

At the End of the Life Cycle of Carbon Nanotubes: An Ecotoxicological Point of View

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH
Aachen University zur Erlangung des akademischen Grades eines Doktors der
Naturwissenschaften genehmigte Dissertation

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Tag der mündlichen Prüfung: 16. Juli 2014

Diese Dissertation ist auf den Internetseiten der Hochschulbibliothek online
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Acknowledgement

First and foremost, I would like to thank Prof. Dr. Henner Hollert, Department of Ecosystem Analysis at RWTH Aachen University, my supervisor, who let me gain experience in the laboratory work as much as trusting in my work.

I am no less thankful to Prof. Dr. Andreas Schäffer, the director of the Institute for Environmental Research, for giving me the opportunity to prepare my thesis under his official supervision and, thus, being my second referee and being there whenever I needed help.

I cordially want to thank Dr. Hanna Maes for supporting my laboratory staff, for having an open door whenever I needed new inspirations or when I got stuck. Thank you, Hanna, for commenting and correcting my thesis.

Good scientific work is supported by friendly and helpful people. My special thank go to my dear colleagues Anne Wyrwoll, Andrzej Schiwy, Jochen Kuckelkorn, Matthias Findeiß, and Stefan Rhiem. I am very grateful for all helping heads and hands that passed my PhD way: I thank Simone Hotz for carrying out cell based assays and helping me whenever I needed more than just two hands. Thanks Birgit, Brigitte, Telse and Dennis. Special thanks go to the students that worked in the frame of my PhD topic: Katharina Graf, Markus Kurth, Maximilian Gruber, and Timon Orths.

Thanks to the working group of Dr. Marc Heggen from the Ernst Ruska-Centre in Juelich for the cooperation to compile TEM images and Prof. Dr. Willi Jahnen-Dechent and Dr. Yu Pan, Helmholtz Institute for Biomedical Engineering, for the cooperation resulting in a very good Bachelor thesis by using transgenic zebrafish. Special thanks go to Dr. Oliver Schlüter, Bayer Technology Services, for synthesis of ^{14}C -labelled CNT in relation to the CarboLifeCycle project.

I also like to thank the BMBF for funding this project and, thus, the majority of my PhD thesis.

I would like to specially thank my parents for having supported me throughout. Ohne Eure Liebe, Hilfe, Aufopferung und wärmende Umarmung hätte ich nicht so ein gutes Leben. Danke für Euer Vertrauen in mich und dass ihr mir diesen Weg überhaupt ermöglicht habt. Danke auch all meinen Freunden und Geschwistern, die mein Leben lohnenswert machen.

Zu guter Letzt danke ich Dir, Jens, für deine kompromisslose Liebe und Unterstützung.

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Summary

The increasing production and application of carbon nanotubes (CNT), might enhance the presence of this nanomaterial in the aquatic environment, and hence, the exposure of aquatic organisms. To date, only a few reports about studies on toxic effects of CNT are available and their results are often controversial. Triclocarban (TCC) is a high-production-volume chemical that is widely used as an antimicrobial compound and is known for its toxicity, endocrine disruption, bioaccumulation potential, and environmental persistence. Additionally, CNT may interact with and further influence the fate and transport of other pollutants and personal care products (PCP) such as TCC. As TCC is used since 1957 in huge amounts, and MWCNT is supposed to reach the amount of a large scale production, both substances might involuntarily occur together in the environment.

First of all, three different cell lines (rainbow trout liver cells (RTL-W1), human adrenocortical carcinoma cells (T47Dluc) and human adenocarcinoma cells (H295R)) were exposed to multiwalled carbon nanotubes (MWCNT), the antimicrobial agent triclocarban (TCC) as well as the mixture of both substances in a concentration range of 3.13 to 50 mg CNT/L, 31.25 to 500 µg TCC/L and 3.13 - 50 mg CNT/L + 1 % TCC (percentage relative to CNT concentration). The influence of MWCNT and TCC on various toxicological endpoints was specified: neither cytotoxicity, nor endocrine disruption could be observed after exposure of the three cell lines to MWCNT, but the nanomaterial caused intracellular generation of reactive oxygen species (ROS) in all cell types. For TCC on the other hand, cell vitality of 80 % (NR₈₀) could be observed at a concentration of 2.1 mg/L for treated RTL-W1 cells. A decrease of luciferase activity in the ER Calux assay at a TCC concentration of 125 µg/L and higher was observed. This effect was less pronounced when MWCNT were present in the medium. Taken together, these results demonstrate that MWCNT induce the production of ROS in RTL-W1, T47Dluc, and H295R cells, reveal no cytotoxicity and reduce the bioavailability and toxicity of the biocide triclocarban.

So far, only little is known about the chronic effects of CNT to aquatic organisms. In an additional study, the population test with *Daphnia magna* was performed over a period of 93 days. The long-term exposure of multiwalled carbon nanotubes (MWCNT) was examined with a concentration of 1 mg CNT/L as of the beginning as well as of day 14, to ensure a stable population at the equilibrium. Additionally, a control population was treated with a 2-day pulse of TCC three times (d 14, d 54, d 68) following exposure to 25 µg TCC/L, 41 µg TCC/L, and 61 µg TCC/L, respectively. Furthermore, a continuously MWCNT treated population was exposed to the same pulses with TCC. The influence of MWCNT and TCC on population level was specified. The total abundance of treated populations did not differ from the control population. Whereas, significant changes in all three size classes could be observed as a result of the long-term exposure to 1 mg CNT/L: increase of neonate, and decrease of juvenile and adult daphnids. Moreover, significantly smaller daphnids were determined when they were exposed to MWCNT from the beginning of the population experiment. The exposure with TCC leads to size-dependent mortality in *Daphnia magna* populations and a subsequently recovery. The mixture of MWCNT and TCC results in a lower toxicity of TCC up to 41 µg TCC/L. Furthermore, the effects of TCC on population level were compared by using an individual-based *Daphnia magna* population model (IDamP). The IDamP model with an integrated toxicity module was able to predict the effects of TCC on the population level (in equilibrium) under constant laboratory conditions. The use of a modeling approach offers a tool to extrapolate from effects derived from laboratory experiments to effects on the population level and can improve the ecological risk assessment of chemicals.

