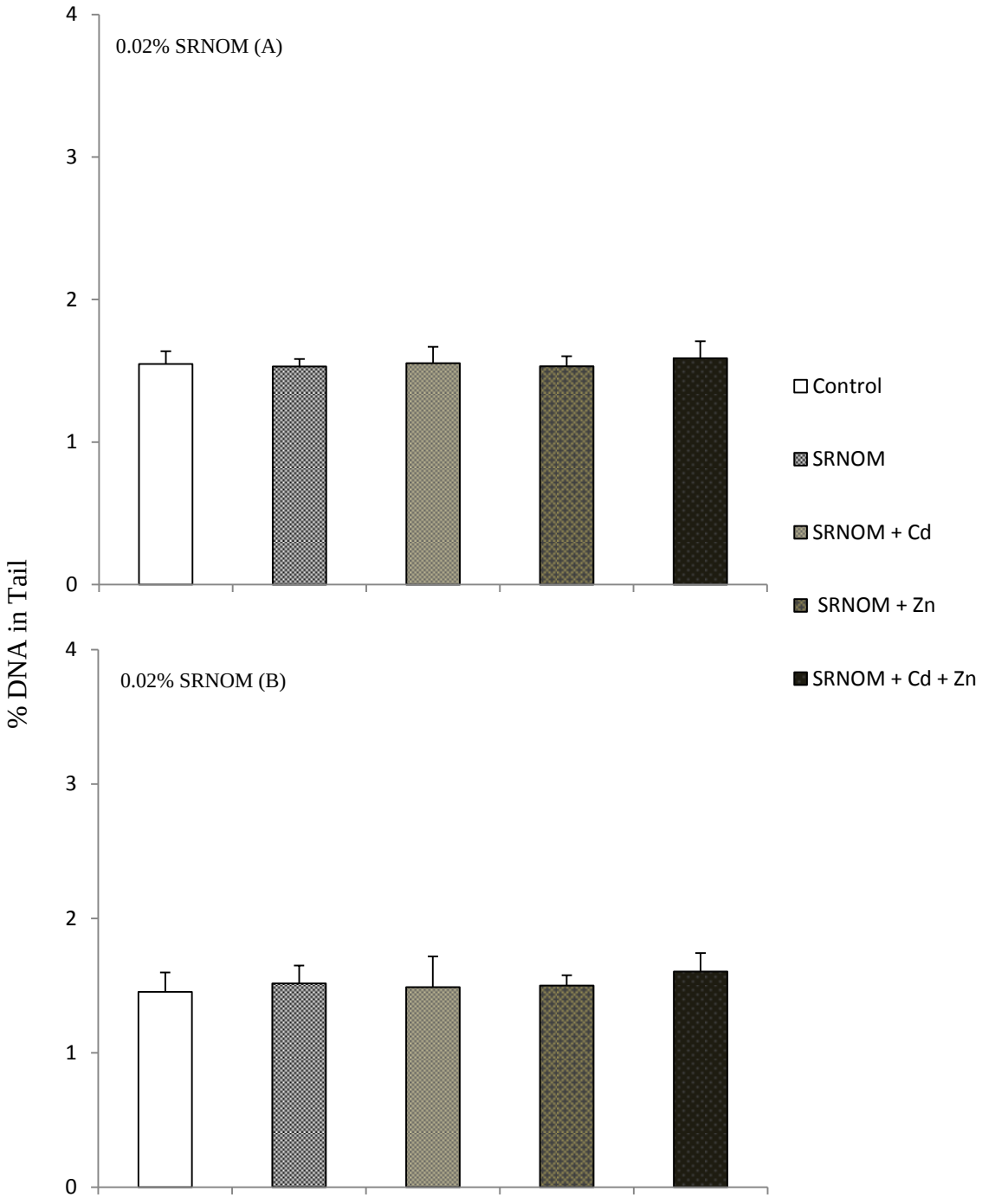

Figure 3.25 DNA damage, expressed as percentage of DNA in the tail, in (A) gill cells and (B) haemocytesof *M. edulis*. Statistically, there is no significant difference in increase DNA damage was measured in gill and haemocytes between control and treatment. ($p = 0.862$; $p = 0.889$; means ± standard deviation, n=5).

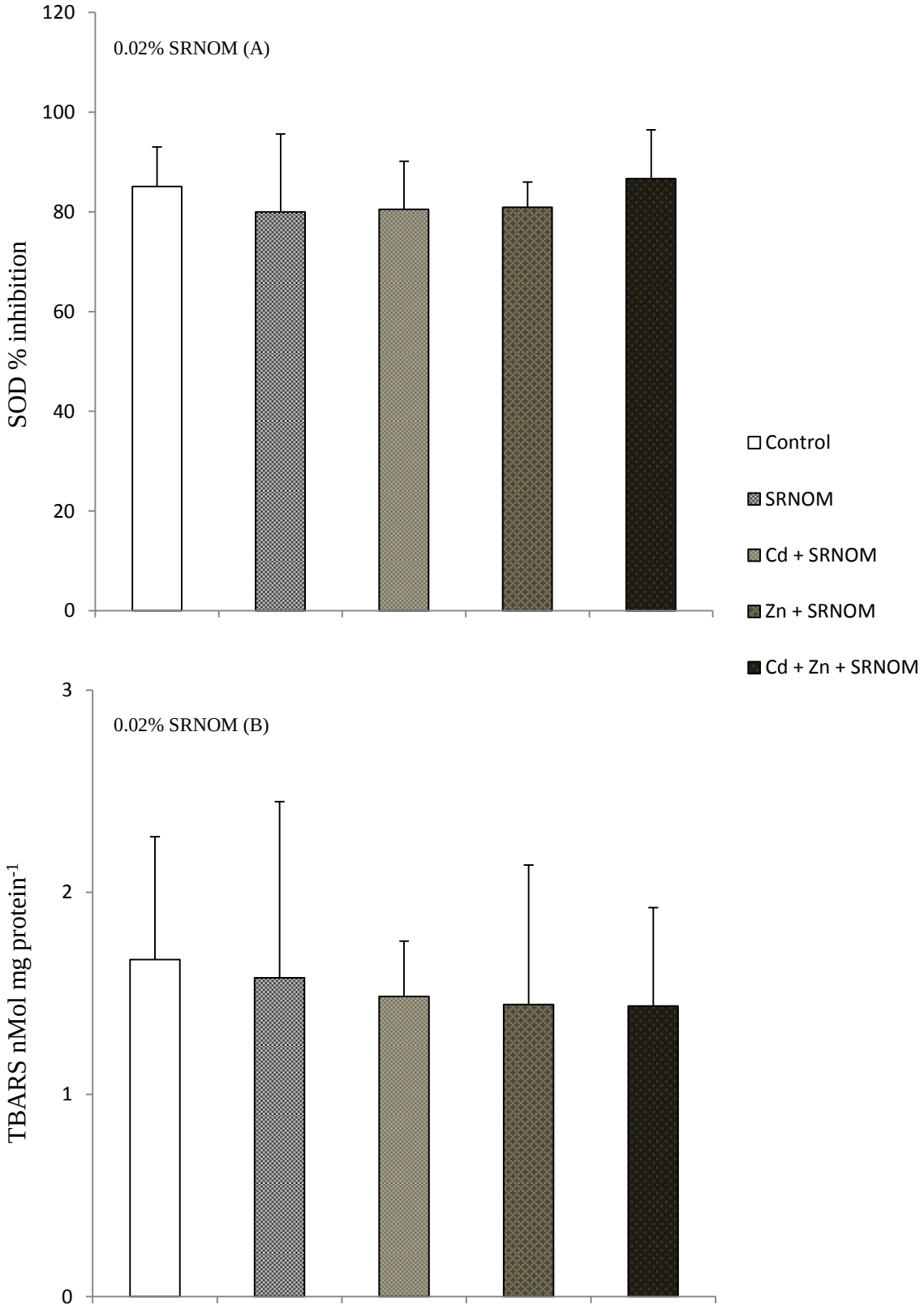

Figure 3.26 Superoxide dismutase activity expressed as percentage of inhibition (A) and (B) thiobarbituric acid reactive substances expressed as nanograms per milligram protein. Statistically, there is no significant differencebetween control and treatments groups ($p = 0.939$; $p = 0.999$; means ± standard deviation, n=5).

Group 7. *Mytilus edulis* exposed *in vivo* to residual metal impurities (RMI): (Fe [0.013 µM] + Mn [0.009 µM] + Ni [0.006 µM]) alone, Cd (0.001 µM) + RMI, Zn (1 µM) + RMI, and Cd (0.001 µM) + Zn (1 µM) + RMI for 72 hours.

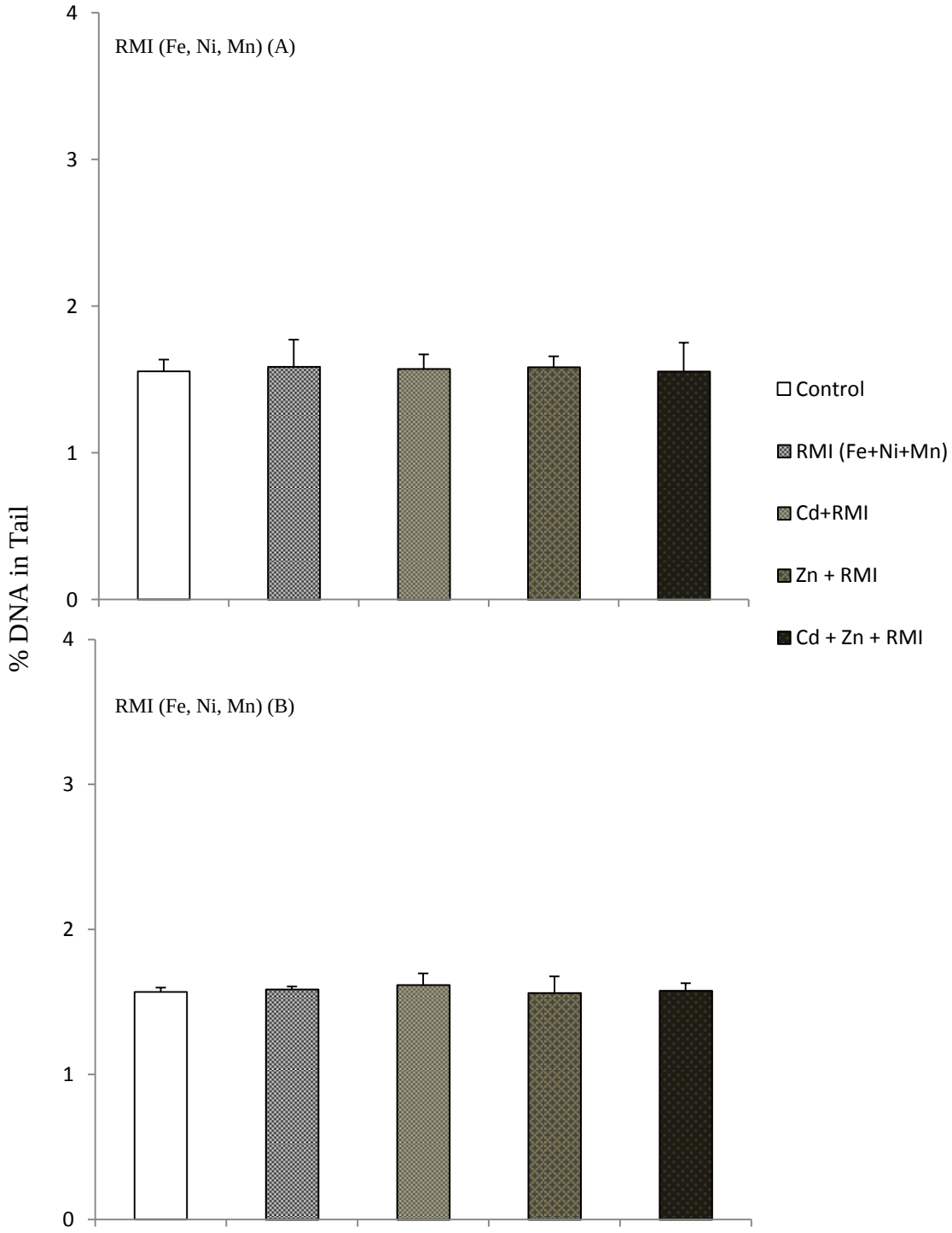

Figure 3.27 DNA damage, expressed as percentage of DNA in the tail, in (A) gill cells and (B) haemocytes of *M. edulis*. Statistically, there is no significant difference in increase DNA damage was measured in gill ($p = 0.997$) and haemocytes ($p = 0.865$) between control and treatment.

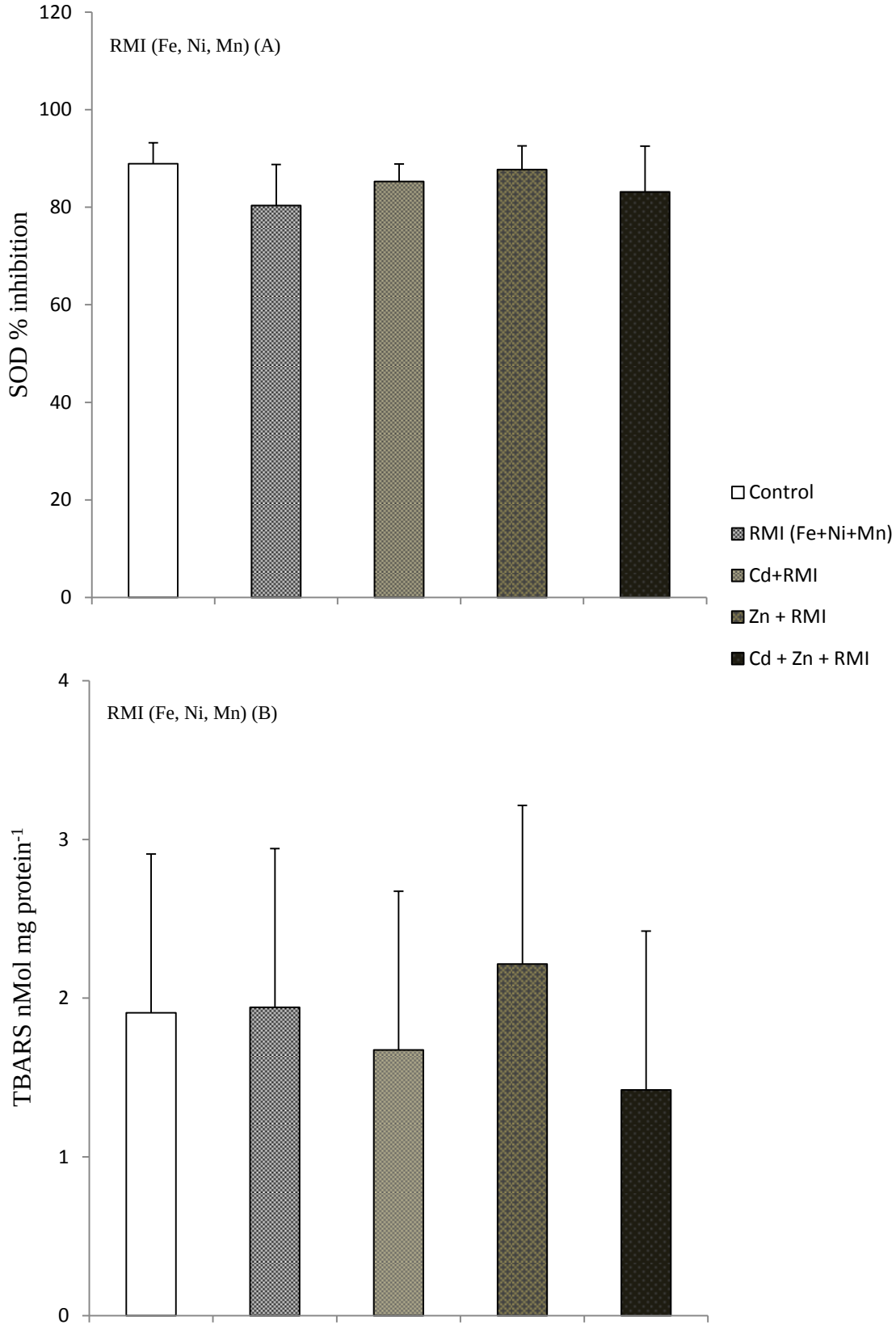

Figure 3.28 Superoxide dismutase activity expressed as percentage of inhibition (A) and (B) thiobarbituric acid reactive substances expressed as nanograms per milligram protein. Statistically, there is no significant difference between control and treatments groups ($p = 0.600$; $p = 0.900$; means ± standard deviation, n=5).

3.3 Impact of agglomerated single-walled carbon nanotubes on the marine green algae, *Tetraselmis suecica*

3.3.1 Light microscope

In order to detect the agglomeration of SWCNT in algae culture media, algae *Tetraselmis suecica* were exposed to SWCNTs at nominal concentrations (100µg L^{-1}, and 500µg L^{-1}). Light microscopical observation was used as an initial method to observe the adherence of SWCNT agglomerates to algal cells (Figure 3.29).