The final study investigated the uptake and toxicity of MWCNT in the extended fish embryo toxicity test (FET) using wild type and transgenic zebrafish. Additionally, the mixture toxicity of MWCNT and the antimicrobial agent triclocarban was considered using wild type zebrafish. Uptake of the nanomaterial in wild type embryos was not observed. Hatching time

and hatching success were not affected and no mortality or deformities occurred. These nanoparticles and/or aggregates mainly accumulated on the bottom of the test vessels and remained sedimented in the water phase. No induction of heat shock protein (Hsp) promoter by MWCNT in transgenic zebrafish as well as no toxicity and no adverse effects on development could be determined after exposure to up to 100 mg CNT/L. For TCC on the other hand, mortalities in wild type zebrafish up to 90 % could be observed at the highest concentration of 111 µg TCC/L. This mortality was significantly reduced in appearance of 10 mg CNT/L. These results demonstrate that MWCNT are not toxic for zebrafish embryos up to 100 mg CNT/L and reduce the bioavailability and toxicity of the biocide TCC.

The findings of this study document that CNT are not acute toxicants but lead to long-term effects in aquatic organisms. This clarify the necessity to investigate not only test endpoints such as the acute toxicity but also focusing systematically on chronic effects after the exposure to this nanoparticles.

Zusammenfassung

Die steigende Produktion mit einhergehender Anwendung von Kohlenstoffnanoröhren (CNT) führt zu einer Anreicherung in der Umwelt und somit zu einer verstärkten Exposition von aquatischen Organismen gegenüber diesen Nanomaterialien. Bislang sind wenige Studien zum toxischen Potential der CNT verfügbar und die Ergebnisse sind kontrovers. Triclocarban (TCC), eine Chemikalie mit hoher Massenproduktion, wird oft aufgrund seiner antimikrobiellen Wirkung eingesetzt und ist bekannt für seine Toxizität, endokrine Wirksamkeit, Bioakkumulation und Persistenz in der Umwelt. Zudem können CNT mit organischen Schadstoffen und Inhaltsstoffen von Körperpflegemitteln, wie dem TCC, interagieren und folglich deren Verbleib und Transport in der Umwelt beeinflussen. Beide Substanzen treten zwangsweise gemeinsam in der Umwelt auf, da TCC seit 1957 in großen Mengen genutzt wird und mehrwandige CNT (MWCNT) im großtechnischen Maßstab produziert werden.

Zunächst wurden drei verschiedene Zelllinien (Leberzellen der Regenbogenforelle (RTL-W1), genetisch modifizierte humane Mammakarzinom-Zelllinie (T47Dluc) und humane Nebennierenrindenkarzinomazellen (H295R)) gegenüber MWCNT (3,13-50 mg CNT/L), TCC (31,2-500 µg TCC/L) und einer Mischung aus beiden Substanzen (3,13-50 mg CNT/L + 1 % TCC; Prozentsatz relativ zur CNT Konzentration) exponiert. Der Einfluss von MWCNT und TCC auf verschiedene toxikologische Endpunkte wurde bestimmt: Weder Zelltoxizität, noch endokrin wirkende Eigenschaften konnten nach der Exposition gegenüber MWCNT festgestellt werden, jedoch bewirkten die Nanomaterialien in allen drei Zelllinien eine intrazelluläre Produktion von reaktiven Sauerstoffspezies (ROS). TCC hingegen führte bei einer Konzentration von 2,1 mg TCC/L zu einer verringerten Zellvitalität der RTL-W1 Zelllinie von 80 % (NR80). Eine Abnahme der Luziferase-Aktivität im ER Calux Assay wurde ab einer Konzentration von 125 µg TCC/L beobachtet. Dieser Effekt war weniger stark ausgeprägt wenn MWCNT ebenfalls im Medium enthalten waren. Zusammenfassend konnte gezeigt werden, dass MWCNT die Produktion von ROS in RTL-W1, T47Dluc, und H295R Zellen induzieren, keine Zelltoxizität herbeiführen und die Bioverfügbarkeit und Toxizität des Biozids Triclocarban reduzieren.