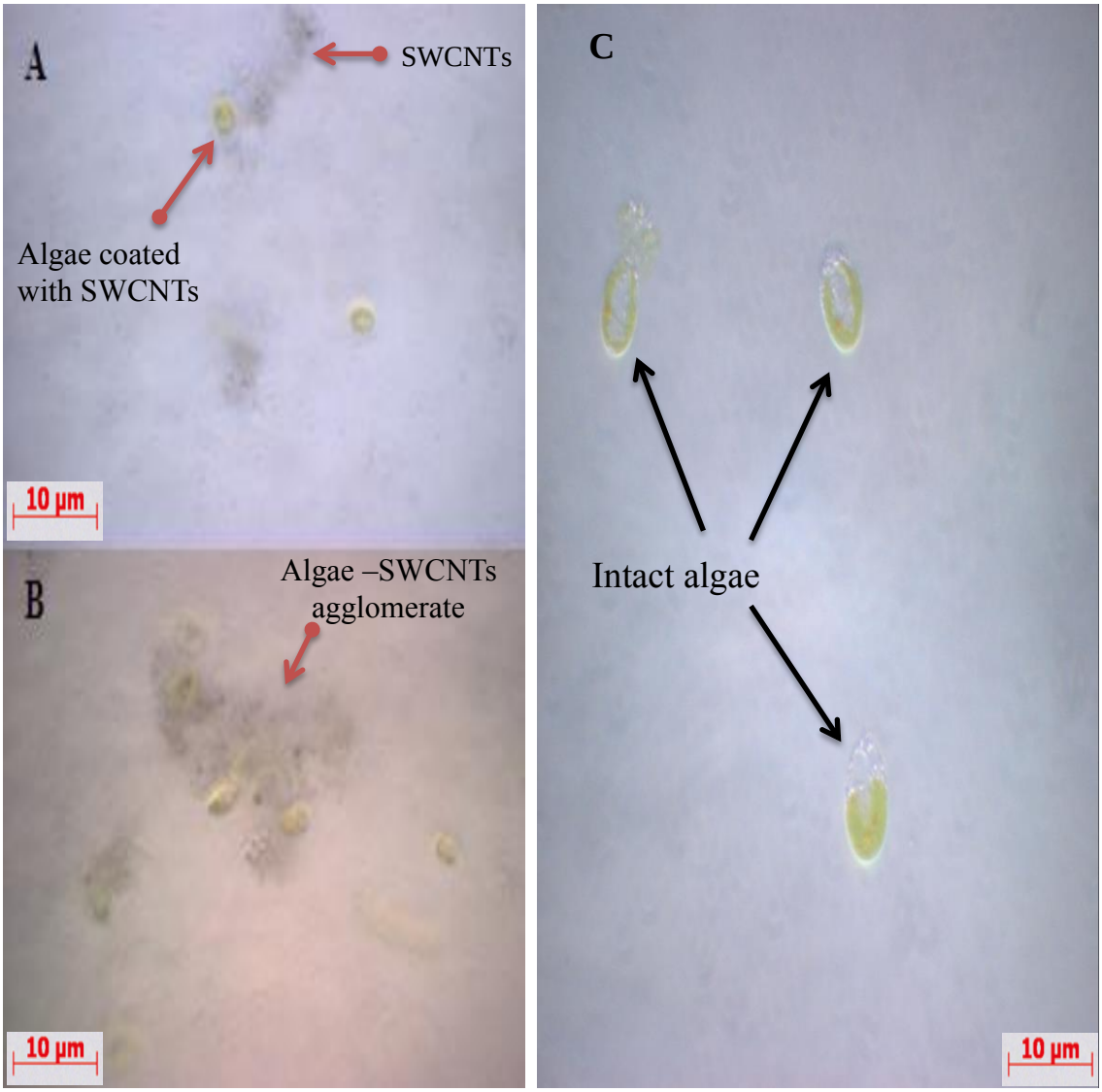

Figure 3.29 *Tetraselmis suecica* in the presence and absence of SWCNTs; light micrographs for SWCNT-exposed (A) 100µg L^{-1}, (B) 500µg L^{-1}, and Control (C). Images show a dark colouration on the surface of treated algae (A&B) compared to the clean surface in the control algae (C).

3.3.2 Raman microspectroscopy

Raman microspectroscopy was applied to confirm the adhered structures as SWCNTS (Figure 3.30).

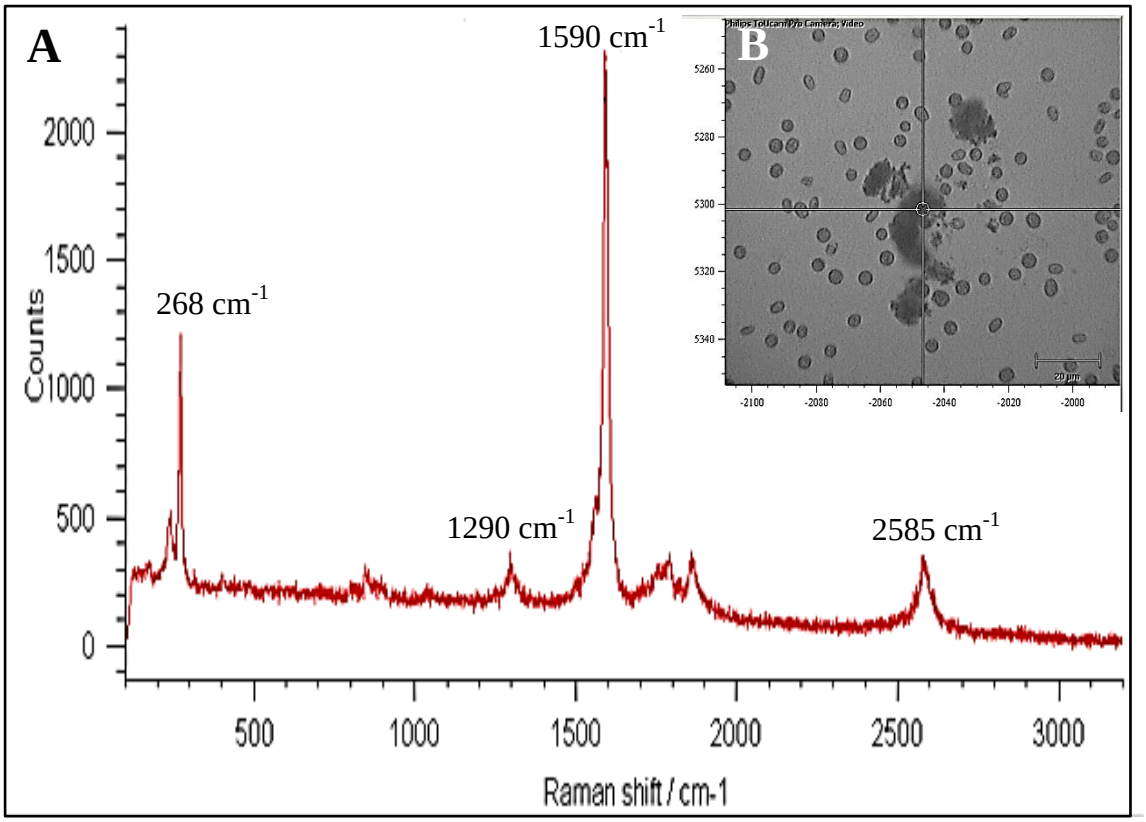

Figure 3.30 Raman spectrum of Algal-SWCNT interaction, (A) Magnification (x 20) of algal cells exposed to 500µg L^{-1} single-walled carbon nanotubes (SWCNTs) for 24hrs. The white circle in the centre of (B) marks the position of the Raman excitation beam spot, which was focused to a diameter of 1.7µm. A representative Raman spectrum was acquired from SWCNT stock (Figure 3.5), algae cells spiked with 500µg L^{-1} SWCNT for 24hrs (A). The spectrum was collected using a 50x, 0.75 numerical aperture microscope objective lens. The spectrum from spiked sample clearly shows the characteristic peaks of SWCNTs: radial breathing mode at 268 cm^{-1}, D band at 1290 cm^{-1}, G band at 1590 cm^{-1}, and G´ band at 2585 cm^{-1}.

3.3.3 Scanning electronic microscopy (SEM)

Algae *Tetraselmis suecica* were exposed to SWCNTs 500µg L^{-1}. SEM showed SWCNTs can adhere to the external algal cell wall (Figure 3.31D-F).

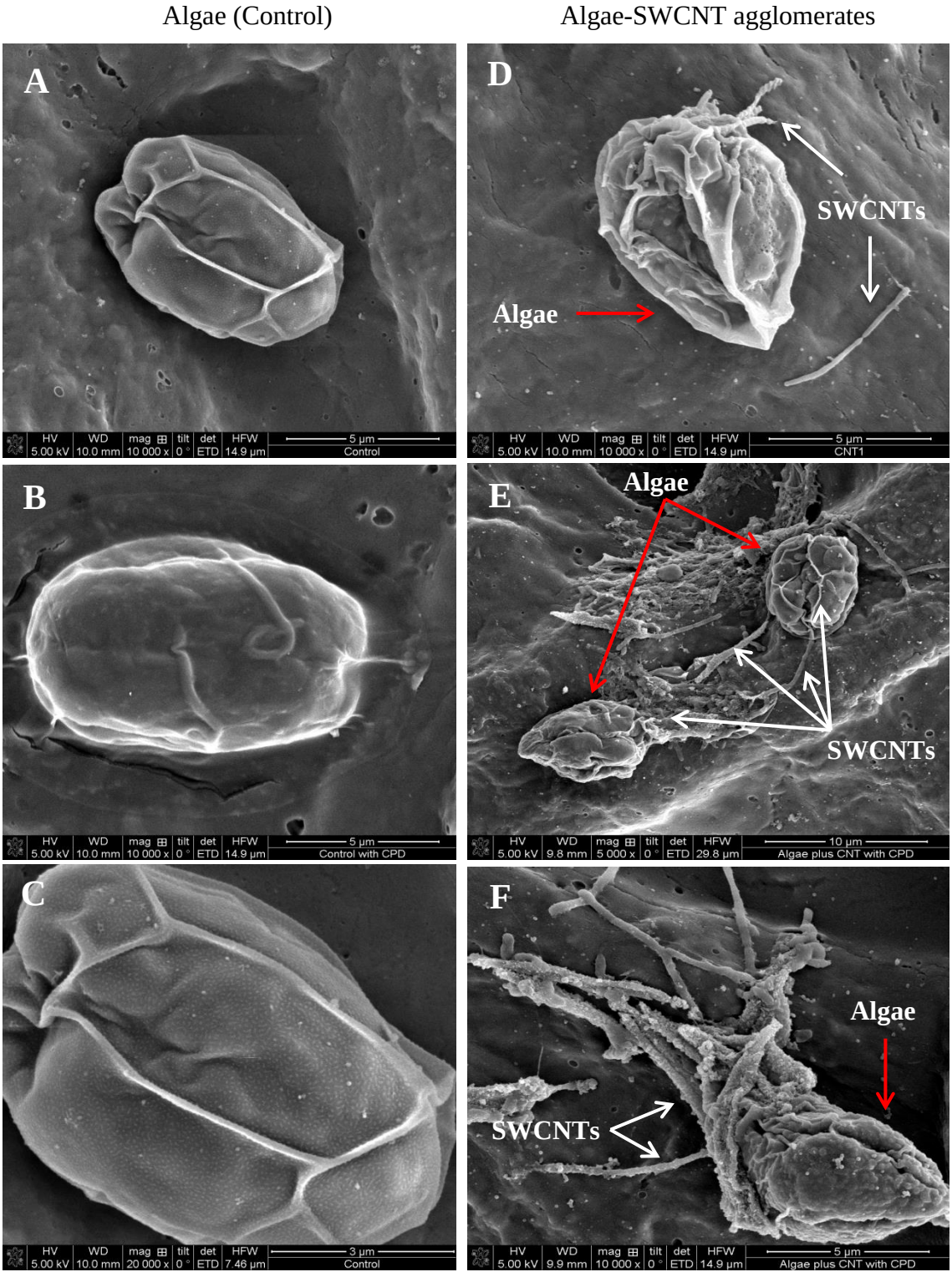

Figure 3.31 SEM images of *T. suecica* from control samples (A-C) and from culture medium containing final 500µg L^{-1} SWCNTs (D-F); algae (red arrows) appear surrounded by SWCNTs agglomerates (white arrows).

3.3.4 Transmission electronic microscoye (TEM)

Algae *Tetraselmis suecica* were exposed to SWCNTs 500µg L^{-1}. TEM showed SWCNTs can be internalized by the *Tetraselmis suecica* (Figure 3.32D-F).

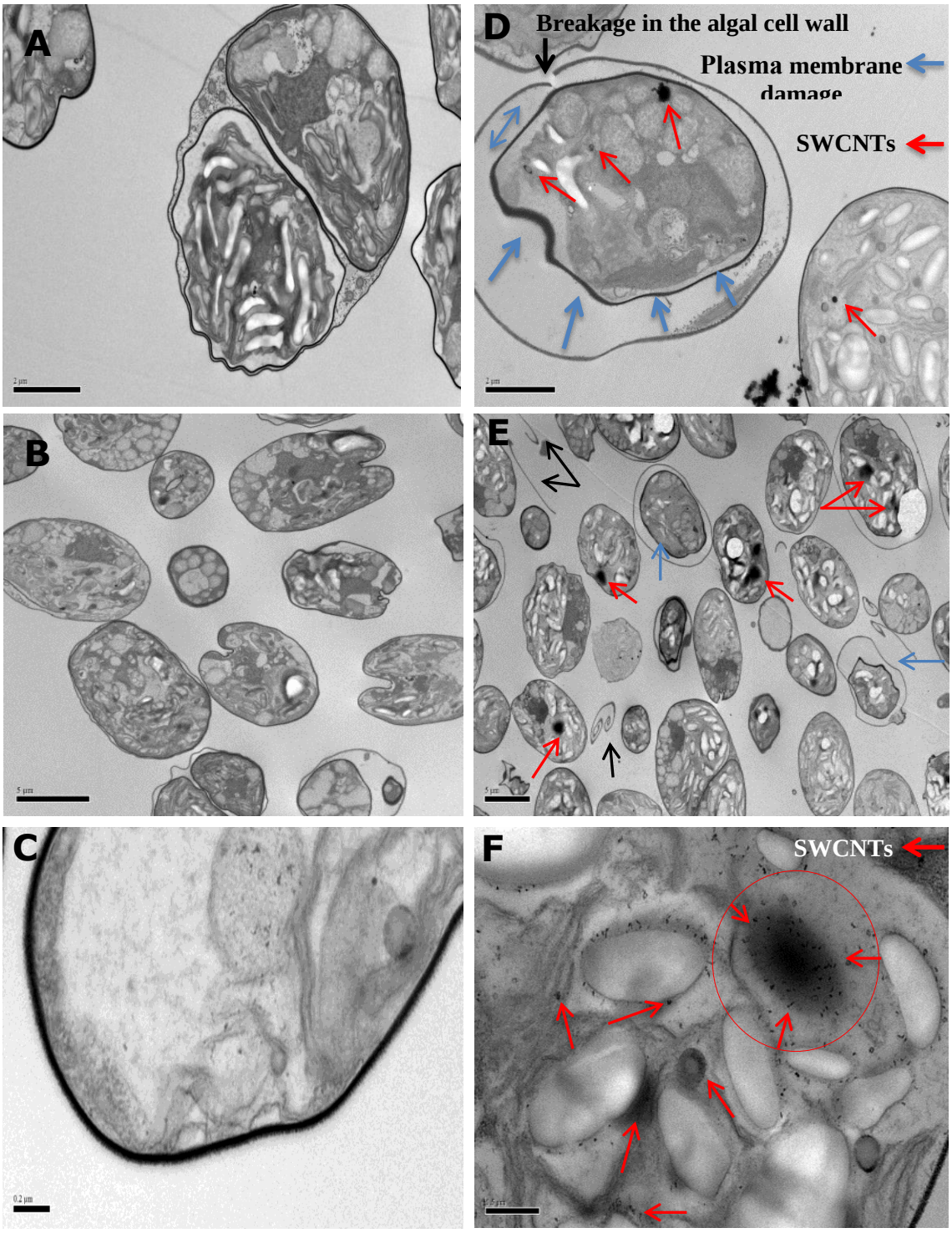

Figure 3.32 TEM images of control cells with intact algal cell wall and plasma membrane (A-C) and from culture medium containing final 500µg L^{-1} SWCNTs (D-F); cell wall breakage (black arrows); plasmolysis (blue arrows) and internalization of the SWCNTs (red arrows).

3.3.5 Cell counting using a haemacytometer (growth rate)

A significant negative effect of SWCNT exposure on algal growth was observed at concentrations >100µg L^{-1} Figure (3.33; P<0.001).

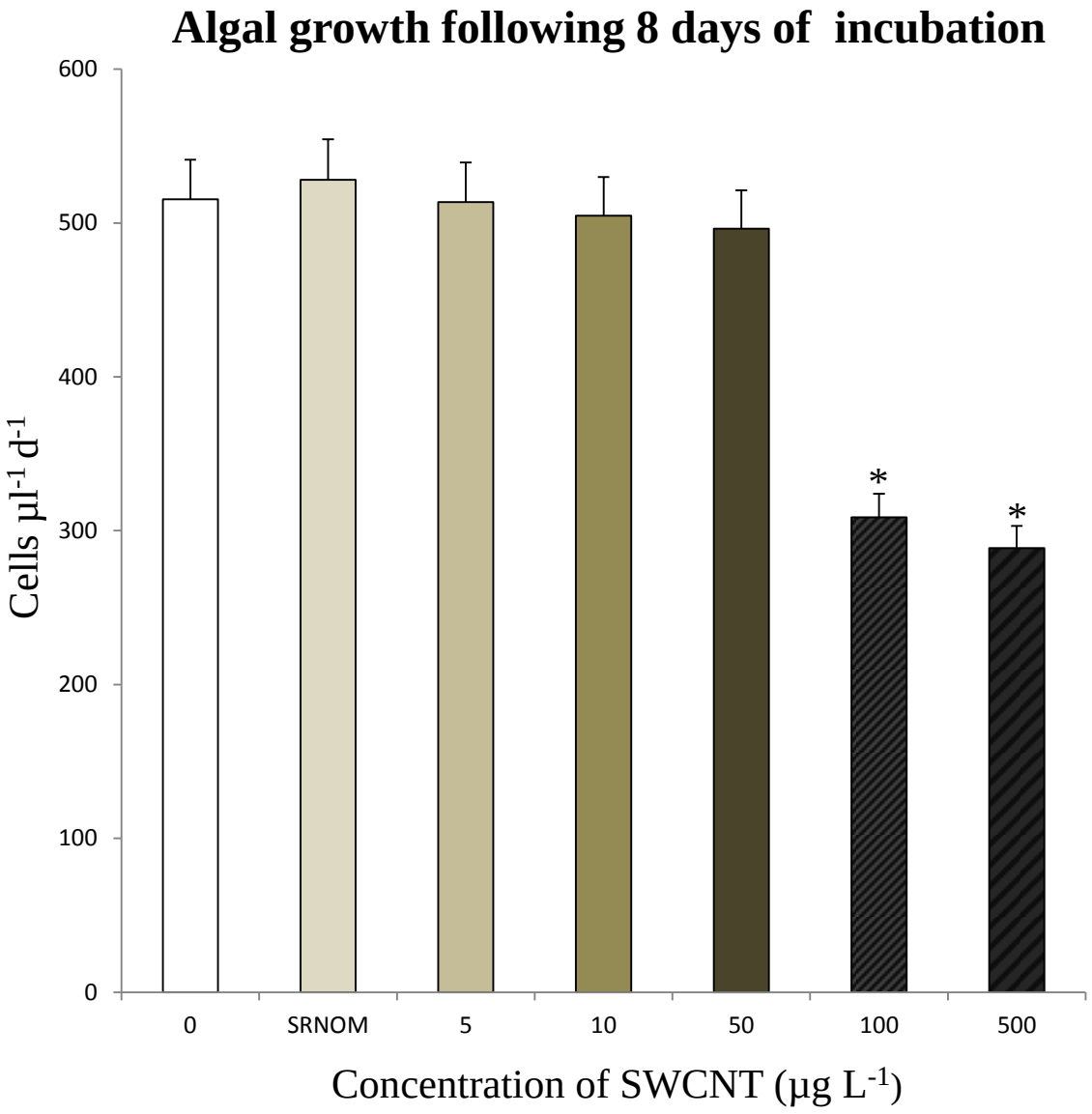

Figure 3.33 *Tetraselmis.suecica* were exposed to SRNOM and SWCNTs at nominal concentrations (5µgL^{-1}, 10µgL^{-1}, 50µgL^{-1}, 100µgL^{-1}, and 500µgL^{-1}) for 8 days. Statistically, there was no significant difference between SRNOM, 5µgL^{-1}, 10µgL^{-1}, 50µgL^{-1} and control groups; however, significant growth inhibition occurred >100µgL^{-1} (P<0.001; n=3).

3.3.6 Cell viability

The cell viability of algal cells was assessed following exposure to the highest concentration of SWCNTs 500µg L^{-1}. By day 8, both control and treatment were analysed, and chlorophyll *a* concentration was slightly fluorescent when excited by 488nm blue laser using flow cytometry. Figure 3.34A-B shows the amount of viable algal cells in either control or treatment.

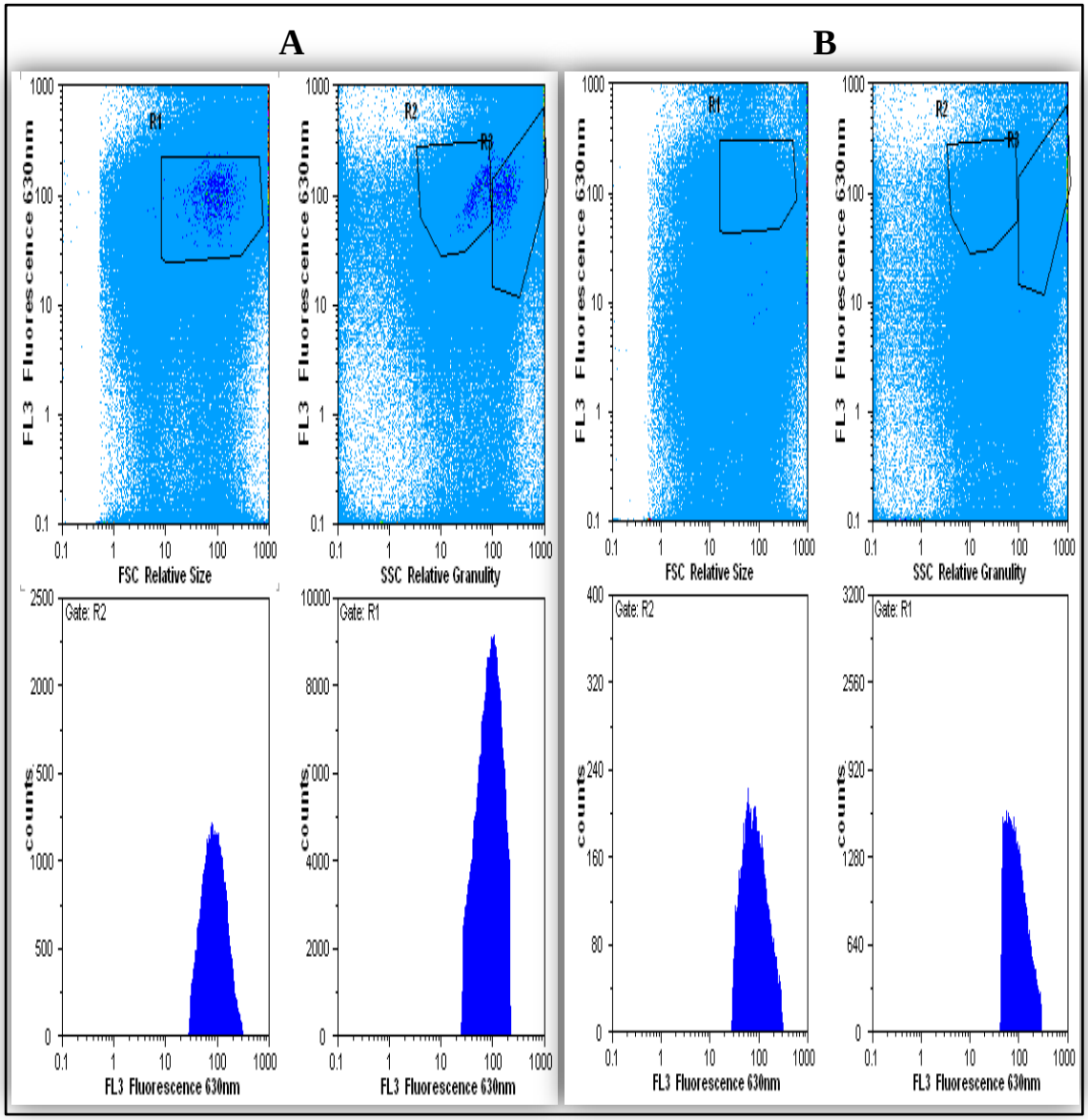

Figure 3.34 Peaks (A) control and (B) 500µgL^{-1} SWCNT show the viable *T. suecica* cells using Cyflow. Statistically, there is a significant difference between 500µgL^{-1} SWCNT and control groups ($P<0.001$; n=3) ANOVA.

3.3.7 Chlorophyll measurement

The aim for measuring the chlorophyll *a* concentration of green algae *Tetraselmis suecica* was to estimate the abundance of phytoplankton in algal culture media. The chlorophyll *a* concentrations following exposure to 500µg L^{-1} were significantly reduced (P<0.001; Fig 3.35).

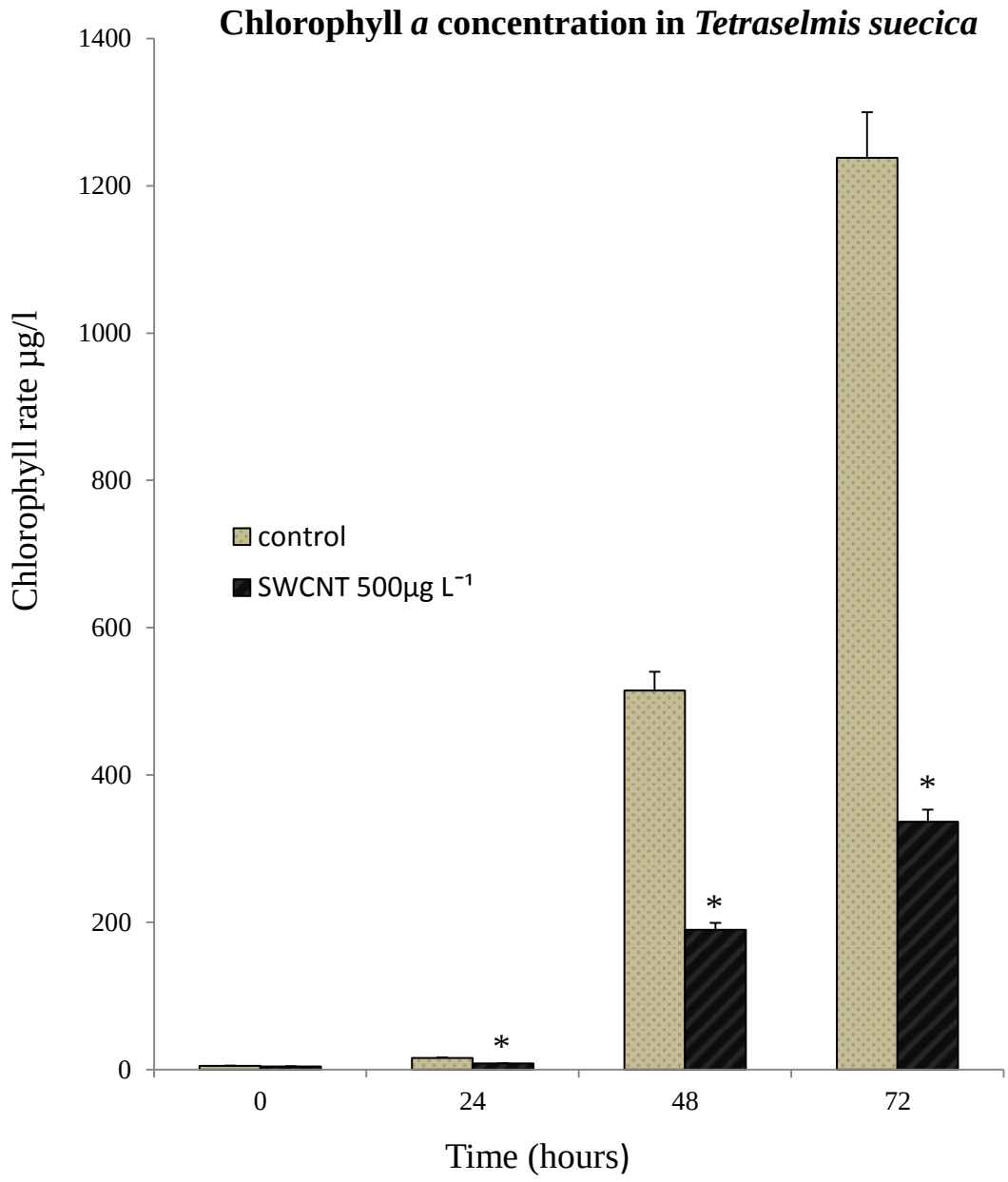

Figure 3.35 *Tetraselmis suecica* were exposed to three replicates of 500µg L^{-1} SWCNTs. Statistically, there was no significant difference between control and SWCNTs at t_0 (P = 0.312). However, between $t_1 \rightarrow t_3$ chlorophyll *a* concentrations were significantly decreased compared to the control (P<0.001; n=3).

3.4 Effect of the interaction of Green algae with single-walled carbon nanotubes on the feeding behaviour of mussels, mitigating nanotube toxicity

3.4.1 Pseudofaeces

In order to study the feeding behaviour of mussels (*Mytilus edulis*) presented with algae in the presence of SWCNTs, known algal concentrations and SWCNT 500µg L^{-1} alone and combined were applied to the tanks, and mussels left to feed for 10 minutes, after which mussels were transferred to clean seawater and left to depurate for 24hrs. Copious amounts of pseudofaecal material were expelled by the exhalant siphon of the mussel when fed the SWCNTs + *Tetraselmis suecica* (Figure 3.36A-E).The SWCNTs were clearly seen to be discarded by the mussels bound up in pseudofaeces.

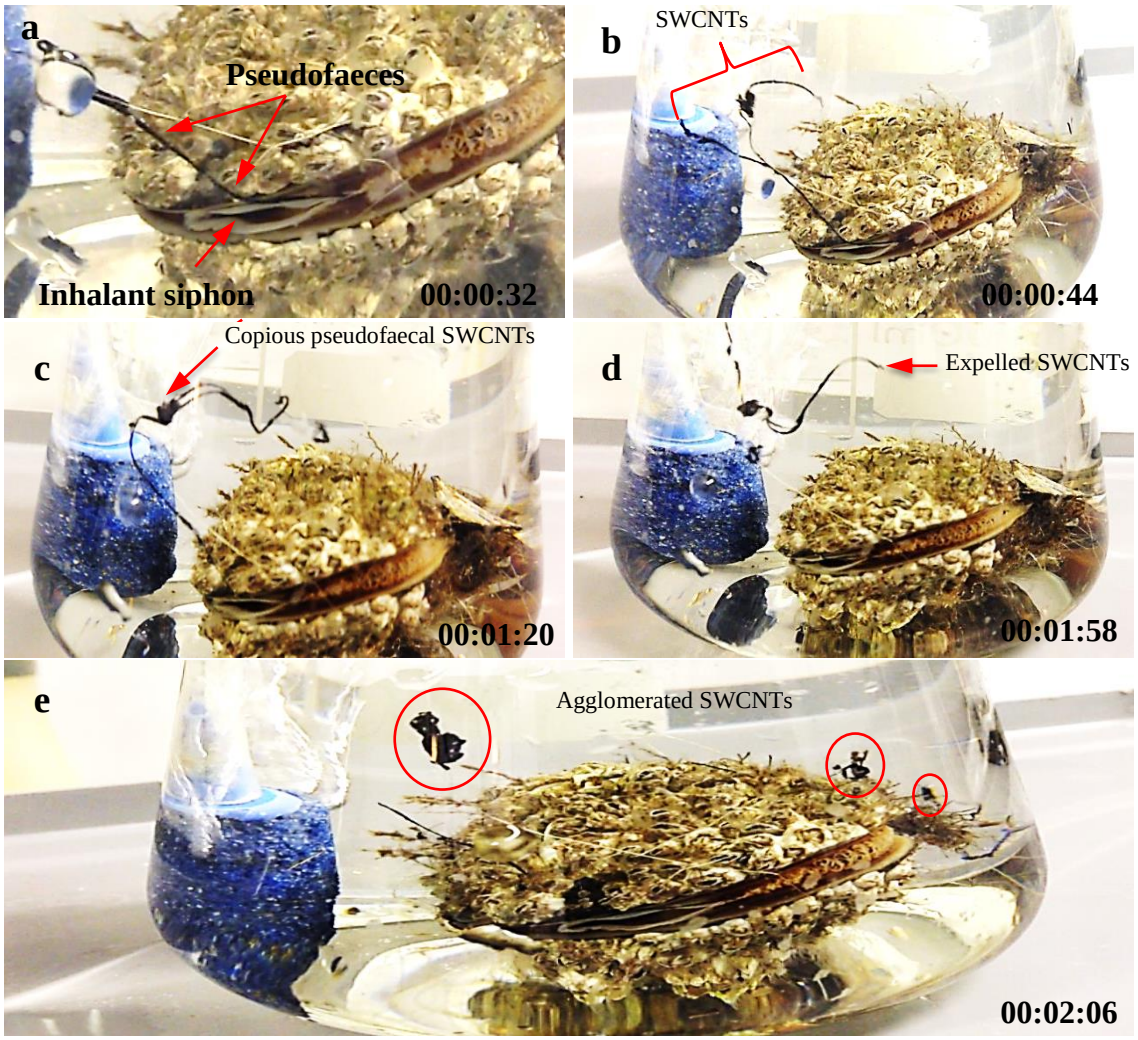

Figure 3.36 Feeding behaviour of mussels when fed SWCNTs + algae: (a) mussel starts to expel SWCNTs; (b, c) long black nanotubes still attached to the inhalant siphon of mussel; and (d, e) SWCNTs have become agglomerated or aggregated in seawater.

After 24hrs faecal and pseudofaecal material were observed to be expelled by the exhalant siphon of the mussels when fed the algae, SWCNTs 500µg L^{-1} alone and SWCNTs 500µg L^{-1} + algae (Figure 3.37A-E). However, copious pseudofaecal SWCNTs were expelled by the exhalant siphon of the mussel when fed SWCNTs + algae.

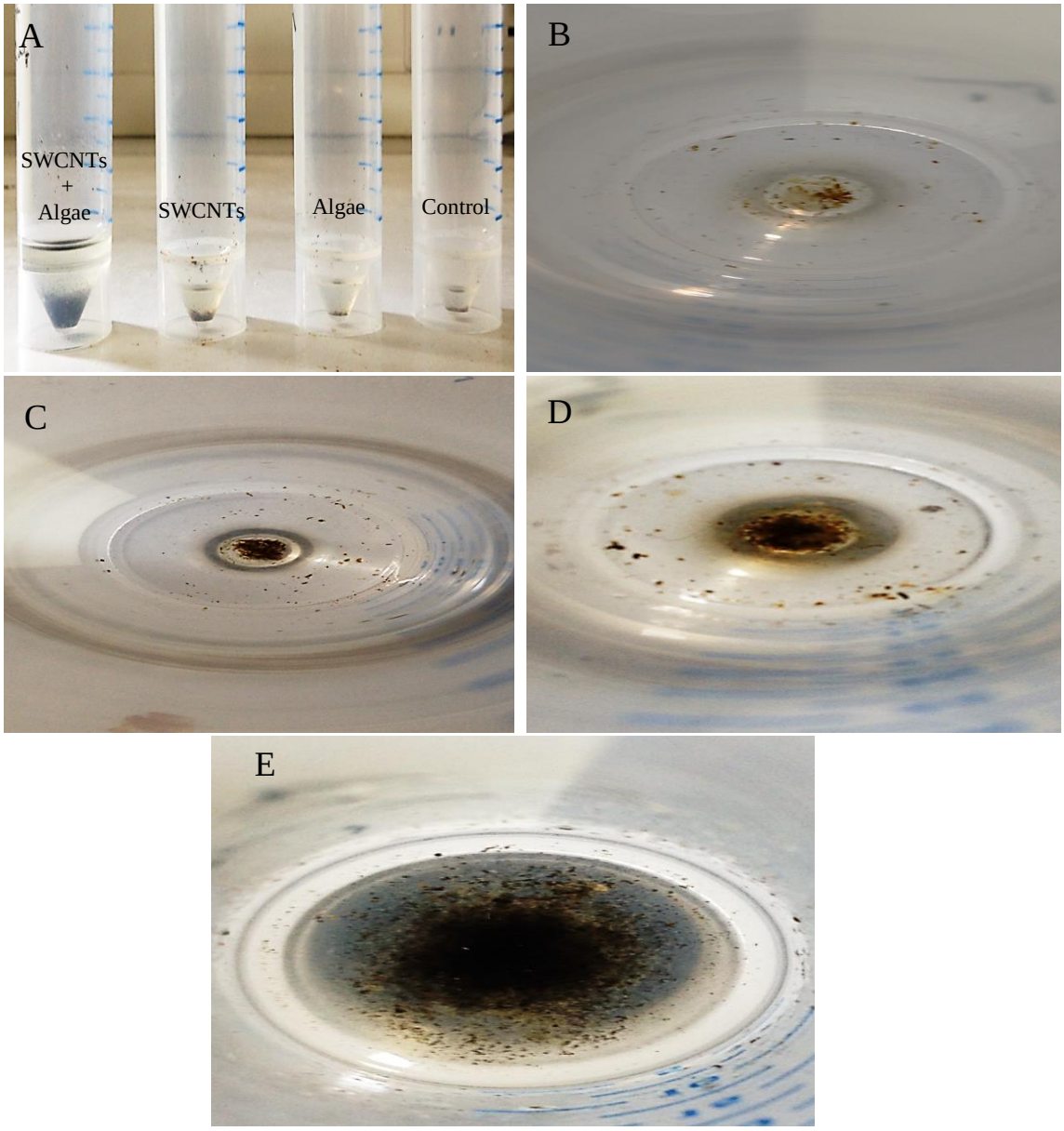

Figure 3.37 Feeding behaviour of the mussel *Mytilus edulis*. (A) Faecal and pseudofaecal material expelled by the exhalant /inhalant siphons of the mussels when fed the algae, SWCNTs 500µg L^{-1} alone and SWCNTs 500µg L^{-1} + algae. (B, C) Faecal material expelled by the exhalant siphon of the mussel when fed the *Tetraselmis suecica* alone. (D) Pseudofaecal material expelled by the inhalant siphon of the mussels when fed the SWCNTs 500µg L^{-1}. (E) Copious pseudofaecal material expelled by the exhalant siphon of the mussel when fed the SWCNTs with *Tetraselmis suecica.*

The selected faecal and pseudofaecal algal cells were microscopically observed (Figures 3.38A-J). These observations of pseudofaecal algal cells in mussel pseudofaeces were more than the faecal algal cells that produced from mussels. These observations were later confirmed by flow cytometry (Figure 3.39).