Bislang ist nur wenig über die Langzeitwirkung von CNT gegenüber aquatischen Organismen bekannt. In einer weiteren Studie wurden die chronischen Effekte von 1 mg CNT/L auf den Wasserfloh *Daphnia magna* im Populationstest über einen Zeitraum von 93 Tagen untersucht. Die Langzeitexposition wurde ab Tag 0, wie auch ab Tag 14 untersucht, um eine stabile Population im Equilibrium zu gewährleisten. Zusätzlich wurde eine Kontrollpopulation an Tag 14 (25 µg TCC/L), 54 (41 µg TCC/L) und 68 (61 µg TCC/L) jeweils einer zweitägigen TCC Exposition ausgesetzt. Weiterhin wurde eine von Beginn an gegenüber CNT exponierte Population denselben TCC-Impulsen ausgesetzt. Die Gesamtabundanz der exponierten Populationen differenzierte sich nicht von der Kontrollpopulation. Jedoch wurden signifikante Veränderungen in allen drei Größenklassen der Daphnien nach Langzeitexposition gegenüber 1 mg CNT/L beobachtet: Zunahme der neonaten, und Abnahme der juvenilen und adulten Daphnien. Zusätzlich wurden signifikant kleinere Daphnien gemessen nach einer konstanten Exposition gegenüber CNT von Tag 0 bis Tag 93. Die Exposition gegenüber TCC führte zu einer größenabhängigen Mortalität der Daphnien mit einhergehender Erholung der Populationen. Die Mischungen mit MWCNT und TCC führten zu einer reduzierten Toxizität des TCC bis zu einer Konzentration von 41 µg TCC/L. Weiterhin wurden die Effekte des TCC auf Populationsebene mit einem Individuen-basierten *Daphnia magna* Populations-Modell (IDamP) simuliert. IDamP, mit einem integrierten Toxizitätsmodul, war in der Lage die Effekte des TCC auf Populationsebene (im Equilibrium) unter konstanten Laborbedingungen zu prognostizieren. Der Gebrauch solcher Modellierungen ermöglicht eine Extrapolation von Effekten aus Laborexperimenten auf Effekte einer Populationsebene und kann die ökologische Risikoabschätzung von Chemikalien verbessern.

Die letzte Studie untersuchte die Aufnahme und Toxizität von MWCNT im verlängerten Embryotoxizitätstest (FET) mit dem Zebrafährbling (*Danio rerio*), sowohl mit dem Wildtyp, als auch mit transgenen Embryonen. Zusätzlich wurde auch hier die Mischungstoxizität von MWCNT und TCC mit Embryonen des Wildtyps durchgeführt. Eine Aufnahme der Nanopartikel konnte nicht beobachtet werden. Ebenso wurden weder Schlupfzeit noch -erfolg durch MWCNT beeinträchtigt und es traten keine Deformationen in der Entwicklung der Embryonen, sowie keine signifikanten Mortalitäten gegenüber der Kontrolle auf. Die Nanopartikel und/oder Agglomerate akkumulierten größtenteils auf den Boden der Testgefäße und verblieben dort in der Wasserphase. Des Weiteren konnte keine Expression des Hitzeschockproteins Hsp70 nach Exposition gegenüber MWCNT festgestellt werden, sowie keine schädigende Auswirkung auf die Entwicklung der transgenen Embryonen und keine Toxizität bis zu einer Konzentration von 100 mg CNT/L. TCC wiederum führte zu Mortalitäten bis zu 90 % bei einer Konzentration von 111 µg TCC/L. Diese Sterblichkeit konnte signifikant reduziert werden bei gleichzeitiger Anwesenheit von 10 mg CNT/L. Diese Ergebnisse zeigen, dass MWCNT bis zu einer Konzentration von 100 mg CNT/L nicht toxisch für Zebrafährchembryonen sind und dass sie die Bioverfügbarkeit und Toxizität des TCC reduzieren.

Die Ergebnisse dieser Studie zeigen, dass CNT nicht akut toxisch auf Wasserorganismen wirken, aber Langzeiteffekte auftreten. Dies verdeutlicht die Notwendigkeit chronische Effekte nach Exposition dieser Nanopartikel zu untersuchen und Studienendpunkte nicht nur auf Effekte nach direkter Exposition zu fokussieren.

List of abbreviations

ANOVA	Analysis of variance
AR	Androgen receptor
ATCC	American Type Culture Collection
CNT	Carbon nanotubes
DCA	Dichloroaniline
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DOM	Dissolved organic material
DWCNT	Double-walled carbon nanotubes
E2	17 β -estradiol
EC ₅₀	Median effect concentration. Concentration of a compound that affects 50 % of the organisms introduced in a test after a given exposure duration
EDA	Electron-donor-acceptor
EDC	Endocrine-disrupting compounds
ELISA	Enzyme linked immunosorbent assay
EN	European norm
ENP	Engineered Nanoparticles
ER	Estrogen receptor
ER Calux	Estrogen receptor mediated chemical activated luciferase gene expression assay
EtOH	Ethanol
F12	Nutrient mixture F-12
FBS	Fetal bovine serum
FET	Fish embryo toxicity test
GFP	Green fluorescent protein
GUTS	General unified threshold model for survival
H295R	Human adrenocarcinoma cell line
H ₂ DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
hpf	Hours post fertilization
HSF	Heat shock factors
Hsp	Heat shock proteins
IDamP	Individual-based <i>Daphnia magna</i> population model
ISO	International organization for standardization
K _{OW}	Octanol-water partition coefficient, indicator for the hydrophobicity of a compound

LC ₅₀	Median lethal concentration. Concentration of a compound that leads to 50 % mortality of the organisms introduced in a test after a given exposure duration
LC/MS-MS	Liquid chromatography-tandem mass spectrometry
LOEC	Lowest observed effect concentration
LSC	Liquid scintillation counter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay
MWCNT	Multi walled carbon nanotubes
NC	Negative control
NM	Nanomaterials
NOEC	No observed effect concentration
NOM	Natural organic matter
NP	Nanoparticles
NR	Neutral Red retention assay
NR ₈₀	Concentrations resulting in cell vitality of 80 %
ODD	Overview, design concepts, details
OECD	Organization for economic cooperation and development
PAH	Polycyclic aromatic organic compounds
PBS	Phosphate buffered saline
PC	Positive control
PCP	Personal care products
RLU	Relative light units
ROS	Reactive oxygen species
RTL-W1	Rainbow trout liver cell line
SC	Solvent control
SD	Standard deviation
S _w	Water solubility
SWCNT	Single-walled carbon nanotubes
T47Dluc	Human breast adenocarcinoma cell line
TCC	Triclocarban
TEM	Transmission electron microscopy
Tg	Transgenic
Tg(Hsp70:GFP)	Transgenic heat shock reporter zebrafish
TKTD	Toxicokinetic-toxicodynamic
TPP	Techno Plastic Products
US EPA	United States Environmental Protection Agency