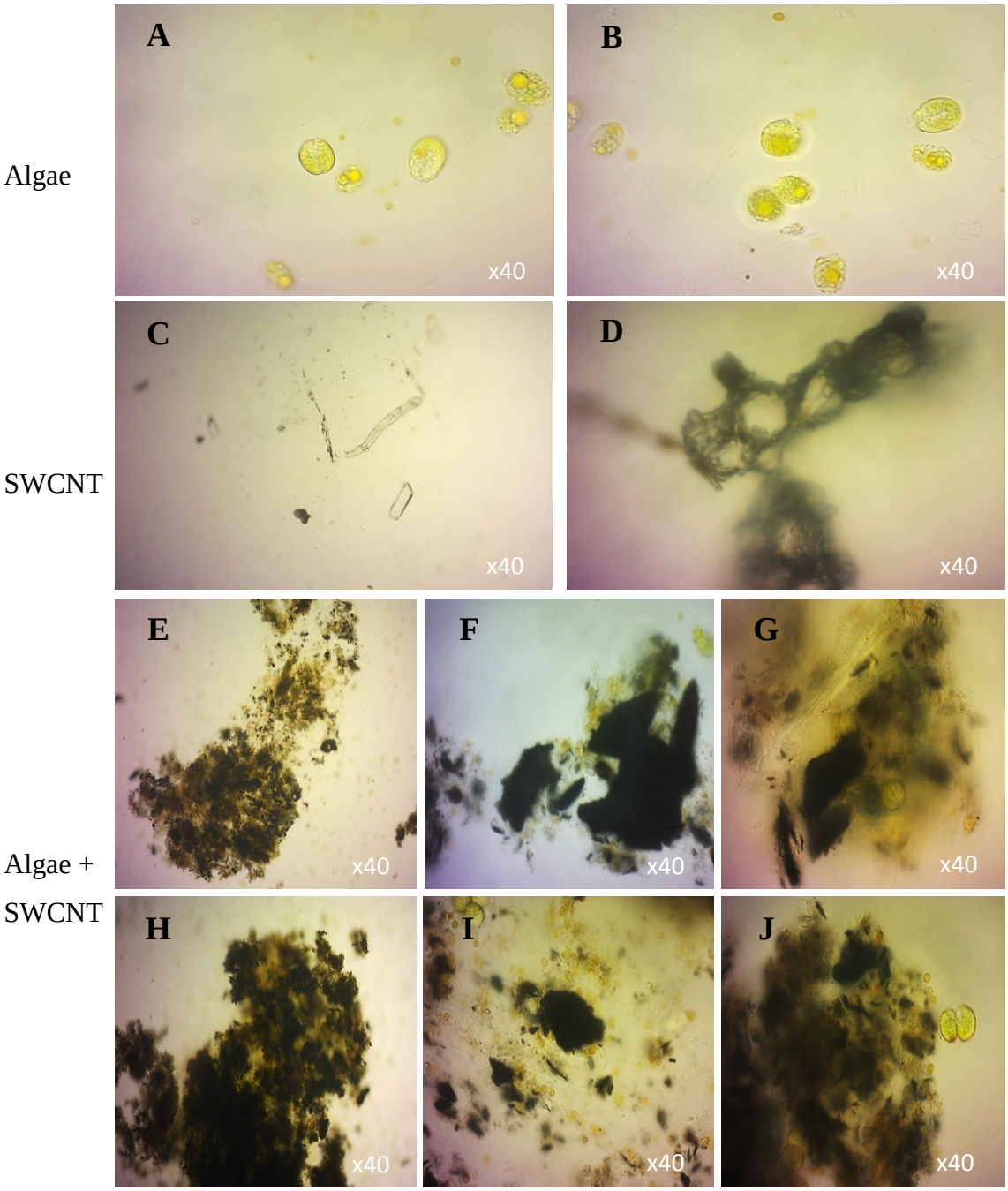

Figure 3.38 Faecal and pseudofaecal algal cells were observed clearly by optical microscopy. (A,B) show faecal algal cells in the absence of SWCNTs, (C, D) Pseudofaecal material expelled by the exhalant siphon of the mussels when fed the SWCNTs 500µgL^{-1} alone, while figures (E-J) show copious pseudofaecal algal cells and SWCNTs expelled by the exhalant siphon of the mussel when fed the SWCNTs with *Tetraselmis suecica.*

Flow cytometry (Figure 3.39) shows the number of the pseudofaecal algal cells produced by mussels in the presence and absence of SWCNTs. Statistically, there was a significant difference (P=0.008) in the number pseudofaecal algal cells between the treatment and control groups

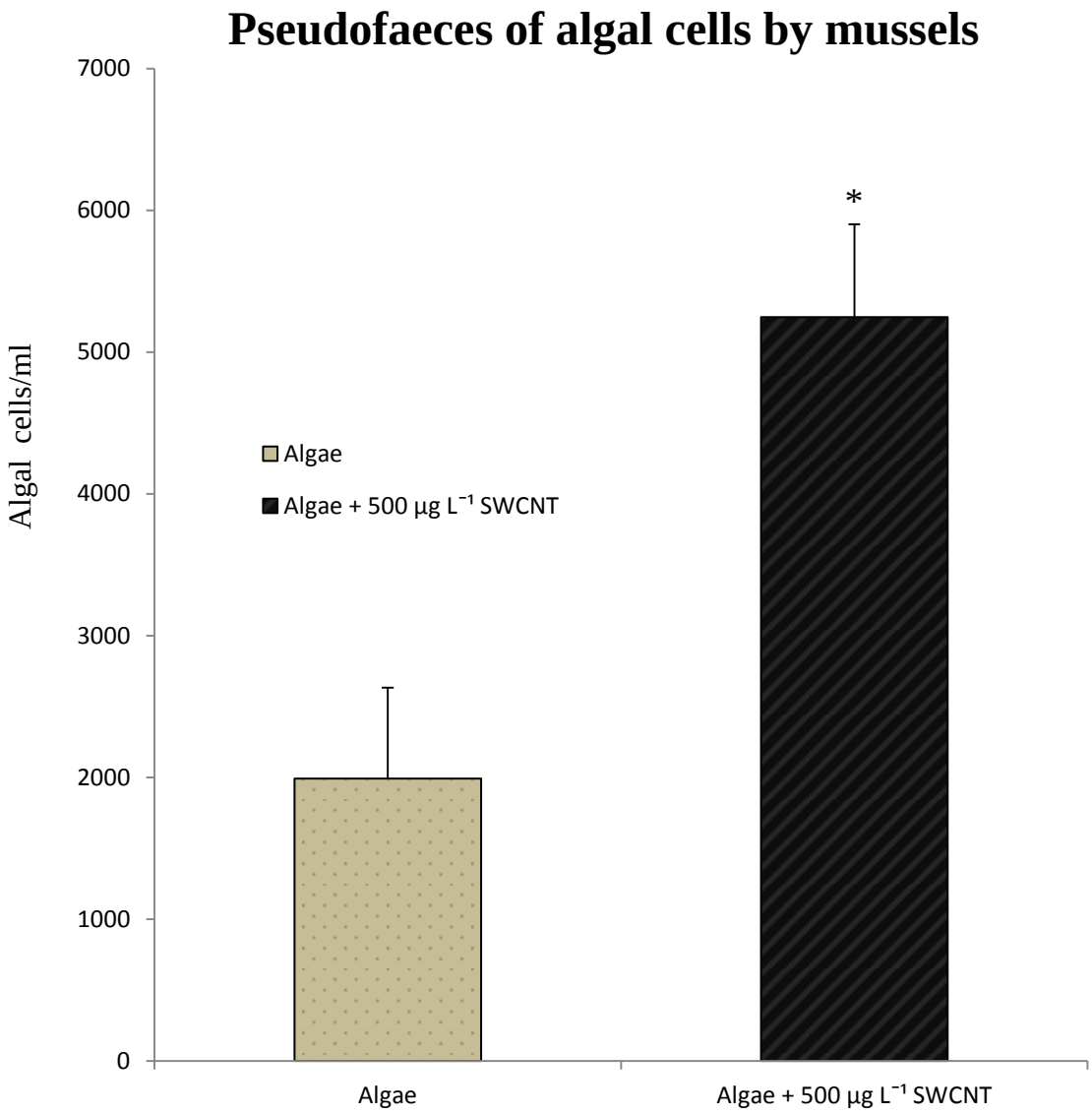

Figure 3.39 Shows mussels fed algae with and without 500µg L^{-1} SWCNTs, exhibited significantly increased pseudofaeces production (P=0.008) under combined algae and SWCNT exposure.

3.4.2 Comet assay and oxidative stress

In this study, Mussels were fed algae (*Tetraselmis suecica*), SWCNT 500µg L^{-1} alone and *T suecica* + SWCNTs 500µg L^{-1} for 24hrs. A 24hrs exposure to 500µg L^{-1} SWCNTs showed significantly increased DNA strand breaks in both gill cells and haemocytes (P<0.001; Figure 3.40A and B), and significantly increased oxidative stress, expressed as superoxide dismutase (SOD) activity (P<0.001) and lipid peroxidation, in gills (P=0.032; Figures 3.41A and B). However, when SWCNTs were presented together with algae, DNA damage in haemocytes and gills (P=0.534; P=0.998) or oxidative stress were not significantly increased above control levels (P=0.981; P=0.999).

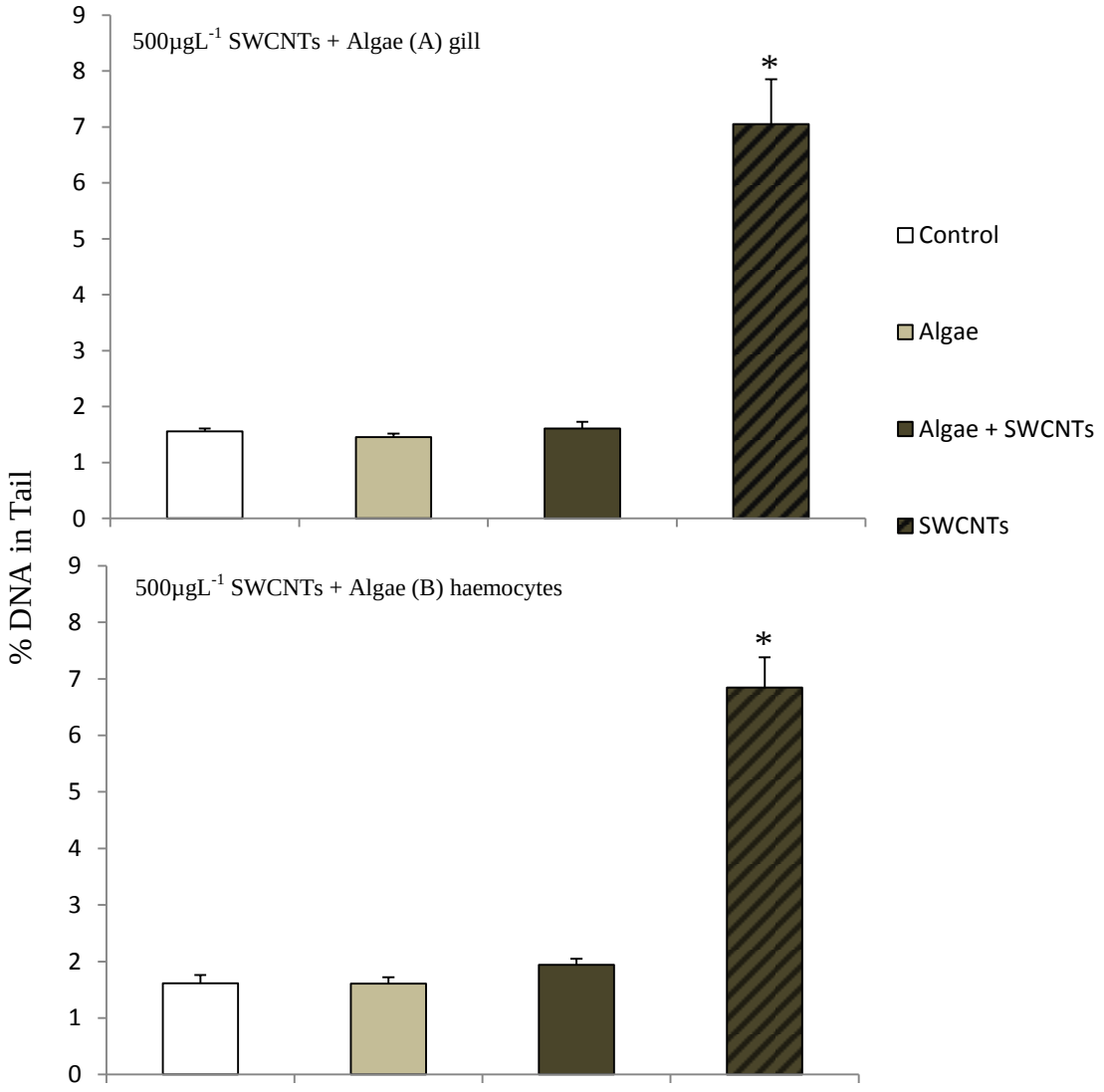

Figure 3.40 Mussels were fed algae (*Tetraselmis suecica*), SWCNT 500µg L^{-1} alone and *Tetraselmis suecica* + SWCNTs 500µg L^{-1} for 24hrs. * significantly different from control, algae and algae + SWCNTs 500µg L^{-1} (P<0.001).

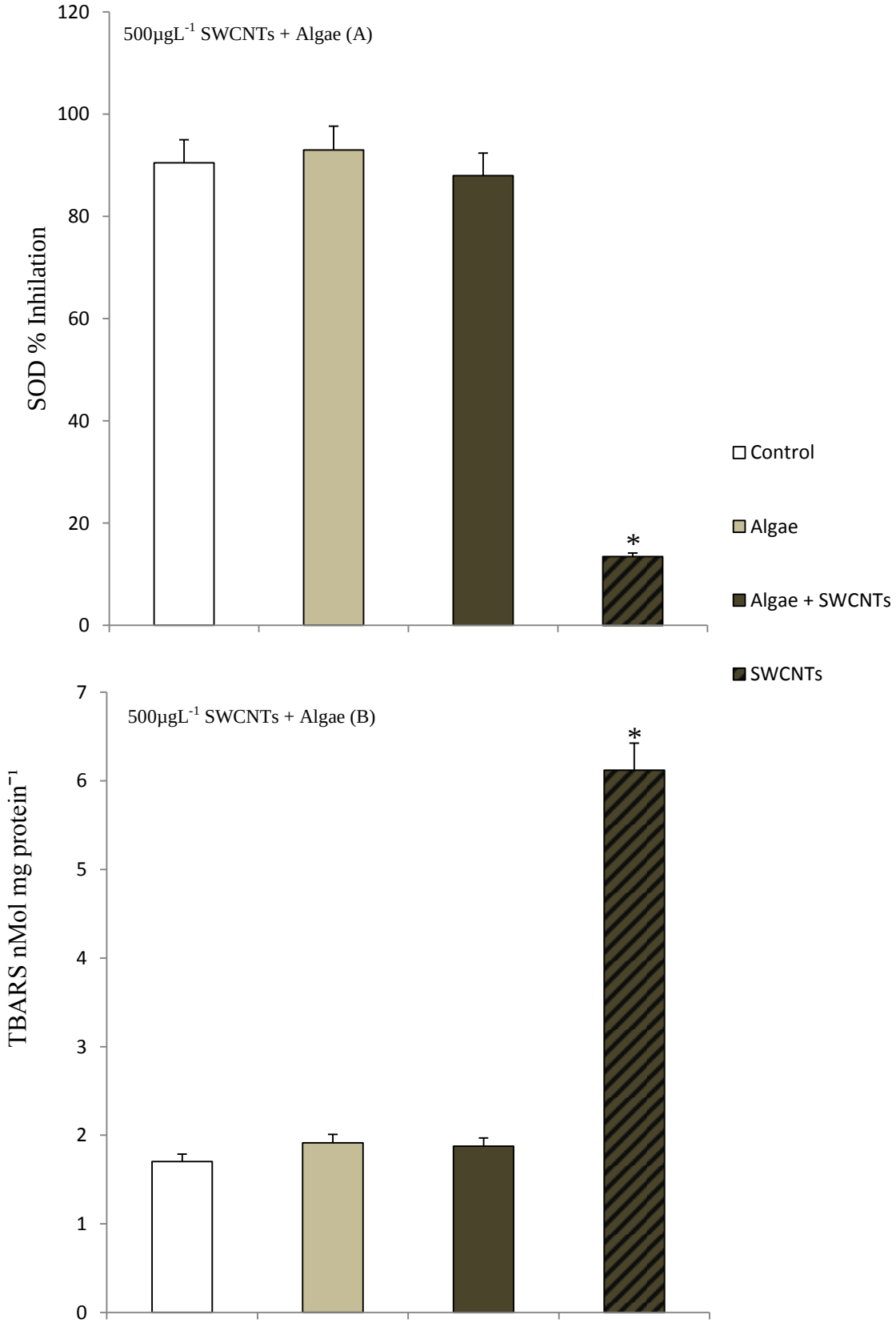

Figure 3.41 Mussels were fed algae (*Tetraselmis suecica*), SWCNT 500µg L^{-1} alone and *Tetraselmis suecica* + SWCNTs 500µg L^{-1} for 24h. * significantly different from control, algae and algae + SWCNTs 500µg L^{-1} ($P<0.001$).

3.5 Trophic transfer of SWCNTs from algae to mussels

3.5.1 Preliminary light-microscopic observations of the trophic transfer of SWCNTs

A direct observation by the light microscope was used here to detect any visible physical interaction between algae and mussels (Figures 3.42A-F), a prerequisite for the uptake and trophic transfer of SWCNTs from algae to mussels.

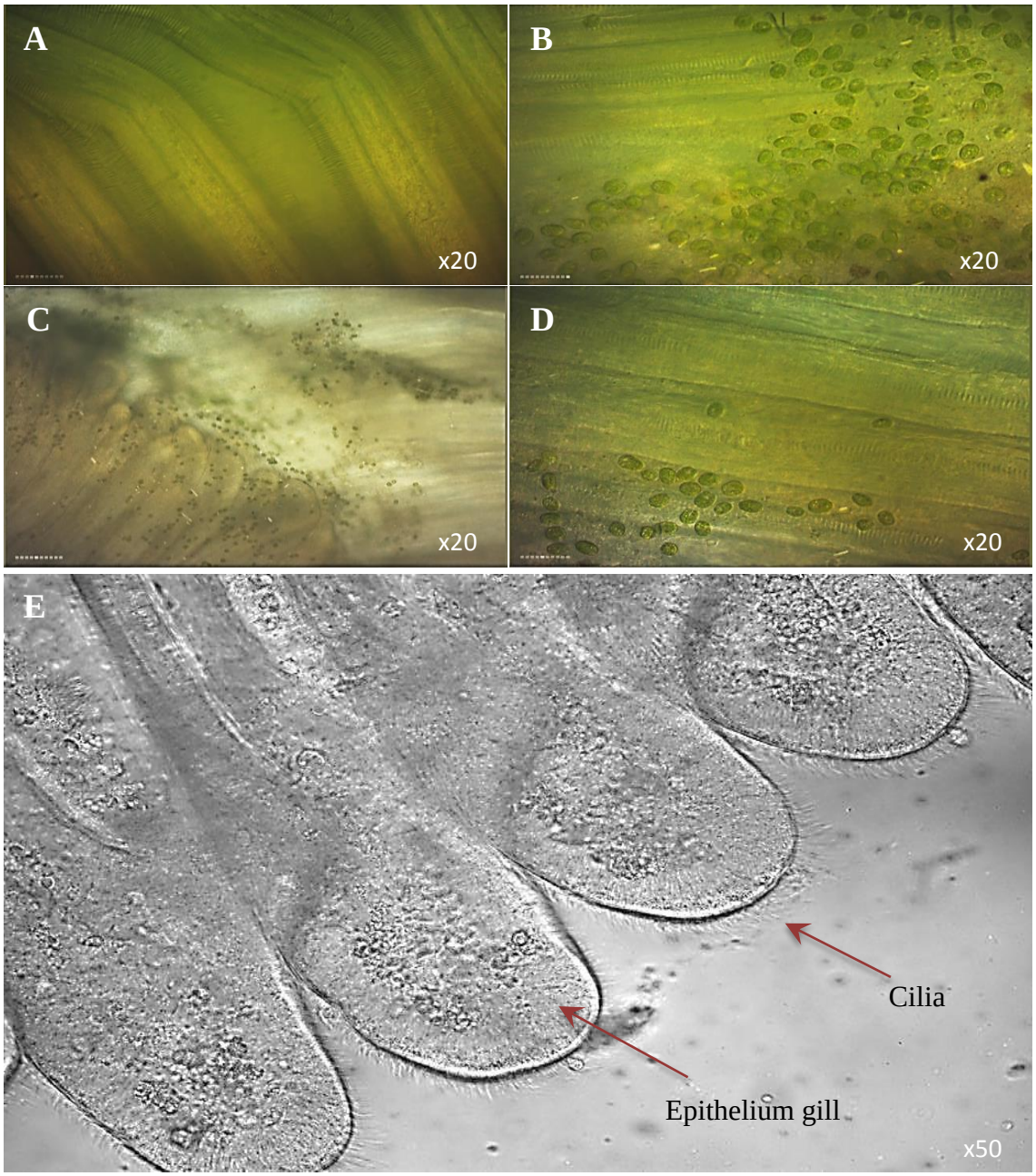

Figure 3.42 Light micrographs showing (A) control epithelium gill mesh, Figures (B-D) show the preliminary observation of physical interaction between algae and mussels, while (E) shows cilia on the gill epithelia that can be used for capturing food or other substances.

3.5.2 Histological observation of the trophic transfer of SWCNTs from algae to mussels

Histological sections of the mussel digestive gland following exposure to algal cells-SWCNT 500µg L^{-1} showed evidence of algal cells including SWCNTs (Figures 3.43-46A and B).

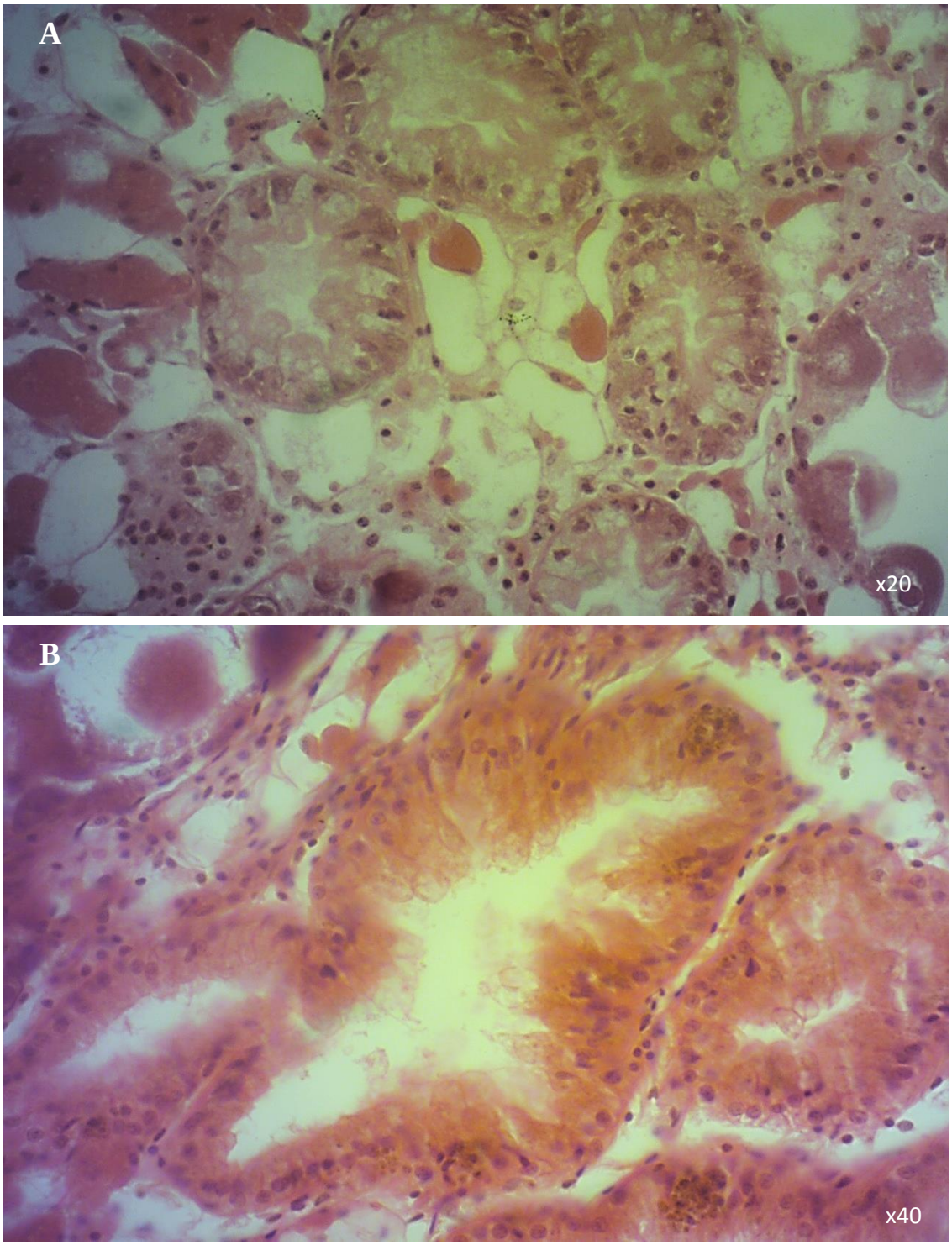

Figure 3.43 Histological sections of digestive gland from mussels. (A, B) Gut sections from control.

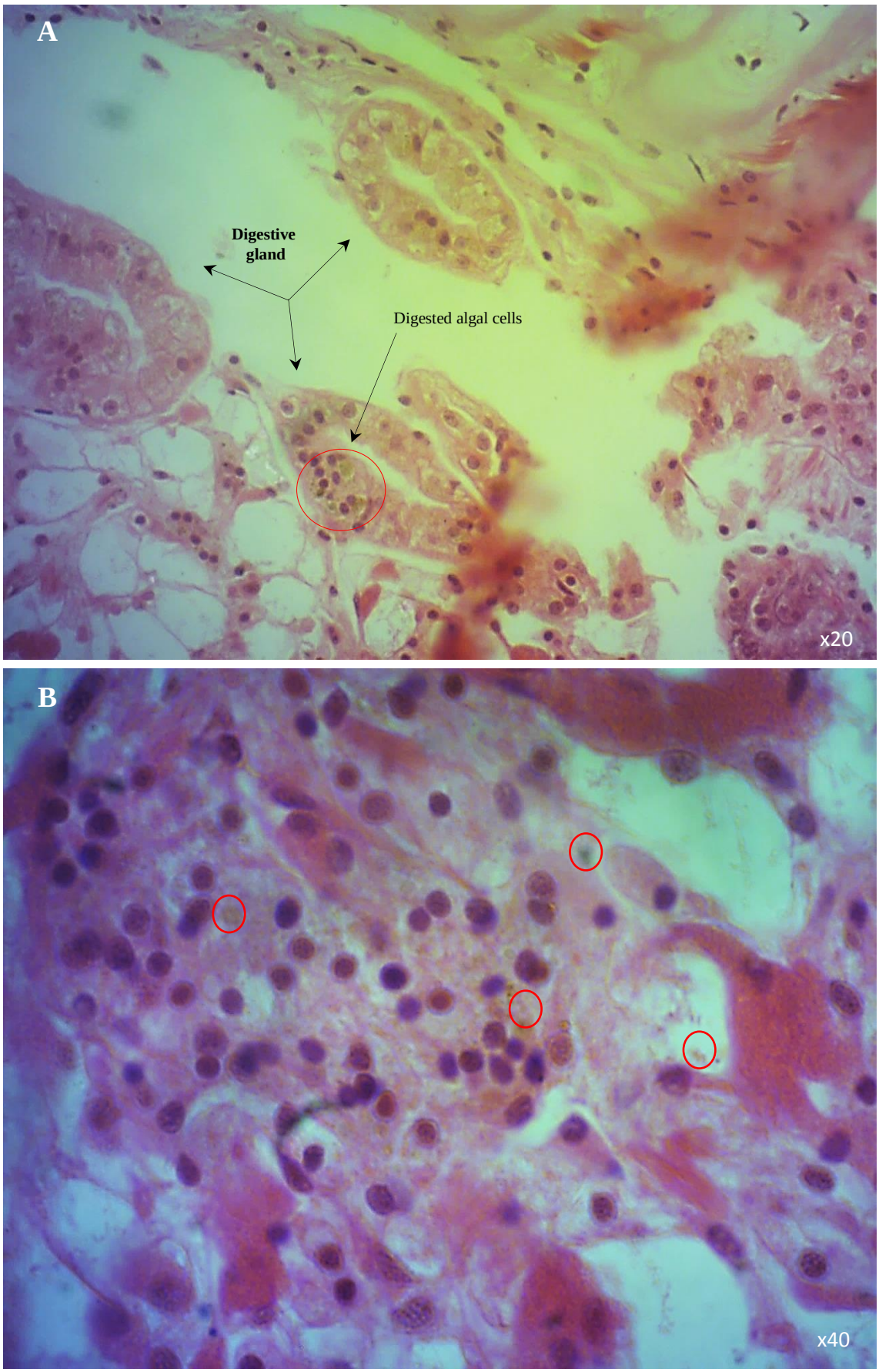

Figure 3.44 Histological sections of digestive gland from mussels. (A, B) Gut sections from mussels that were left to feed algae for 10 minutes.

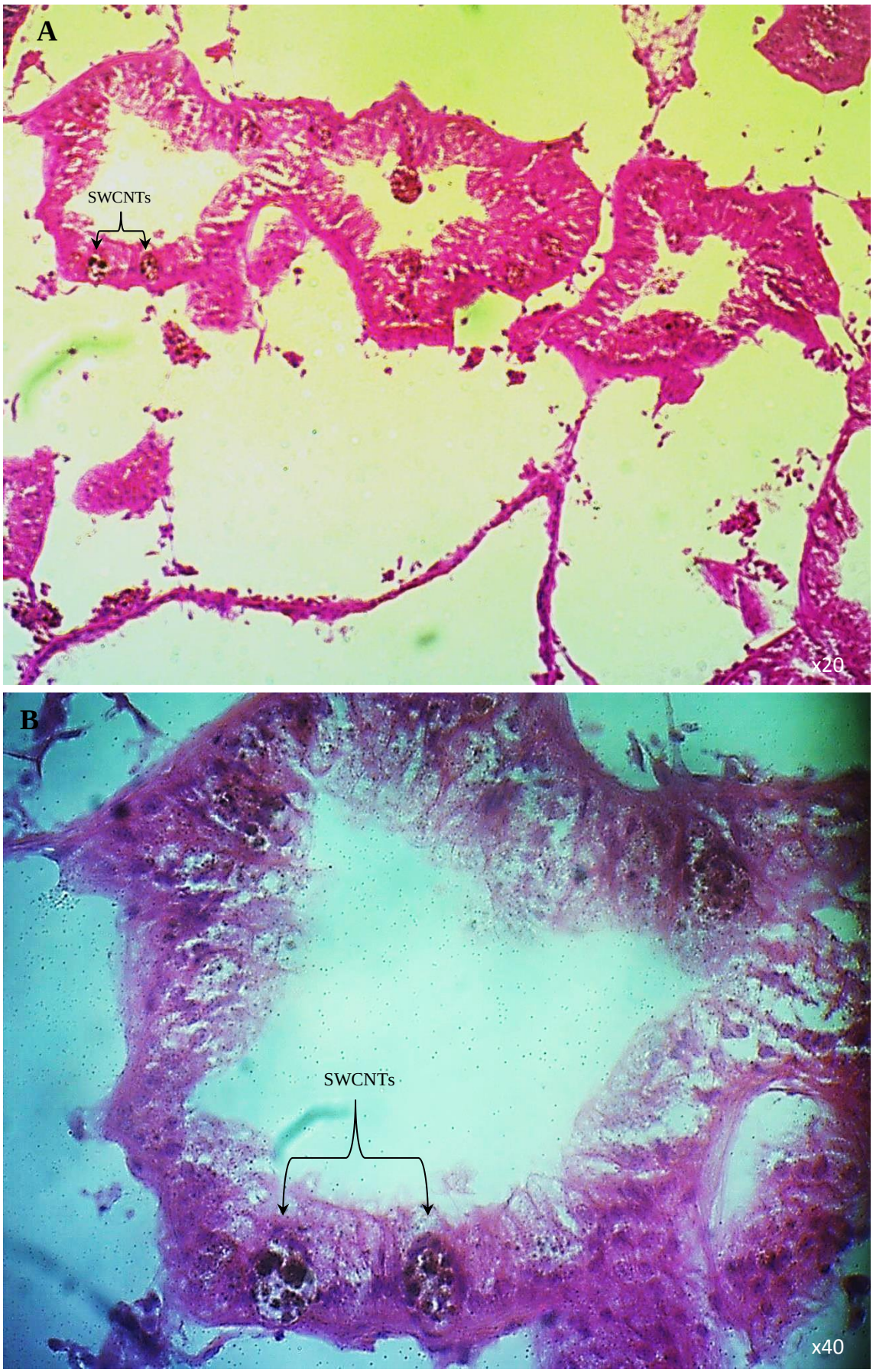

Figure 3.45 Histological sections of digestive gland from mussels. (A, B) Gut sections from mussels that were left to feed algae for 10 minutes.

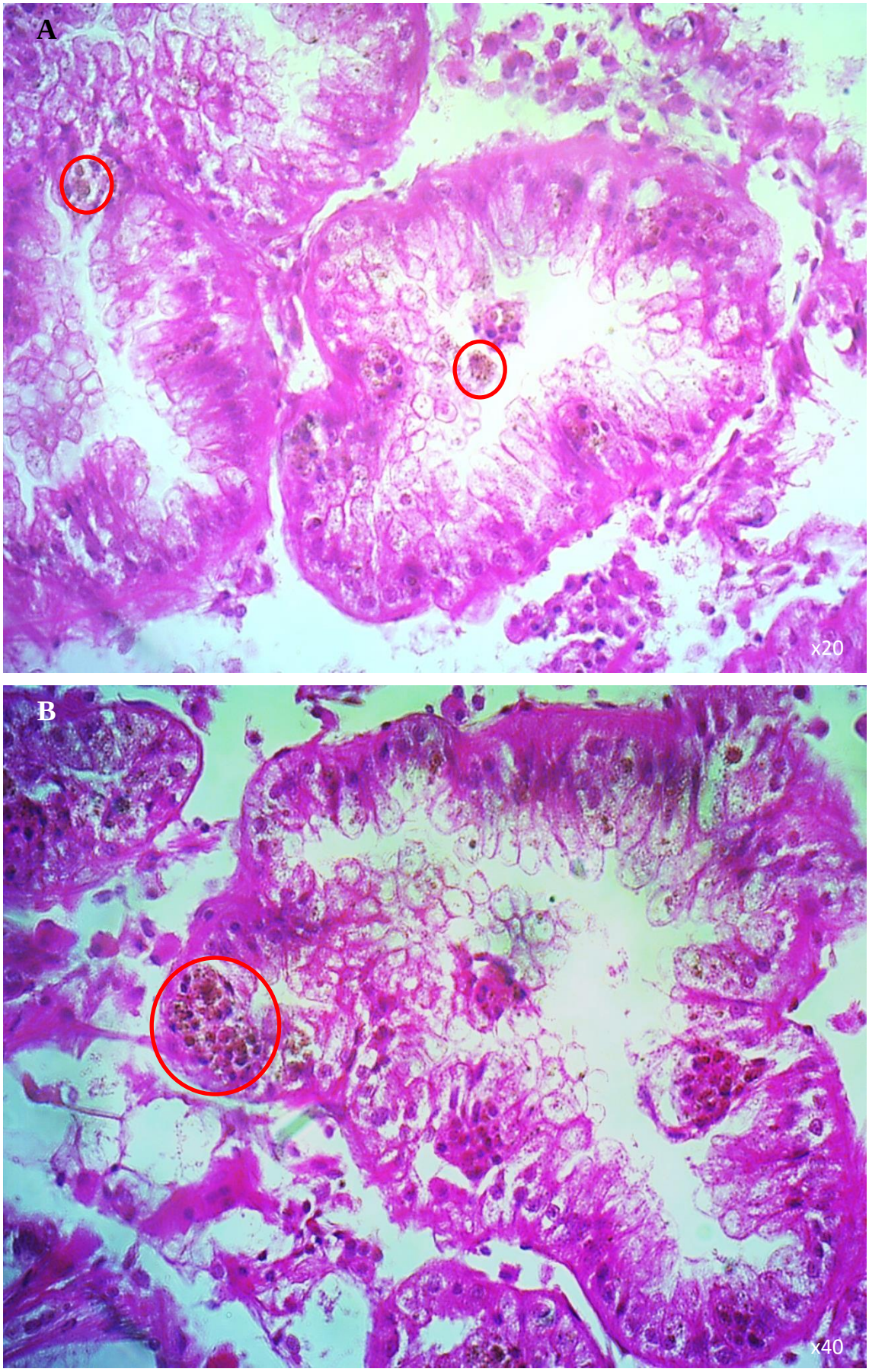

Figure 3.46 Histological sections of digestive gland from mussels. (A, B) Gut sections from mussels that were left to feed algae for 10 minutes.