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Chapter 1

General introduction

1.1 Nanotechnology

Nanotechnology is a rapidly expanding and advancing field in technology of global economic importance. Exploiting the novel characteristics of materials manufactured at the nanoscale and has already yielded a variety of commercially available products including cosmetics, suntan lotions, paints, self-cleaning windows and stain-resistant clothing^[1,2]. In October 2011, the European Union defined nanomaterials (NM) as natural, incidental or manufactured materials containing particles, in an unbound state or as an aggregate or agglomerate, where 50 % or more of the particles exhibited one or more external dimension in the size range of 1-100 nm^[3]. Several classes of nanoparticles (NP) have been defined since they consist of very different compounds, such as inorganic metaloxide based nanoparticles (i.e. TiO₂, ZnO, SiO₂), carbon-based nanoparticles (fullerenes, carbon nanotubes, carbon black), zero-valent and metal nanoparticles (i.e. nanoiron). According to 'The Nanotechnology Consumer Products Inventor'^[4] the most common material mentioned in product descriptions was carbon, e.g. fullerenes and nanotubes.

Three key elements of NP toxicity screening strategies have been outlined by Oberdörster et al.^[5]: (i) physicochemical characterization (size, surface area, shape, solubility and aggregation), (ii) elucidation of biological effects involving in vitro and (iii) in vivo studies. These three key elements, formulated mainly from the point of view of potential effects of NP on humans, will be investigated in the present study. Even though nanoparticles are prevalent in worldwide research and development, gaps still exist on the environmental hazard identification and effects/ exposure assessment of NP. However, experience in nanoecotoxicology laboratories is improving and recommendations for systematic and comparable evaluations are revised regularly^[6-8].

1.2 Carbon nanotubes

Carbon nanotubes (CNT) represent one of the most promising nanomaterials for various applications^[9-12] and were first described in 1991^[13]. The structure of CNT originated from graphite sheets rolled up into a cylinder (Figure 1). Graphite, consists of several layers of single-atom width, planar sheets of hexagonal bonded carbon in a honeycomb crystal lattice. It is known as graphene when present as single sheets. Single or multiple graphene sheets can be folded into cylindrical structures to give single-walled carbon nanotubes (SWCNT) or multiwalled carbon nanotubes (MWCNT), which exhibit unique and novel properties like for example a high mechanic stability, good electric conductivity, very low density, and a large surface area^[14-19].

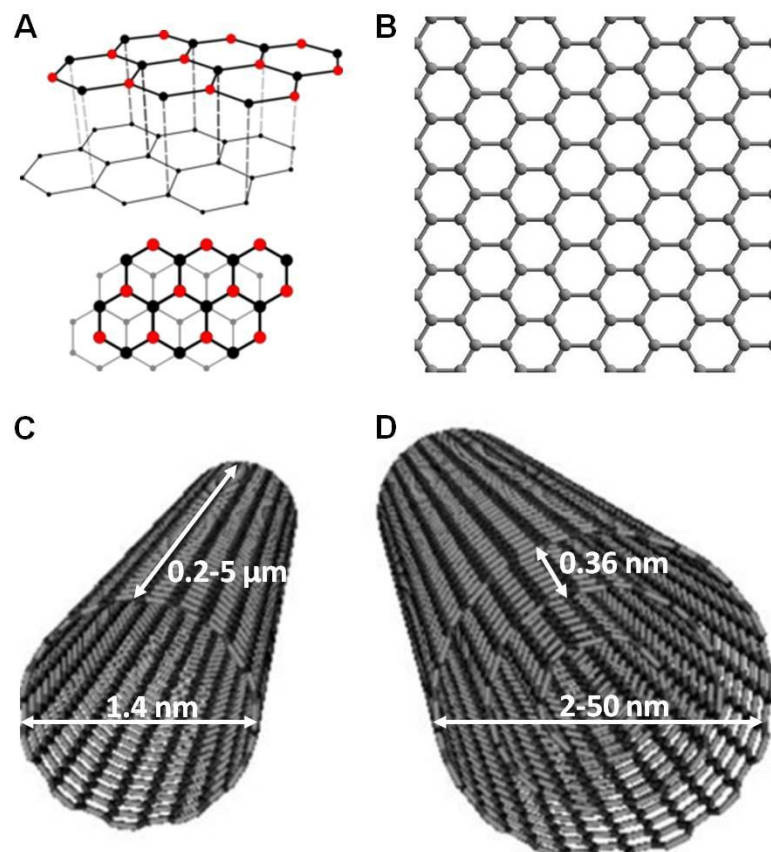


Figure 1. Structure of graphite (A), graphene (B), and different types of CNT (C: single-walled carbon nanotubes; D: multiwalled carbon nanotubes) with possible scales^[14,20,21].

Distinguished by their wall number, CNT dimensions range from 1.4 nm SWCNT up to 50 nm (MWCNT) in diameter and up to several micrometers in length^[13,15,16,22]. In addition to variations in their wall number, CNT can differ in their length, shape, surface modification, purity, and their propensity to form agglomerates and aggregates^[23]. CNT also exhibit a large surface area due to their high surface to volume ratio^[18]. All of these factors influence the physicochemical properties of CNT and consequently affect their biological behavior. However, these properties that make NP useful in a wide range of industrial applications have led to concerns regarding their potential impact on human and environmental health^[1]. The estimated world-wide production is increasing rapidly and the production capacity is now exceeding several thousand tons per year^[10]. In the future, CNT are expected to be used in drug delivery or in a broad range of environmental applications, such as sorbents, filters, antimicrobial agents, environmental sensors, renewable energy technologies, and pollution prevention strategies^[10,18]. While CNT have great potential to contribute to environmental protection, more widespread use and higher volumes will inevitably contribute to the unwanted release into environment.