4. DISCUSSION

4.1 Synthesis and characterization of stock SWCNT

As outlined above, our understanding of the genotoxic effect of nanomaterials to organisms in the marine environment has hitherto been limited mainly to the effects of metallic nanoparticles, with very little equivalent information available regarding the toxicity of SWCNTs in seawater. In part, this situation has arisen from the expectation that insoluble carbon nanomaterials simply precipitate, accumulating in sediments, and have thus been perceived to pose no threat. The characterization of SWCNTs in the present study was carried out using a range of methods. TEM micrographs showed SWCNTs agglomerate in water (Figure 3.2). Accordingly, dispersants are used to keep uncoated SWCNTs in suspension during toxicity experiments. For instance, sodium dodecyl sulphate has previously been used as a dispersant of SWNCTs in aqueous exposure experiments (Smith *et al.*, 2007). However, the observed toxic effects have subsequently been attributed, at least in part, to the sodium dodecyl sulphate component (Handy *et al.*, 2008; Boyle *et al.*, 2014; Petersen *et al.*, 2014). Therefore, Suwannee River natural organic matter, although not as good a dispersant as sodium dodecyl sulphate or other detergents (Alpatova *et al.*, 2010), was chosen in the present study because it is far less toxic but still able to maintain the SWCNTs suspended in seawater long enough for mussels to clear them from the water column (Figure 3.11). Furthermore, dispersing SWCNTs prepared in 0.02% Suwannee River natural organic matter (SRNOM) produced a pH–zeta potential dependence as well as a concentration-dependent sized spectrum of SWCNT agglomerates similar to those previously described in the literature for other aqueous media (Heo *et al.*, 2012; Dresselhaus *et al.*, 2002). However, natural organic matter is known, under certain conditions, to undergo complex reactions with dissolved metals, influencing the bioavailability, route of exposure, and thus, potentially, the toxicity of metals (Macdonald *et al.*, 2002). SEM images in Figures 3.3a,b show the matrix of SWCNTs dispersed in 0.02% SRNOM, which may possibly coat the SWCNTs (Brady-Estevez *et al.*, 2010), therefore, the thickness of the SWCNT layer may increases (Figure 3.3). However, in the present study, SEM images, similar to those obtained by Rao et al. (2012), show what appear to be individual SWCNTs (Figures 3.3c-f); however, it cannot be completely ruled out that this observation is the result of an alignment of SWCNTs, as has been demonstratedby Chinnapongse et al. (2011) in the presence of humic acids. However, the ability of SRNOM to disperse SWCNTs and keep them in suspension was clearly demonstrated.

Further ultrastructural assessment in seawater was impossible owing to the development of salt crystals.

Therefore, other techniques had to be employed to characterize the physiochemical properties of SWCNTs in seawater. DLS was used to approximate the size of the agglomerates Figure 3.4. To the naked eye SWCNTs seemed to disperse well with SRNOM in seawater. In fact, although they remained in suspension for longer than without SRNOM, SWCNTs became agglomerated, an observation confirmed using DLS. As indicated above various organic dispersants, such as SDS (and SRNOM), have been shown to cause CNTs to align themselves in a polarization-dependent manner (Lee *et al.,* 2005). In the present study, the resulting SWCNT agglomerates in seawater may have been the result of such scenario. Nanotubes may have aligned themselves in the presence of SRNOM; it appears that under stable pH conditions (pH 8) increases in the agglomerate size of SWCNTs may be dependent on SWCNT concentrations as well as their SRNOM-influenced alignment. In seawater, the increased ionic strength on carbon nanotubes has been shown to have an effect on surface area and the charge of the SWCNTs (Joseph *et al.,* 2011). These physicochemical conditions are likely to have affected the chemical interactions between SWCNTs and ionic compounds in seawater; these process, in particular how they affect the characterisation techniques in environmentally relevant media need to be better understood.

Raman spectroscopy is a technique suitable for identifying CNTs in complex media. In the radial breathing mode (RBM) the characteristic G-band peaks for carbon nanotube are known to have strong Raman intensity in lower (100-450 cm^{-1}) and higher 1500-1605 cm^{-1} frequency region (Saito *et al*., 2001), indicating the sp^2-hybridized bonding nature of the SWCNTs. The G-band refers to whether the nanotube is semiconducting or metallic; the red laser excitation (785nm) is used to obtain a signal from semiconducting nanotubes and the green laser shows whether the nanotubes are metallic. SWCNT aggregation has often been observed to induce changes in the G-band peaks, which may interfere with the metallicity identification by resonance Raman spectroscopy (Heller *et al.,* 2004). Owing to the strong resonance effect in SWCNT Raman peaks (Kataura *et al.,* 1999), performing a complete analysis of all (n,m) species would require continuous laser excitations (785-nm) to match resonance energies of every (n,m) species in a SWCNT (Wanga, 2012).However, the electronic states, phonon and the resonance Raman effect are highly dependent on nanotube diameter (Saito *et al.,* 2001), and under these resonance conditions, the Raman spectrum or cross section

observed becomes very large due to the strong coupling that occurs between the electrons and phonons of SWCNTs (Dresselhaus *et al.,* 2002). The resonance Raman spectroscopy is important for understudying the geometrical structure of individual SWCNTs (Dresselhaus *et al.,* 2002), and providing an important tool for biological detection (Alivisatos, 2004). For instance, Raman detected SWCNT in the intestine, kidney, and bladder of mice (Liu *et al.,* 2008), and in the gill *Daphnia magna* and mussels (Petersen *et al.,* 2013; Al-Shaeri *et al.,* 2013). This shows that, SWCNTs have distinctive resonance-enhanced Raman signatures with large scattering cross-sections for single nanotubes, which makes Raman a suitable system for SWCNTs identification in biological systems, (Liu *et al.,* 2009). Meanwhile, the peaks occurring in D-band at 1250-1450 cm^{-1} and in G′-band at 2500-2900 cm^{-1} are also characteristic of CNTs (Dresselhaus *et al.,* 2002). In the present study the spectrum of SWCNT stock clearly showed peaks at (RBM) 268 cm^{-1}, D band at 1290 cm^{-1}, G band at 1590 cm^{-1}, and G´ band at 2585 cm^{-1} characteristic for 1.1 to 1.5 nm diameter SWCNTs produced here (Dillon *et al.,* 1999; Al-Shaeri *et al.,* 2013) It appears that, in resonance Raman spectroscopy conditions, the enhancement of the Raman intensity depends on the G-band phonon symmetries (Tang and Albrecht, 1970), especially in the case of SWCNTs (Saito *et al.,* 2001).

The main source of problems associated with carbon nanotube toxicity studies originate in residual metals used as catalysts during their synthesis (Pumera, 2007; see review in Chapter 1, section 1.2 and the discussion further down). It was therefore important to assess the potential presence of residual metal impurities (RMI) in stock SWCNTs before subjecting the SWCNTs to ecotoxicological studies. The main RMI in stock SWCNTs were found to be iron, manganese and nickel (Table 3.2). The reason for not choosing more RMI in SWCNTs was that SWCNTs are known to contain a high percentage of iron, nickel and manganese (Chiang *et al.,* 2001; Baron *et al.,* 2003; Arepalli *et al.,* 2004; Shvedove *et al.,* 2005; Kumar, 2006; Kumar and Ando, 2010). It would be better to state that only extremely low trace levels of other metals are usually associated with pristine SWCNTs and are unlikely to cause any problems (Hurt *et al.,* 2006; Tian *et al.,* 2010; Adeleye and Keller, 2014; Rosenzweig *et al.,* 2014).Single-walled carbon nanotubes have been shown to attract metal ions in the aquatic environment (Rao *et al.,* 2007; Lu and Chiu, 2006), a property that is increasingly being exploited in the development of remediation technologies (Choi *et al.,* 2011). However, the process by which this occurs can be complicated by the surface properties of the

SWCNTs as well as the ionic strength and pH of the medium. In the case of purified, uncoated SWCNTs, the charge is negative at pH 8 (Rao *et al.*, 2007), a condition similar to that found in full-strength seawater and the seawater used in the present study (salinity 32%, pH 8.4). It was not necessary or practical to directly measure the surface charge here, because (1) the measurement of the solution pH is an effective method of determining the proton-induced surface charge, (2) the pH measurement can be effected by (a) 'excess' or 'missing' H^+ in solution during a potentiometric pH titration of an oxide powder suspension, (b) incorporating an activity coefficient model or any background contamination, and (c) non-Nernst equation characteristics of the pH and reference electrodes. In summary, the proton-induced surface charge density is directly related to this solution excess or deficit (Wesolowski *et al.,* 2008). Furthermore, there exists no practical method for directly measuring the surface charge of individual surfactant-wrapped SWCNTs (White *et al.*, 2007).

However, the expected inverse relationship between pH and zeta potential was observed, giving rise to negative zeta potentials under slightly alkaline conditions in seawater (Table 3.1). For the dispersant charge (SRNOM), measurements showed that, SRNOM had a negative charge, caused due to the presence of functional groups such as carboxyl and hydroxyl, forming the major components of NOM in water (Kim *et al.*, 2010). From the observed results a negative surface charge of SRNOM and SWCNTs can be assumed (Malvern Instruments, 2004). This assumption was further supported by the majority of the recoverable Cd^{+2} and Zn^{+2} being found to partition on to the dispersed SWCNTs rather than remaining dissolved in the aqueous phase (Table 3.2).

Qiao and Aluru (2003) and Rao *et al*. (2007) report that divalent metals ions, such as Cd^{+2}, Zn^{+2}, Cu^{+2}, Pb^{+2} and Ni^{+2}, may be sequestered by CNTs and thereby removed from aqueous solutions and concentrated on the large surface area of CNTs. The adsorption of divalent metal ions (Zn^{+2}) onto SWCNT increased with the increase of pH in the pH range of 1-8 (Lu and Chiu, 2006), and in the present study metal scavenging behaviour of SWCNTs was observed for Cd^{+2} and Zn^{+2} under exposure conditions (Tables 3.3-3.10). As can be seen, the amounts of Cd^{+2} and Zn^{+2} adsorbed onto SWCNTs in pellets were higher than those in the supernatants. This chemical interaction between metals and SWCNTs may be considered very important in gaining an understanding of nanomaterial behaviour in the environment and their potential impact on biological systems, particularly where there is DNA damage. It suggests that the wall of the SWCNT surface may play the main role in adsorbing the other

substances, such as metals, from seawater, so that these substances may combine to cause additive, synergistic or potentiating effects on exposed organisms, greater than those of the individual substances. If this behaviour is indeed occurring in natural aquatic media, it could have implications for the role of CNTs in environmental metal dynamics (Kleiner and Hogan, 2003). In the environment, it has been observed that free NMs tend to form aggregates/agglomerates which may be eliminated or trapped and precipitated out of suspension (Farrē *et al.,* 2009). This suggests that SWCNTs may adsorb dissolved metals (Cd^{+2} and Zn^{+2}) and other contaminants, and this may increase toxicity to the point that these substances are more toxic than the metals or SWCNTs alone. Despite recent progress in understanding the fate of ENMs in model environments and aquatic organisms, there remains a large need for fundamental information regarding releases, transformations, persistence, distribution and bioavailability of NMs in complex media (Sahu, 2009). Moreover, research into transport, fate, bioaccumulation, and ecological impacts is needed, using environmentally relevant concentrations and forms of ENMs (functionalized and weathered as opposed to pristine) in representative environmental media and with a broader range of organisms (Lowry *et al.,* 2010; Al-Shaeri *et al.,* 2013).

4.2 Potentiating toxicological interactions of single-walled carbon nanotubes with dissolved metals

The mussel gill is a very effective filter-feeding apparatus that is able to remove particulate matter of submicrometre size from the water column, although the efficiency increases above 1.5µm (Lei *et al.,* 1996). Therefore, the size range of the SWCNT agglomerates formed under exposure conditions in the present study (Table 3.1) was easily removed by filter-feeding mussels, the vast majority of which were excreted as pseudofaeces (Figure 3.11). However, an unquantified proportion of the SWCNTs was retained by the gill and remained attached to the epithelium for at least 24hrs following the initial exposure (Figure 3.12A). Several possible causes of DNA damage have been hypothesised: NPs adhered to the surface, altering the membrane properties, therefore affecting the permeability and the respiration of the cell; they can penetrate inside bacteria and cause DNA damage (Klaine *et al.*, 2008). Examination of the retained black agglomerates using Raman spectroscopy displayed the characteristic and unique signatures of carbon nanotubes, the radial breathing mode at low frequencies (Figure 3.12A and C). Similar results were shown by Roberts et al. (2007), who exposed *Daphnia Magna* to 500µg L^{-1} SWCNT for to 20hrs. No peaks characteristic of carbon

nanotubes were detected in unexposed (control) gill tissue (Figure 3.12B and C). Based on this information and the from the observations made in the present study it is reasnobale to conclude the presence of SWCNT agglomerates on the gill epithelium of mussels is visible evidence confirming direct interaction of mussels with SWCNTs through their filtration system. This observation is consistent with previous studies by Hull *et al*. (2013), who observed the uptake of 50 nm FeOxNPs by mussels (*Mytilus galloprovincialis*) during water column exposures and Tedesco *et al.* (2010), who reported the uptake of 5 nm AuNPs by mussels (*M. edulis*), finding that following termination of the exposure, 95% of the AuNPs take up by the mussels were localized in the digestive gland. The uptake of spherical, polystyrene latex particles varying in size from 40 to 1000 nm in the American oyster *(Crassostrea virginica)* was described by Haye et al. (2006), who observed that particles were taken up and digested by the oysters. These results provide an indication of the potential for accumulation of SWCNTs to occur in marine bivalves (mussels) (Hull *et al*., 2013). Therefore, it appears likely that, if SWCNTs accumulated in the mussel body, the SWCNTs concentrations in the mussel body would also increase, which might result in consequences effecting the biological system; i.e. increasing the susceptibility to disease, which may lead to death of the animal. The majority of CuO NPs and SWCNTs have been shown to have accumulated in the mussel gill epithelium, which is considered to be the main interface between the organism and the surrounding environment (Gomes *et al.,* 2011; Al-Shaeri *et al.,* 2013).

In addition to confirming the presence of SWCNT using Raman, the presence of metals (Cd^{+2} and Zn^{+2}) on the gill epithelium were confirmed by atomic absorption spectrometry (AAS). Mussels have been used in environmental monitoring studies as biological "sentinels", because of their propensity to take up and concentrate metals from the surrounding media (Bellotto and Miekeley, 2007; Goldberg, 1975). The uptake of Cr, Mn, Ni, Cu, Zn, Cd, Ag, Pd, Sb, Pb, Fe, Pt and Rh in tissues of mussels and their shells has been confirmed in controlled laboratory studies (Bellotto and Miekeley, 2007; Zimmermann *et al.,* 2002). In the present study, the chemical analysis of whole-tissue homogenates from mussels exposed *in vivo* to Cd^{+2} and Zn^{+2} showed higher body burdens compared with the control, demonstrating the bioavailability of the metals under exposure conditions. Although the presence of SWCNTs had no measurable effect on the overall metal burden in the gill tissue, Cd^{+2} and Zn^{+2} levels associated with SWCNTs incubated with the respective metals were higher than with SWCNTs in the

absence of spiked metals (Table 3.11). It appears that, SWCNTs are able to adsorb metals with high adsorption capacity (Mubarak *et al.,* 2014), subsequently, metals can be accumulated in mussel's tissue (Kournoutou *et al.,* 2014), which might result in consequences biological effect such as DNA damage and oxidative stress (Faria *et al.,* 2014).

Cell viability as a biomarker is important in cytotoxicity studies, firstly because these levels are excellent indicators of cell health, and secondly, because their measurement is an important step to take to determine if treatment is killing cells or alternatively keeping cells alive; and finally, it may be difficult to continue performing research on dead cells for other endpoints, such as comet assay, SOD activity and lipid peroxidation. There are several accepted assays for cell viability, such as propidium iodide (Pi) using flow cytometry and Trypan blue, which uses a light microscope that utilise the exclusion of certain dyes by live cell membranes, neutral red (NR), and crystal violet (CV) stain, primarily lysosomes and live cell membranes, while tetrazolium bromide (MTT) dyes measure the mitochondrial dehydrogenases activity in cells (Jones and Senft, 1985; Pulskamp *et al.,* 2007). However, in the present study, flow cytometry was used to assess cell viability in mussel haemocytes and gill cells, because this is a rapid, economical and readily available method. Furthermore, it has been shown that flow cytometry is more accurate than Trypan blue, because flow cytometry can count around 50,000 cells per second, while Trypan blue tens cells can be manually counted per minutes. Pulskamp *et al.* (2007) incubated human lung cells with commercial SWCNTs and MWCNTs, using carbon black and quartz as reference particles, as well as acid-treated SWCNTs with reduced metal catalyst content. They did not produce any observable acute toxicity affecting cell viability when human lung cells were incubated to CNTs with 100g/ml for up to 72hrs, and cell viability was still between 70–80%. Tejral et al. (2009) observed no effect on cell viability in human lungs exposed to 100µg/ml CNT (Binelli *et al.,* 2009). For *in vivo* exposures, a sub-lethal concentration of 0.001µM Cd was used in the present study, based on the findings of George and Coombs in 1977 (Pruksi & Dixon, 2002). After the 72hrs exposure period to SWCNTs 100µgL^{-1}, Cd 0.001µM, Cd 0.001µM + SWCNTs 100µgL^{-1}, Zn 1.0µM, Zn 1.0µM + SWCNTs 100µgL^{-1} and Cd 0.001µM + Zn 1.0µM + SWCNTs 100µgL^{-1}, and following isolation, no significant effect on cell viability was observed in exposed mussels in either gill cells or haemocytes (Tables 3.12-3.13). The cell viability

of haemocytes of bivalves exposed to heavy metals has also been successfully measured using flow cytometry by Brousseau *et al*. (1999).

However, to date there has been a lack of quantitative ecotoxicity data generated, and recently there has been great scientific concern about the possible adverse effects of dissolved metals or other substances that may become associated with engineered NMs (Farré *et al.,* 2009). The combined effect of *in vivo* exposure of mussels to subthreshold concentrations of metals and SWCNTs on the degree of DNA damage in both gill cells and hemocytes was significantly ($p<0.001$) elevated compared with exposure to the respective metals or SWCNTs alone (Figures 3.15, 17, 19, 21, 23A and B). Pruksi & Dixon (2002) exposed *M. edulis* to 0.001µM Cd for 4 weeks, followed by a 1 day exposure to 0.001µM $ZnCl_2$ or 0.02µM $MgCl_2$ and did not observe significant increases in the level of DNA strand breaks in gill cells. In the present study there were no effects on DNA damage observed in either gill cells or hemocytes following exposure to 0.001µM $CdCl_2$ or 1.0 µM $ZnSO_4$, or combined, in the absence of SWCNTs (Figures 3.15, 17, 19, 21, 23A and B). While Cd is a known genotoxicant and carcinogen (Zhang and Xiao, 1998), including in marine species (Hartl *et al.,* 2004), Cd at a concentration ≤0.001µM is not genotoxic to mussels. Zinc is known to be less genotoxic than Cd (George and Coombs, 1977; Pruksi and Dixon, 2002), which fits well with the results of the current study, showing no toxicity at concentrations as high as 1µM (Figures 3.15-3.24A and B).

In terms of SWCNT concentration, it has been observed that there was no effect on the mortality of the shrimp *Thamnocephalus platyurus*, copepod *Amphiacus tenuiremis* and *Hydra attenuata*, which were exposed to 10mg L^{-1} (Cattaneo et al, 2009). It has also been demonstrated that 500µg L^{-1} SWCNT was a sublethal concentration to mussels (Woods *et al.,* 2009). Accordingly, the present mussels were exposed to 5µg L^{-1}, 10µg L^{-1}, 50µg L^{-1}, 100µg L^{-1} and 500µg L^{-1} single-walled carbon nanotubes (SWCNTs), and the study has shown that the SWCNT concentration ≤50µg L^{-1} SWCNTs alone did not cause DNA damage, oxidative stress or mortality in *Mytilus edulis*. However, some studies have shown that NMs can be toxic to aquatic organisms, such as fish or *Daphnia*, as well as unicellular organisms, such as protozoa or bacteria (Zhu *et al.,* 2006), SWCNTs have been shown to be ingested by various species, accumulating in the intestine in the mussel, *Villosa iris*; Mwangi *et al.,* 2012) and oligochaete (*Lumbriculus variegatus*; Petersen *et al.,* 2008). This is very different from other nanoscale particles, such as CuO NPs, which were found to accumulate in gills,

affecting the ability of mussels to cope with low O_2 levels, thereby inducing oxidative stress (Hu *et al.,* 2014). ROS such as hydrogen peroxide, hydroxyl radicals, superoxide anions and other oxygen radicals are capable of directly oxidizing DNA, amino acids in proteins and polyunsaturated fatty acids in lipids (Yoshida *et al.,* 2004). Several toxic affects arise at the cellular level, caused by environmental pollutants, and can be induced by reactive oxygen species (ROS) (Livingstone, 2003), and subsequently cause oxidative stress (Valavanidis *et al.,* 2006). According to several studies of reactive oxygen species (ROS) and oxidative stress as the main effects of SWCNTs exposure in mammals (Pacuari *et al.,* 2008), toxicity mechanisms require further clarification in invertebrate species (Unfried *et al.,* 2007; Mortimer *et al.,* 2010; Gomes *et al.,* 2011). ≥150µg L^{-1} SWCNTs have been shown to induce DNA damage and increase SOD activity and lipid peroxidation in the hepatopancreas cells of snail (*Lymnea luteola*) and mussels (*Mytilus galloprovincialis*) (Ali *et al*., 2014; Moschin *et al.,* 2014). In the present study, a 72hrs exposure to ≥ 100µg L^{-1} SWCNTs alone showed significantly increased DNA strand breaks in both gill cells and haemocytes (ANOVA, $P<0.05$), and significantly increased oxidative stress, expressed as superoxide dismutase (SOD) activity and lipid peroxidation in gills ANOVA, $P<0.05$). Murray et al. (2009) measured oxidative stress in epidermal JB6 P+ cells, and the skin of SKH-1 mice exposed to SWCNT, by evaluating decreases in antioxidants and increases in lipid peroxidation. They hypothesized that SWCNT may be toxic to the mouse epidermis, with the toxicity dependent upon the formation of free radicals antioxidant defences. It appears that the increase of SOD activity, lipid peroxidation in mussel gill and DNA damage in mussel haemocytes and gill cells are in all probability a result of the formation of ROS. The SOD activity in gills increased after exposure to ≥ 100µg L^{-1} SWCNTs, showing that these SWCNTs may have potent redox properties (Moschin *et al.,* 2014), with the capacity to generate ROS. Furthermore, it has been suggested that CNTs also have a ROS scavenging effect (Petersen *et al.,* 2013; Fenoglio *et al.,* 2006), and may be indicative of the formation of superoxide anions in mussel gills (Gomes *et al.,* 2011) (Figure 3.22, 3.24A and B). In the present study, SOD activity significantly decreased in 72hrs when mussel's were exposed to ≥100µg L^{-1} SWCNTs. This adverse result may produce oxidative stress in mussel gills, as evidenced by the breakdown in the antioxidant defence system and lipid peroxidation. It has been reported that exposure to CNTs has caused increased generation of ROS in different types of cultured cells (Shvedova *et al.,* 2008; Pulskamp *et al.,* 2007). It has also been suggested that, if antioxidant defences are deficient, then damage may occur, affecting a variety of tissues

(Betteridge, 2000). Oxygen and nitrogen free radicals are continuously produced in living cells, and are considered fundamental to the physiological control of cell function in biological systems (Halliwell and Gutteridge, 1985).

The physicochemical properties (e.g., structure, size distribution, and surface area, surface chemistry, surface charge, agglomeration state) of CNTs dictate their behaviour (Pérez *et al.,* 2009), much of which is still relatively unknown in environmental media (Stone et al., 2014). A particular important physiochemical property is surface change, which in the case of SWCNTs in seawater is negative (Table 3.1). As a consequence Zn^{+2} was shown in the present study to associate with SWCNTs, leading to a significant genotoxic insult (Figures 3.15, 17, 19, 21, 23A and B). This is particularly remarkable as Zn^{2+} alone at concentrations as high as 1 µMol failed to induced an increase in DNA strand breaks compared to the control (Figures 3.15, 17, 19, 21, 23A and B). Furthermore, the lack of increased DNA damage following combined Zn^{+2} and Cd^{+2} exposure in mussel hemocytes and gill cells in the absence of SWCNTs, suggests that the observed effect was not additive or synergistic, as the concentrations (0.001µM Cd^{+2} and 1µM Zn^{+2}, respectively) of the individual constituents were below the endpoint thresholds for the respective substances. Instead, effects observed following the addition of SWCNTs at concentrations lower than 100µg L^{-1} to the metal spiked seawater, were of a potentiating nature. Potentiation, where toxicity occurs following exposure to a mixture of substances and one or more of these substances induce no intrinsic toxicity when administered alone, has been observed in other carbon nanomaterials, such as the interaction of ultrafine carbon black particles with transition metals *in vitro* (Wilson *et al.,* 2002). *In vivo* inhalation experiments have also shown that multi walled carbon nanotubes can potentiate airway fibrosis in murine allergic asthmatic mice (Ryman-Rasmussen *et al.,* 2009). The observations made in the present set of experiments lend weight to the model that uncoated SWCNTs could be acting as sequestration agents and vectors (Christian *et al.,* 2008) for otherwise benign concentrations of dissolved metals, delivering a concentrated toxic dose to localized areas of gill epithelia, thereby increasing their toxicity. Association of metals with carbon nanotubes has been shown to cause biological effects, such as decreases in cell viability and glutathione levels in human keratinocyte cells (Pulskamp *et al.,* 2007; Shvedova *et al.,* 2003). Lam *et al.,* (2004) reported that the association of Ni with SWCNTs caused an increased mortality rate in mice, and they also observed SWCNTs associated with Ni were more toxic than SWCNTs with associated quartz. This suggests that the presence of the divalent ions

Cd^{+2} and Zn^{+2} associated with SWCNT may be able to increase DNA damage more than with the individual substances alone.

Stable agglomeration/aggregation or colloidal suspensions of nanomaterials are a prerequisite for efficient interactions with some aquatic organisms, such as mussels and algae, which may lead to their uptake or toxic effects (Pérez *et al.,* 2009). Suwannee River natural organic matter (SRNOM)–dispersed SWCNTs alone, without metal co-exposure, showed increased DNA damage only at concentrations above 50µg L^{-1} in both gill cells (Figures 3.15, 17, 19A) and hemocytes (Figures 3.15, 17, 19B). Dispersant control experiments in the present study using 0.02% Suwannee River natural organic matter spiking volumes equivalent to those used for the 500µg L^{-1} SWCNT exposures with and without metals, but in the absence of SWCNTs, showed no increased DNA damage above that of the negative control (Figure 3.25), suggesting that the dispersant, or any potential interactions between dispersant and spiked metals, did not influence the observed toxicity. Among other mechanisms, oxidative stress has widely been proposed as the causative agent for observed toxicity response to nanomaterial exposure, especially carbon nanotubes (Kagan *et al.*, 2006; Pulskamp *et al.*, 2007).

In the present study, superoxide dismutase activity, expressed as percentage inhibition, and the generation of lipid peroxidation, expressed as TBARS nMol mg protein (Figures 3.26A and B), in gill cells of mussels exposed to 0.001µM $CdCl_2$ alone, 1µM Zn alone, 0.001µM $CdCl_2$ + 1µM Zn and SWCNTs alone, at concentrations of up to 50µg L^{-1} was not significantly increased above the control (Figures 3.16, 18, 20, 22, 24A and B). The process of ROS formation can lead to oxidative damage, such as lipid peroxidation and genotoxicity (DNA damage) (Gagné *et al.,* 2008). However, in the present study, the above results report no increase in SOD activity and lipid peroxidation, subsequently no ROS was produced; meaning no DNA damage was detected when the mussel was exposed to metals 0.001µM $CdCl_2$, 1µM Zn with and SWCNTs $\leq$50µg L^{-1}. Furthermore, superoxide dismutase activity, expressed as percentage inhibition, and the generation of lipid peroxidation, expressed as TBARS nMol mg protein (Figures 3.26A and B) in gill cells of mussels exposed to SRNOM–dispersed SWCNTs responded in a SWCNT concentration-dependent manner. The superoxide dismutase and TBARS responses correlated well with the DNA damage observed in gill cells exposed to SRNOM–dispersed SWCNTs. However, this correlation did not hold once mussels were exposed to SRNOM–dispersed SWCNTs in

the presence of cadmium and/or zinc, suggesting that the increased toxicological response at lower exposure concentrations was attributable to the interaction of metals with SWCNT rather than the dissolved metals or SWCNTs on their own.

Hull *et al.* (2009) discussed the need to review the potential impact of the ENM manufacturing process and demonstrated that the toxicity of CNTs can be explained by the release of divalent metals, synthesis by-products of the nano-manufacturing process. Some released NPs or NMs can be harmful to aquatic organisms (Maurer-Jones *et al.,* 2013); with regard to environmental fate, the aquatic sediments tend to be the ultimate repository for particulate bound contaminants (Chen and White, 2004). Thus, it is reasonable to assume a similar fate for NPs or NMs (Keller *et al.,* 2010), which can be taken up by organisms, subsequently resulting in a biological effect. SWCNT and MWCNT are known to contain residual metal impurities (RMI) (Arepalli *et al.,* 2004), and have been to be linked to toxic effects SWCNTs in human health and the environment (Tejral *et al.,* 2009). Many residual catalyst metals such as Co, Si, Cu, Ni, Mo and Fe are used in the synthesis process of SWCNTs, and can be present even after purification treatments (Arepalli *et al.,* 2004; Tejral *et al.,* 2009). These RMI present a toxic threat to cells; however, the RMI release, its release mechanism and behaviour in the environment have not yet been fully understood (Tejral *et al.,* 2009; Pruski *et al.,* 2002; Petersen *et al.,* 2014). As previously mentioned, the observed catalytic properties of carbon nanotubes and their related toxic effects have been attributed to metal residue impurities from the synthesis process, in particular iron, manganese, and nickel (Oberdörster *et al.,* 2005; Banks *et al.,* 2006), which were also detected in trace amounts in the present study, at less than 1% w/w (SWCNTs; Sigma, catalog 704121; Table 3.2). However, no increases in DNA damage or oxidative stress was observed in the present study following exposure of mussels to $\leq 50\mu g\ L^{-1}$ SWCNT without Cd^{+2} and Zn^{+2}, nor were any effects observed following exposure to representative concentrations of RMI alone (Figures 3.27-28A and B). The lack of DNA damage and oxidative stress in mussel haemocytes and gill cells at these SWCNT concentrations may be because the very low concentrations of RMI were unable to induce measureable genotoxicity. This suggests that metal catalysts, unlike metals adsorbed under environmental conditions from the surrounding medium, may not have the same importance for interpreting toxicological data as indicated in previous *in vitro* studies (Oberdörster *et al.,* 2005; Banks *et al.,* 2006), although they may become significant at higher carbon nanotube concentrations.

4.3 Impact of agglomerated single-walled carbon nanotubes on the marine green alga, *Tetraselmis suecica*

Engineered nanomaterials (ENMs) are used in a wide range of commercial products that may enter the marine environment through a variety of routes and come into contact with marine algae, important primary producers at the base of the aquatic food chain (Kroll *et al.,* 2013). Although there are some reports that NPs can adhere to algal cell surfaces, and hence restrict light accessibility to the algal cells, subsequently reducing photosynthesis and resulting in growth inhibition (Hund-Rinke and Markus, 2006; Kwok *et al.,* 2010), questions remain regarding the fate, adherence and effect of SWCNTs during interaction with algal cells (Fabrega *et al.,* 2010). The majority of algal cells were shown to be located inside >25 mg L^{-1} CNT agglomerates, (Schwab *et al.,* 2011). In the present study, *Tetraselmis suecica* was exposed *in vivo*, in triplicate, to 5µg L^{-1}, 10µg L^{-1}, 50µg L^{-1}, 100µg L^{-1} and 500µg L^{-1} single-walled carbon nanotubes (SWCNTs) for 8 days. The agglomeration process for agglomerated CNT was positively correlated to the time dependent growth inhibition (Schwab *et al.,* 2011). The growth inhibition is pronounced in the first 150hrs of SWCNT exposure (% growth decreased below ~60% of growth observed in control samples) (Youn *et al*., (2011). SWCNTs adhered to the external algal cell walls were observed using light microscopy (Figure 3.29A-C) and SEM (Figure 3.31D-F), and confirmed by Raman spectroscopy (Figure 3.30). Long et al. (2012) showed the algal cells were largely entangled with the MWCNT agglomerates, showing no sign of mitotic divisions. They concluded that, most of the algal cells trapped in the MWCNT layer lost their cellular integrity, with cytoplasm outflow onto the MWCNT layer, indicating irreversible cell damage. Schwab *et al.,* (2011) reported a shading affect attributed to the agglomeration of CNTs, leading to a reduced algal growth rate. Similar observations were made in the present study, where, at the end of the exposure to ≥100µg L^{-1} SWCNTs, most of the algal cells were visibly entrapped within the SWCNT agglomerates (Figure 3.29A, B). They caused a decrease in algal cell viability (Figure 3.34) and chlorophyll *a* concentrations (Figure 3.35), and an inhibition of algal growth was observed (Figure 3.33), possibly owing in part to a shading effect afforded by the attached CNT (Wei *et al.,* 2010).

The shaking speed during the incubation of the algal cell played a role in reducing the shading density of the aligned SWCNTS (Long *et al.,* 2012; Liu *et al.,* 2009). Long et al. (2012) reported that since increasing shaking speed may increase the possibility of physical interaction between multi-walled carbon nanotubes (MWCNTs) and algal cells,

they tested the toxicity of MWCNTs at different shaking speeds. The toxicity (i.e. growth inhibition) remained largely unchanged at shaking speeds <180rpm. A similar result has been shown in the present study, using a shaking speed of 120rpm and light intensity of 115 ± 15 μEm^{-2} s^{-1}. However, the reduced MWCNT toxicity on algae at ≥180 rpm could have been caused by the increased light availability, which could have stimulated algal growth (Long *et al.*, 2012; Hu *et al.*, 2010). Although the current study did not vary shaking speeds as a confounding factor of SWCNT toxicity on algae, the hypothesis put forward by Long *et al.* (2012), that "algal growth was inhibited at ≤120rpm, which could have been caused by the decreased light availability", seems plausible. This hypothesis was previously formulated by Hund-Rinke and Simon (2006) and Kowk et al. (2010), who stated that "CNTs can adhere to algal surfaces, and hence restrict light accessibility to the algal cells, resulting in the inhibition of growth". As carbonaceous CNTs are opaque, it has often been speculated that shading may explain reduced cell viability, growth rate and chlorophyll *a* concentration of phototrophic biota exposed to CNTs (Schwab *et al.*, 2011; Long et al., 2012; Baun *et al.*, 2008; Wei *et al.*, 2010). Furthermore, SWCNTs were observed to attached and adhere above, below and in between algal flagella, diminishing the flagellar motility, and thus reducing the mobility of algal cells. In the present study, physical interaction between SWCNTs and *T. suecica* was directly observed using LM, Raman spectroscopy, SEM, and TEM. Raman spectroscopy has confirmed the presence SWCNTs on algae, through the peaks in the D-band at 1290-1300 cm^{-1} and G-band at about 1590-1600 cm^{-1}, and it is thus concluded that the aggregation/agglomeration of SWCNT on cells may have detrimental effects on the growth rate of algae. Similarly, in the present study, Raman spectroscopy confirmed that *T.suecica* had been covered or removed by SWCNTs from the seawater, through the peaks in the D-band at 1290 cm^{-1} and G-band at 1590 cm^{-1} (Figure 3.30). Youn et al. (2011) exposed freshwater green algae (*Pseudokirchneriella subcapitata)* to 500µg L^{-1} SWCNTs dispersed in gum arabic (GA). Comparing the presence of SWCNT in *P.subcapitata* and *T.suecica* at 1590 cm^{-1}, it seems that, although there are different chemical compounds between the two dispersions, 1% GA and 0.02% SRNOM with SWCNTs, the Raman spectrum of SWCNTs is still constant at G-band at 1590 cm^{-1}. Furthermore, the Raman spectroscopy data (Figure 3.30) shows that the stock SWCNTs are similar in surfactant coverage and agglomerate state throughout algal growth (Youn *et al*., 2011).