1.2.1 Fate and behavior of CNT in the environment

Carbon nanotubes may enter the environment directly during intentional release as well as unintentional release during use and consumption of CNT containing goods or as a waste from sewage treatment plants, waste incineration plants, and landfills^[24,25]. In the future, CNT may be released intentionally as they are been explored for remediation and water cleaning purposes^[26-31]. Based on a preliminary product life cycle analysis, CNT were characterized as 'rather safe for the environment'^[32], because hazardous effects^[33] are not expected at current predicted exposure concentration (5 ng CNT/L) modeled by Mueller et al. and Gottschalk et al.^[34,35]. However, there is still a great lack of data in estimating the entrance of nanotubes to the environment, such as the production scale up in the future, the ways of exposure and the toxicological relevance of this material. Except for this modeling approach, no reliable data on environmental concentrations of CNT are available and up to now no monitoring studies have been performed. Therefore, it is highly necessary to determine the fate and behavior of this

manufactured NM in the environment. However, CNT quantification in natural systems is still not possible due to missing detection methods^[36,37]. Furthermore, CNT may be removed during waste incineration since they have been found to be completely destroyed at temperatures between 600-850°C^[38,39], assuming proper burning. However, CNT entering lakes, ponds or rivers, outline a potential risk for aquatic organisms, which is a main subject of this study. Water was identified as one of the key media to distribute CNT in the environment^[40]. Due to their high hydrophobicity, pristine CNT are poorly dispersible in water and immediately form agglomerates. These aggregates show fast sedimentation and the adsorption to sediment particles display a sink for NP^[41].

In recent years, much discussion has centered on the relation between the physicochemical properties of NP and their behavior in aqueous systems. It is almost universally agreed that the following properties are an essential requirement: chemical composition, mass, particle number and concentration, surface area concentration, size distribution (including polydispersity of the primary particle and the nature of any aggregates), specific surface area, surface charge/zeta potential, surface contamination and the nature of NP (and any capping agents), stability, and solubility^[25]. However, other properties may also be of importance.

Since nanotubes do not behave like other known chemicals, it is necessary to gain data on the effects and behavior of CNT and to further develop characterization and detection techniques for CNT in the environmental samples.

1.2.2 Bioavailability of CNT in the environment

Bioavailability is a dynamic approach considering physical, chemical, and biological processes of contaminant exposure and dose. Bioavailability incorporates concepts of environmental chemistry and ecotoxicology, integrating contaminant concentration, fate, and the behavior of organisms present in the environment. Bioavailability of NP depends on the physicochemical properties of the particles (aggregation, solubility), on the nanoparticle-organism contact environment, but also on the target organism (i.e. particle-ingesting or not)^[2].

CNT will interact with dissolved organic material (DOM) in the aquatic environment and this interaction will affect their presence in the water column^[42]. MWCNT ingested by *Stylonychia mytilus*, a unicellular protozoan, were excreted as granules in micron size and sedimented^[43]. Uptake of MWCNT by *Daphnia magna* and obstruction of the material in the gut was also described by Petersen et al.^[44]. They showed that the addition of uncontaminated food led to facilitated excretion of the nanomaterial but they could not find the entrance of CNT in tissue cells. Kennedy and coworkers^[41] described the uptake of different CNT (raw, hydroxylated and carboxylated) in the gut of another cladoceran *Ceriodaphnia dubia*, as all three types of MWCNT were visible in the gastrointestinal tract. Individuals appeared to have difficulties in clearing their guts, which was hypothesized to be one reason for the observed mortality and immobilization^[41]. Additionally, biomodification of lipid coated SWCNT by *D. magna* was observed^[45]. The daphnids used the lipids as food source and excreted precipitated SWCNT to the medium. Hence, the daphnids were able to modify nanotubes and change their chemical and physical properties in aqueous dispersions. Furthermore, Porter et al.^[46] found C₆₀ (carbon-based NP) to accumulate at several unexpected sites within human monocyte macrophages, in particular along the nuclear membrane and within the nucleus.

Although CNT are difficult to disperse in water, they might reach sites of possible toxic action in organisms, depending on its bioavailability. However, whatever constitutes the apparent route of exposure and the mechanisms of toxicity, bioavailability remains a key factor for the hazard evaluation of synthetic NP.

1.3 Toxicology of CNT

Despite of a growing understanding that synthetic NP should be evaluated for their potential environmental hazard prior their use in products and subsequent inevitable release into the environment, there are currently few data available on the toxicity of nanomaterials to environmentally relevant species, limiting the quantitative risk assessment of NP^[2]. NP exhibit, in contrast to other contaminants with unique physicochemical properties, quite different

toxicities when exposed to aquatic organisms during their existence in the aquatic environment^[47]. Therefore, special attention has to be drawn at different parameters such as the dispersion process and the agglomeration state in the test system^[48]. This is the main reason, why such a heterogeneous picture of results can actually be found in the literature for CNT nanotoxicity^[25,49-52]. Moreover, SWCNT were described to be more toxic than MWCNT, which may contribute to their higher surface area and smaller size. However, all these characteristics and the behavior of CNT has to be considered for CNT toxicity testing.

Generally addressed toxic mechanisms of nanoparticles toward aquatic organisms are released metal ions, oxidative stress, shading effects, and physical damage^[1]. Increasing evidence indicates that NP can generate reactive oxygen species (ROS), and consequently induce oxidative injury, cell damage, and even apoptosis^[53-57]. Toxic metal ions released from metal NP^[58] and carbon nanomaterials^[59] can cause toxicity to aquatic organisms. Moreover, physical contact, including adsorption and internalization, is another mechanism contributing to the nanotoxicity^[60,61].

Up to now, chronic exposure data are very limited and cover exclusively a period of 21 days, whereas many researchers occupied the acute toxicity of CNT. As the present study deals with the bioavailability of CNT in the aquatic environment as well as cell lines, reported effects of CNT to cell lines, *Daphnia magna* and *Danio rerio* are briefly summarized in the following sections.

1.3.1 Effects on cell lines

The influence or possible toxic effects of CNT on cells are an essential focus in evaluating and understanding CNT compatibility versus toxicity^[62-66]. The effect of CNT on the cellular level has been repeatedly evaluated in the past to assess possible risks of CNT to human tissues. Previous researchers have explored the toxicity of carbon nanomaterials to animal and human cells^[67-73]. Main endpoints in these studies were uptake of CNT into cells and further processing of CNT by different routes, membrane perturbations, effects on cell signaling, production of chemokines, cytokines and reactive oxygen species, cell apoptosis, overt toxic

reactivity, and no obvious toxicity^[74]. It was suggested that the toxicity of CNT may also be caused by sorption of toxic substances to their surface^[75-77]. Therefore, knowledge of toxic compound adsorption by carbon nanomaterials is critical and useful for risk assessment of these nanomaterials because in the environment both, nanomaterials and chemical pollutants, are present as complex mixtures.

To date, many studies on the safety of different CNT materials have been conducted but the results are often controversial and depending on the appearance of the applied CNT material.

Aggregation state and peptide coating seemed to influence the toxic impact of CNT^[78,79]. Wick and coworkers^[79] found that suspended CNT-bundles were less cytotoxic than rope-like agglomerates for human lung cancer cell lines. Additionally, serum proteins adsorbed on CNT were found to attenuate the inherent cytotoxicity of CNT, and the extent of toxicity attenuation increased with increasing amounts of serum proteins adsorbed on CNT^[80]. It has been reported that CNT, modified with peptides or DNA fragments, could enter cells, and therefore, are promising to be used as specialized drug carriers e.g. in cancer therapy^[81-83]. CNT may adsorb a wide range of chemicals, including pollutants, and thus may transport exogenous substances into cells, similar to the case of drug carriage. After internalization, CNT were found to differently distribute inside of the cells, depending on their characteristics. Differently modified nanotubes were suggested to accumulate in diverse parts of cells, such as the cytoplasm, nucleus or organelles, thereby most probably inducing varying toxic effects^[62,82,83].

For other types of cell lines, Belyanskaya et al.^[84] measured the toxicity to neurons and glial cells and found SWCNT significantly decreased the overall DNA content, related to the NP agglomeration state. Moreover, it is recognized that nanoparticles produce ROS^[25,85] inside and outside the cell, which has to be considered as one of the key factors inducing toxicological effects^[86]. Thereby, oxidative stress triggers inflammation via the activation of oxidative stress-responsive transcription factors^[23]. It was suggested that nanotubes may cause membrane damage during possible perturbation or piercing of single CNT through the outer cell membrane. This may result in novel transport ways for pollutants from the

extracellular environment into the cell^[87]. Moreover, leaching of molecules out of the cell is assumed to be possible^[88].

In summary, cytotoxicity following CNT exposure might be a result of direct or indirect impact of the material on the cell by contact and internalization or by disturbance of chemical reactions in the cell, but more research is needed for an entire picture.

1.3.2 Effects on pelagic invertebrates: daphnids

Invertebrates, including zooplankton and zoobenthos, are regarded as the largest community in water environments. Even though some zooplanktons, such as *Daphnia magna*, it is quite easy for the suspending NP to enter the organisms via the water phase through certain dietary pathways. Different entrances and routes for the uptake of NP can lead to various distributions of the NP inside the invertebrates (Figure 2). The majority of existing studies has indicated that NP taken up by daphnids mainly remain in the guts with limited absorption into the gut tissue^[41,44,89-91], suggesting ingestion of NP to be the most likely route for uptake. This includes active selection by the feeding apparatus, as well as passive diffusion or uptake alongside larger particles, which is consistent with the feeding behavior of daphnids. Given that daphnids can alter particles with a maximum size of 70 μm ^[92], most of the retained materials are likely aggregates of NP.