Concerning the shape of the algal cells with or without SWCNTs in SEM (Figures 3.31A-F), both the size and the morphology of algal cells in any of the tested materials (SWCNTs) looked different compared with the controls. The ≥100µg L^{-1} SWCNT agglomerates were shown very clearly to surround the algal cell. This observation is consistent with previous reviews mentioning that agglomerations of SWCNTs played a main role in reducing the light availability, subsequently causing a decrease in the chlorophyll *a* concentration, leading to a decrease in the growth rate of algae (Long *et al.*, 2012; Youn *et al*., 2011; Schwab *et al.*, 2011).

Some life forms, such as plants, including algae, bacteria and fungi possess a semipermeable cell wall that allows small molecules to pass through it. Fabrega *et al.* (2011) reported that Ag NPs agglomerates caused cell wall damage in algae, and were able to pass through cell wall to reach the plasma membrane. There are conflicting views regarding the ability and mechanisms for the uptake of NPs and NMs by cells, although several studies have shown uptake using TEM. For instance, bacteria have been shown by TEM to take up NPs through their semipermeable membranes (Fabrega *et al.*, 2009; Huang *et al.*, 2008), and for vertebrates, Lee et al. (2007) showed Ag NPs passing through the pores of the chorion in Zebrafish embryos, using TEM. Our findings are in agreement with the previous literature (Long *et al.*, 2012). In the present study, TEM images (Figures 3.32D-F) show algal cells exposed to 500µg L^{-1} SWCNTs become subject to cell wall breakage, plasmolysis and internalization of SWCNTs. Although cell wall breakage, plasmolysis and internalization of SWCNT were observed in algal cells in the present study, it cannot be completely ruled out that this is an artefact. Coupling Raman to Confocal microscopy would allow a 3D scan through the fresh algal cells without the need for potentially damaging sample preparation and enable a conclusive assessment of SWCNT internalization.

In comparison with cells from control samples in TEM, there are significant changes in the morphology of cell wall/membranes: their size has been reduced and cell membranes deformed. These impacts could be attributable to the destructive effects of ROS, due to the fact that membrane integrity is a primary target (Cabiscol *et al.*, 2000). These results suggest that the SWCNTs in this study may have been internalized by the algae and may also have played a role in reducing the algal viability and growth, although the mechanism of SWCNT uptake is at present unknown. Changes in chlorophyll *a* concentrations have been used as an indicator of general biological responses, such as algal growth rate (Youn *et al*., 2011). *Tetraselmis suecica* were

exposed to three replicate 500µg L^{-1} SWCNTs. Statistically, there was no significant difference in the level of chlorophyll *a* concentrations between the control and SWCNTs at t_0 (P = 0.312). However, from $t_1 \rightarrow t_3$ (Figure 3.35) there is significant difference between control and 500µg L^{-1} SWCNTs (P<0.001) in the decrease of the level of chlorophyll *a* concentrations was observed. This result was a strong indicator for the occurrence of algal growth inhibition. It can be concluded that a direct effect of SWCNT exposure on the algae was a significant (P<0.001) decrease in chlorophyll *a* concentrations, accompanied by a significant decrease (P<0.001) in cell viability following a 7 days exposure to SWCNTs concentrations of 500µg L^{-1}.

4.4 Effect of the interaction of Green algae with single-walled carbon nanotubes on the feeding behaviour of mussels

Vanderploeg *et al.* (2001) hypothesised that (i) mussels would continually filter algae in the presence of bacteria *Microcystis aeruginosa*, (ii) mussels would ingest all algae except *M. aeruginosa*, and (iii) mussels would produce loosely consolidated pseudofaeces that would be injected back into the water column. The hypothesis in the present study was that (i) mussels would continually filter algae in the presence of SWCNTs, (ii) mussels would ingest all algae but not SWCNTs, and (iii) mussels would produce loosely consolidated pseudofaeces consisting of algal cells and/or SWCNTs that would be injected back into the water column. The potential connection between mussels and algae (*M. aeruginosa*) has important water quality management implications (Vanderploeg *et al.,* 2001), so, the feeding behaviour of mussels *Mytilus edulis* and other suspension-feeding bivalves have been thoroughly studied in the absence or the presence of algal cells (Riisgård *et al.,* 2011). The interaction of SWCNTs with algae was observed in the feeding behaviour of mussels, video-camera recordings showed copious pseudofaecal SWCNTs expelled by the exhalant siphon of the mussel when fed the SWCNTs in the presence of algae (*Tetraselmis suecica)* (Figure 3.36A-E). Clearly, SWCNTs became agglomerated during the experiment. The feeding behaviour of mussels was effected when fed SWCNTs with and without algae. Faecal material is the result of edible foods expelled by the exhalant siphon following digestion. In contrast pseudofaeces consist of mucus-bound material rejected by the sorting mechanism of the labial palps and excreted by the exhalant siphon without being ingested. Both faecal and pseudofaecal material was observed when mussels were fed algae, SWCNTs 500µg L^{-1} alone and SWCNTs 500µg L^{-1} + algae (Figure 3.37A-E). However, our preliminary observations involving traditional experiments and direct

microscopic observations showed copious pseudofaecal SWCNTs expelled by the exhalant siphon of the mussel when fed SWCNTs + algae (Figure 3.38). Selective bivalve feeding was observed using a newly developed flow cytometry technique with pseudofaeces as a proxy for feeding behaviour in mussels (Figure 3.39). Pseudofaeces containing algal cells increased significantly (P=0.008) under combined algae and SWCNT exposure, suggesting mussels largely rejected algae containing SWCNTs.

4.4.1 Toxicity to mussels

All mussels in the previous experiments have been analyzed using a comet assay to assess DNA damage and activity and lipid peroxidation in gills to assess oxidative stress using the SOD and TBARS assays. DNA damage and oxidative stress were used as ecotoxicological biomarkers of exposure in mussels. A 24hrs exposure to 500µg L^{-1} SWCNTs showed significantly increased DNA strand breaks in both gill cells and haemocytes (P<0.001) (Figures 3.40A-B), and significantly increased oxidative stress, expressed as superoxide dismutase (SOD) activity (P<0.001) and lipid peroxidation in gills (P=0.032) (Figures 3.41A-B). However, when SWCNTs were presented together with algae, DNA damage in haemocytes and gills (P=0.534; P=0.998) (Figures 3.40A-B) and oxidative stress were not significantly increased above control levels (P=0.981; P=0.999) (Figures 3.41A-B). These results illustrate that the presence of algal cells with SWCNTs stimulated mussels to expel copious amounts of SWCNT-containing pseudofaeces. It can therefore be concluded that the presence of algae plays a major role in mitigating the toxicity of SWCNTs.

4.4.2 Trophic transfer of SWCNTs from algae to mussels in a simplified seawater food chain

Nanoparticles can be transferred through planktonic crustaceans who feed on primary producers such as algae, to secondary consumers such as fish, mussels and others, to the next trophic level and possibly even to humans (Baun *et al.,* 2008). Research on the toxicity of SWCNT has focused mainly on human health (Ghafari *et al.,* 2008). However, no studies have demonstrated their indirect impact on humans through the food chain. One of the most significant and not well-understood risks of ENMs is their potential trophic transfer and biomagnification in food webs (Klaine *et al.,* 2008; Zhu *et al.,* 2010). However, Farré *et al.* (2009) and Baun *et al.* (2008) report that nanoparticles could pass from the water column to the aquatic food web through the primary producers such as algae to primary consumers such as, mussels and secondary

consumers such as crabs, fish and others to the next trophic levels. A fundamental concern in nanotoxicology is the possible spread of intact NMs or NPs and their associated ecological effects through food chains (Priester *et al.,* 2009). The risk assessment of NMs or NPs is not only restricted to their concentration in the environment and their toxicity to organisms, but also depends on their bioaccumulation, bioconcentration and biomagnification in the aquatic food chain (Zhu *et al.,* 2010). Despite recent progress in understanding the trophic transfer of SWCNTs in aquatic organisms (from algae to mussels), Werlin *et al.* (2011) have shown that ENMs can be transferred from prey to predator, although the ecological impacts of this are mostly unknown. Together with the lifecycle of CNTs in the environment, these key and emerging knowledge gaps are critical obstacles in understanding the physical interactions between algae and mussels, and the trophic transfer of SWCNTs through the food web (Lowry *et al.,* 2010). In the present study, trophic transfer of SWCNT through the food chain (from algae to mussels) was assessed by exposing algae to SWCNT 500µg L^{-1} for 7 days, and then mussels were fed the exposed algal cells for 10 min. The initial visible physical interaction between algae and mussels is presented in Figures (3.42A-F). Although the physical interaction between algae and mussels did not represent trophic transfer of SWCNTs, it may, nevertheless, be considered as a pre-indicator for trophic transfer. Histologically, *Mytilicola intestinalis* copepods were visibly attached to the mussel gut epithelium (Bignell *et al.,* 2008). Similar histologically analysis in the present study showed algal cells containing SWCNTs in mussel gut epithelium (Figures 3.44-46A and B). Zhu et al. (2010) have observed how *D. magna* ingest and accumulate $nTiO_2$ from water, and they observed that trophic transfer and greater biomagnification of $nTiO_2$ in the food web did occur (from daphnia to zebrafish). In summary, this study provides the first direct evidence for the transfer of SWCNTs from algae to mussels in a simplified seawater food chain. This finding may be able to illustrate that SWCNTs are taken up by algal cells (Figure 3.32D-F) and then transferred through the food web via mussels feeding, which have been observed digesting algal cells (Figures 3.44-46A and B). These results show that mussels can incorporate SWCNTs from algae. Whether or not they can subsequently be transfered to other organisms is a subject for further study.

5. CONCLUSION

One of the most important considerations is to accurately characterise single walled carbon nanotubes (SWCNTs) prior to the ecotoxicological experimentation (Petersen, 2014; Stone *et al.,* 2014). There are several dispersal methods and functionalisations, which can be either enhanced or diminished to influence the toxicity of the CNTs, and to determine their fate. It has been reported, that humic acid (NOM) is a good agent for dispersing CNTs in water (Kennedy *et al.,* 2009). We have shown that the dispersion of SWCNTs in 0.02% SR-NOM is good and relative by stable, lending weight to the hypothesis that the presence of NOM can result in more dispersible and stable suspensions (Bennett *et al.,* 2013). The characterisation of SWCNT is not restricted to their shape and size and surface charge, but their behaviour in the environment in relation to metals or other substances is also very important. The present study has shown that SWCNTs have negatively charged surfaces in seawater, and are able to remove Cd^{+2} and Zn^{+2} from solution. The sorption mechanism appears to depend fundamentally on the chemical interaction between divalent metal ions and the SWCNT's surface functional group in water.

Although previous chemical interactions may elucidate one of the most important risk factors for the SWCNT in the environment, it has been suggested that CNTs are promising sorbents for environmental protection applications (Rao *et al*., 2007); they might also use CNTs to improve the efficiency of wastewater treatment (Ghfari *et al.*, 2008). The surface charge of nanotubes is latterly observed to play a critical role in enhancing the toxicity of SWCNTs when they adsorb dissolved metals (Cd^{+2} and Zn^{+2}) from aqueous solution. In other SWCNT synthesis studies techniques such as TEM and SEM are essential for assessing nanotube size distribution. Furthermore, dynamic light scattering (DLS) and zeta potential, which are straightforward to perform, provide agglomerated SWCNTs size and surface charge. Moreover, Raman spectroscopy and atomic absorption spectrometry are important for detecting SWCNTs and residual metal impurities (RMI). CNTs are often synthesised using a metal catalyst (Petersen, 2014); residual metal impurities (RMI: Fe, Ni and Mn) were found in our SWCNT product used in the current study. It can be concluded that the understanding of SWCNTs functionalisation, synthesis, characterisation, behaviour and interaction with metals provides good evidence that may lead to an understanding of their fate and effect on environment and human health.

The environmental impacts of NPs released from commercial products are not well understood (Benn *et al.,* 2008); despite this, some potential release pathways for SWCNTs into the natural environment are hypothesised in section 1.3. Several studies have observed the uptake of NMs or NPs by aquatic biota, and our direct microscopic observations also show the uptake of SWCNTs by mussels via their food filtration systems, as confirmed by Raman spectroscopy. We also concluded that SWCNTs can be taken up by mussels, and in the presence of SWCNTs the amounts of metals (Cd^{+2} and Zn^{+2}) in mussel gills are greater than those in the absence of SWCNTs. As explained above, this implies that metals (Cd^{+2} and Zn^{+2}) can be strongly adsorbed by the SWCNT's surface.

In the cell viability assay, 100µg L^{-1}, SWCNTs, separately and in combination with metals (Cd^{+2} and Zn^{+2}), showed no effects on either the viability of gill cells or hemocytes. It can be concluded that, at concentrations of 100µg L^{-1}, SWCNTs, even when combined with metals, are unable to affect mussel cell viability. Different scenarios have been observed in a genotoxicity assay: SWCNTs alone at concentrations of up to 50µg L^{-1} are non-toxic; however, concentrations of SWCNT ≤50µg L^{-1}, when combined with metals (Cd^{+2} and Zn^{+2}), become toxic. It can be concluded that chemical interactions between nanotubes and divalent metal ions are playing a major role in increasing the toxicity of metals. Meanwhile SWCNTs at concentrations of ≥100µg L^{-1}, separately or in combination with metals (Cd^{+2} and Zn^{+2}), showed a significantly increased number of DNA strand breaks in both gill cells and haemocytes. These significantly increased oxidative stress, such that the toxicity of SWCNTs when interacting with metals (Cd^{+2} and Zn^{+2}) became higher than when the mussels were exposed to SWCNTs alone. This leads to the conclusion that high concentrations of SWCNTs are harmful to aquatic organisms, and therefore may be harmful to the marine environment. Moreover, SWCNTs, even at low concentrations, when associating or interacting with divalent metal ions, have a potentiating effect. The dispersant media (SRNOM) and all of the RMIs (Fe^{+3}, Ni^{+2} and Mn^{+2}) which were found in SWCNT synthesis are harmless, even when spiked with heavy metals (Cd^{+2} and Zn^{+2}) in the absence of SWCNTs. It can be concluded that there is no chemical interaction between either SRNOM or RMI with divalent metal ions of Cd^{+2} and Zn^{+2}; accordingly, no toxicity was observed under these conditions.

Algal cells can be entrapped and coated by a tangled matrix of ≥100µg L^{-1} SWCNTs. These observations elicited five interrelated results: diminished motility, decreased light availability, decreased cell viability, growth inhibition and decreased chlorophyll concentrations. A chain of events may occur in the case of the release of ENMs into the aquatic environment: if so, the algal distribution and habitat can be gradually reduced; subsequently, this may become one of the main causes of biodiversity loss. SWCNTs, at concentrations of 500µg L^{-1}, were observed by TEM to cause algal cell wall breakage and plasmolysis, leading to the conclusion that high SWCNT concentrations are able to cause excessive loss of algal cell walls, membranes and resulting cell death. Over the long or maybe short term, the SWCNTs appear to have been internalised by *T.sueciac,* possibly allowing the nanotubes to move up the food chain (Ghfari *et al.*, 2008), causing a biological effect at higher trophic levels, including humans. Finally, this part of the study also leads to the conclusion that a high concentration of agglomerated SWCNTs is toxic to algae, and might subsequently cause ecotoxicological effects in the environment.

Feeding behaviour is one of the most important tests for assessing the toxicity of ENMs on animals. Therefore in this study, it was observed using a digital camera that copious amounts of pseudofaecal SWCNTs were expelled by the exhalant siphon of the mussel when fed on SWCNTs in the presence of algae (*Tetraselmis suecica*). Both visibly and microscopically faecal and pseudofaecal materials were observed to be expelled by the exhalant siphons of the mussels, when fed algae, 500µg L^{-1} SWCNTs alone and 500µg L^{-1} + algae SWCNTs. However, the most important point here is that selective bivalve feeding, in which mussels are observed rejecting food (algal cells) in the presence of SWCNTs (using a newly developed flow cytometry technique with pseudofaeces as a proxy for feeding behaviour in mussels), suggests that this feeding behaviour is triggered by SWCNTs. It can be concluded that copious pseudofaecal algal cells and SWCNTs can be expelled by the exhalant siphon of the mussel when fed solely on SWCNTs with algal cells. In a genotoxicity assay, the toxicity of 500µg L^{-1} SWCNTs in the presence of algae was reduced. It can be concluded that selective feeding in mussels is working to mitigate the toxicity of SWCNTs by preventing ingestion

The SWCNTs have already been microscopically observed to be being taken up and internalised into algal cells. Histologically, mussels are able to digest algal cells containing SWCNTs. Under continuous microscopic observation, some algal cells and distributed SWCNTs were observed to be digested in the gut (after the mussels were left

to feed on algae for 10 minutes). SWCNTs can be seen in mussels' gut tissues as small, black grains. In this final part of the current study, the trophic transfer of SWCNTs from algae to mussels was clearly histologically observed, suggesting that the trophic transfer of SWCNTs may also arise between invertebrate to vertebrate organisms. Thus, this scenario may imply a significant risk to human beings and animals when SWCNTs enter the food chain, with potential consequences for human health. In conclusion, in this study we have demonstrated both the direct and indirect toxicity of SWCNTs on marine mussels.

6. Recommendations For Further Research Into Invertebrate Nanoecotoxicology

Future studies should ensure that the behaviour of the physiochemical properties of SWCNTs in the environment is well understood. To the best of my knowledge, the interactive behaviour between dissolved metals and SWCNTs has not been studied previously under present exposure conditions. If laboratory experiments were confirmed in the natural environment, observations would have implications for the role of SWCNTs in environmental metal dynamics and toxicology, and consequently effect regulatory requirements. Trophic transfer of NMs throughout the food chain must be carefully researched, particularly the potential trophic transfer of NMs from animal to human via the food chain. It is recommended that their biological effect must also be carefully assessed.

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