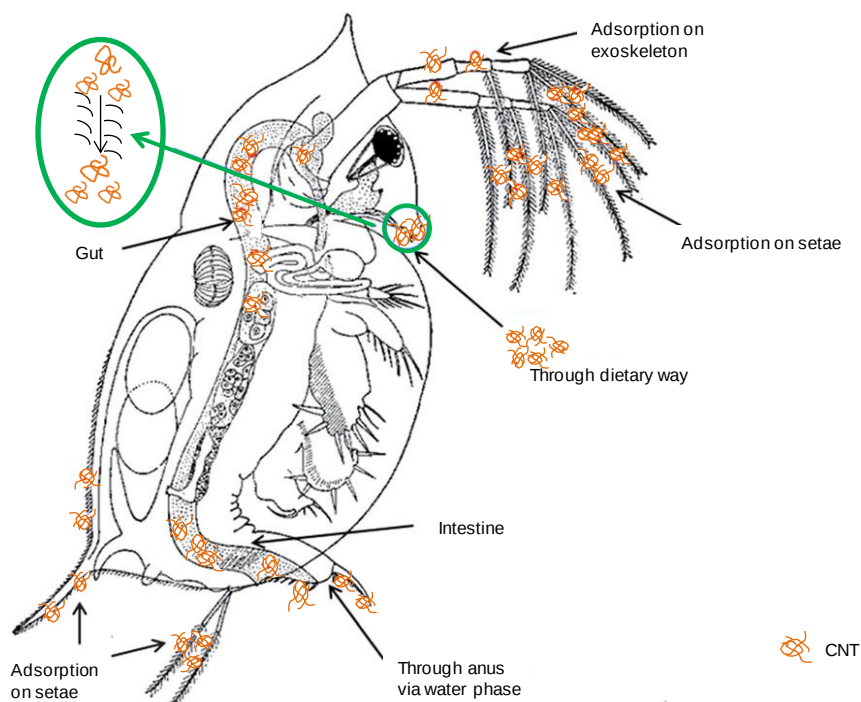


Figure 2. Uptake routes and distribution of CNT in *Daphnia magna* (modified from Ma et al.^[47])

Once ingested, food particles will be digested and then the nutrients will transport across the gut epithelium to storage cells^[93]. However, this appears to contrast with most existing observations for NP that show limited translocations across gut walls. One exception is the work by Rosenkranz et al.^[92] who demonstrated that daphnids take up micron-sized (1000 nm) fluorescent carboxylated polystyrene particles more efficiently than nano-sized ones (20 nm), and some particles of both sizes absorb across gut walls, accumulating in the lipid droplets within storage cells. Following exposure, daphnids are able to purge NP from their bodies. Tervonen et al.^[91] reported that daphnids eliminate 75 % of accumulated nC₆₀ after 48 h of depuration. However, the depuration is not complete and the 25 % of nC₆₀ mass remaining in daphnids is significant. Similar observations have also been reported for CNT^[44,94]. Feeding daphnids during waterborne exposure and depuration impacts the accumulation behavior for CNT^[44,94]. Nevertheless, in all cases the elimination is not complete, suggesting the potential for trophic transfer of CNT (biomagnifications).

It was even suggested that daphnids do not only feed by mechanical sieving but also can gather food particles, even in the ultrafine range, by direct interception^[95,96] or by drinking the

surrounding media to replenish depleted sodium and to facilitate digestion^[97,98]. Both mechanisms, the direct interception as well as the drinking, are possible uptake routes for particles in the nano range. The appearance of ingested matter in the gut is reported to be very rapid after feeding. For example, under optimal conditions, the gut has been reported to fill within 30 minutes of exposure to a food source^[99].

Furthermore, adsorption of NP on the surfaces of aquatic invertebrates has been documented. It should be noted that there are setae on the surfaces of *Daphnia* and NP can likely be adsorbed onto these setae. Additionally, it was observed that NP could gather on the surfaces of *Daphnia* probably due to the adhesion to the exoskeleton of *Daphnia*^[60,100]. As a matter of fact, the interface between invertebrates and NP is not as simple as just a layer or flat surface. For some invertebrates, besides the gills, mantle and labial palps were also the exposure organs contributing to the internalization of NP though they were not the main entrance compared to the ingestion route^[100].

1.3.3 Effects on vertebrates: fish

Being in the higher trophic level of the aquatic ecosystem, fish are susceptible to contaminants in their environment. NP needs to cross several barriers on the fish surface before they may absorb into fish tissues and organs (Figure 3). The epidermis, which consists of several layers of epithelial cells, plays an important role in the interfaces that form when fish meet NP^[47]. Fish gills are generally identified as an important site for xenobiotic exposure, as it is the place where the chemical exchange between fish and the environment occurs^[42,101]. There are also protective covers, like a mucus layer on the gills of some species of fish, in which NP may be trapped^[102], as in the case of the association of SWCNT with the gill mucus in trout^[103]. Another exposure route may includes gut exposure (i.e., ingestion).

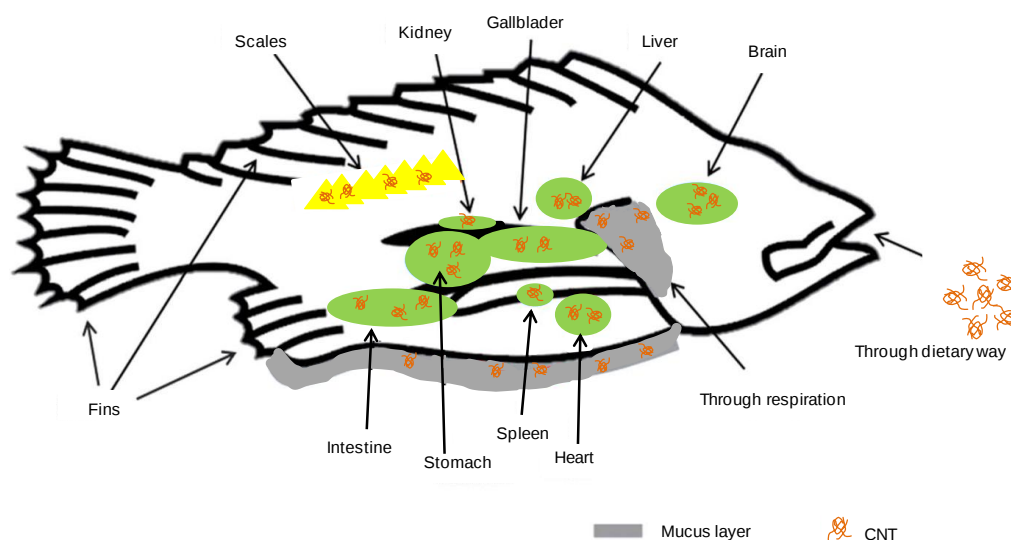


Figure 3. Adsorption of CNT on the outer surfaces and distribution in the main internal organs of fish (modified from Ma et al.^[47])

Only few data is available for the exposure of zebrafish embryos to CNT. Cheng et al.^[104] investigated the impact of SWCNT and double-walled CNT (DWCNT) on the development of zebrafish embryos. They observed delayed hatching of SWCNT exposed embryos but no influence on their development and survival. Exposure to DWCNT induced a shorter hatching delay in zebrafish embryos. It was supposed that the chorion acts as a strong protective barrier preventing the passage of large SWCNT clusters through the pores. Olasagasti and coworkers^[105] exposed zebrafish embryos to different concentrations of functionalized MWCNT (carboxylated), and observed increased mortality compared to the controls. Additionally, clear increase in the expression of several genes, known to respond to toxicant exposure, was observed with increasing MWCNT concentration.

An exposure of MWCNT via microinjection to zebrafish embryos conducted by Cheng et al.^[106] resulted in no significant toxicity. But, contrarily, another study by Cheng and Cheng^[107] showed an increase toxicity depending on the longer sonication time of MWCNT. The test design of both abovementioned studies is questionable though, since the MWCNT were injected into the embryos, which is not a likely environmental scenario. Regarding the exposure of MWCNT to zebrafish embryos, it has to be noted that NP with particle sizes ≤ 45 nm like silver and gold have been shown to pass the chorion and reach the embryo^[33,108],

whereas larger silica NP have been shown to not be able to pass the chorion, and could be found on the chorion surface. MWCNT have particle sizes ranging from 5 - 100 nm, but only reach these small scales in one dimension. Typical lengths of MWCNT are in the μm range, therefore MWCNT should not be able to pass the chorion.

Concluding, current literature suggests that the bioaccumulation potential of NP to fish is relatively low and that the chorion of zebrafish may act as a barrier for CNT exposure and the major route of NP uptake for fish larvae or adults may be oral (i.e., direct ingestion) or food exposure (i.e., ingestion of NP contaminated diets).

1.4 CNT-interactions with pollutants

The use of nanoparticles, in water treatment and purification to remove organic and inorganic contaminants, has advantages over conventional materials. CNT have attracted great attention due to their unique tubular structures and large length/diameter ratio and their chemical, electronic and mechanical properties^[18]. Because CNT surfaces have a strong interaction with other molecules, particularly with those containing benzene rings, they possess excellent adsorption ability and substitute active carbon. Thereby, the first couple of layers interact with the surface, while molecules beyond the first two layers interact with each other. The energy of such process varies depending on the distance between the adsorbed molecules and the nanotube surface^[109,110].

1.4.1 Properties of CNT

The shape and size of CNT are important factors that can likely regulate the nano-bio interactions. The adsorption kinetics may be attributed to their faster particle mobility and lower energy barriers to the target organisms, leading to a faster adsorption rate for smaller CNT as for larger ones^[111]. NP with larger surface areas undoubtedly have more opportunities and sites to come into contact with cells. SWCNT exhibited higher toxicity to the cell membrane of bacteria than MWCNT due to the much larger surface area^[112]. Therefore, CNT with a smaller size have a higher potential to be adsorbed and internalized by aquatic organisms. However,

small-sized NP are prone to aggregation in water and the aggregation can likely influence the nano-bio interactions. When NP aggregate in water, the apparent particle size highly increases; this makes it difficult or even impossible for the NP to pass through the cell wall via natural pores^[113]. Nevertheless, there is very limited information available to date on the size effect of NP as influenced by NP dose, which should be an area of focus for future nanotoxicity research, especially on the microcosmic interactions^[114].

1.4.2 Adsorption of organic contaminants to CNT

The large specific surface area may accommodate pollutant adhesion and thus influence CNT toxicity itself and/or toxicity of co-pollutants^[115]. The surface area, a function of outer diameter and pore volume, may determine the adsorption capacity. Sorption effects of CNT to different pollutants present in the environment has been studied by several authors concluding that CNT are an successful adsorbent media^[26-31,115-117].

CNT have been reported to exhibit a high adsorption capacity. This is due to the presence of high energy adsorption sites, like defects functional groups and interstitial and groove regions between nanotubes' bundles. Multilayer adsorption so called surface condensation, occurs when organic chemicals were adsorbed on CNT surfaces^[118]. The adsorption of NP on the organisms by electrostatic forces can be regulated by surface charges of the organisms and NP. Therefore, the surface charge is one of the key characteristics of NP that may dominate the nano-bio interactions^[47].

CNT are attracting increasing attention as adsorbent for the removal of organic pollutants because of their characteristic structures which allow strong interactions with organic molecules via non-covalent forces, such as hydrogen bonding, π - π stacking, electrostatic forces, van der Waals forces and hydrophobic interactions. The presence of functionalized CNT allows the possibility of incorporating one or more of these interactions which increase the selectivity and the stability of the system^[119-123].

SWCNT and MWCNT were used as adsorbents for the removal of Reactive Blue 4 textile dye from aqueous solutions^[124]. Moreover, MWCNT show better adsorption removal of Reactive Red M-2BE textile dye from aqueous solutions than powdered activated^[125,126]. Furthermore, CNT have shown potential as adsorbents for removing many kinds of pollutants such as heavy metals, organics, and biological impurities^[127,128]. As adsorbent media, they are able to remove heavy metals such as Cr^{3+} ^[129], Pb^{2+} ^[130], and Zn^{2+} ^[131], metalloids such as arsenic compounds^[132], organics such as polycyclic aromatic organic compounds^[117,133,134], pesticides^[135], and a wide range of biological contaminants including bacteria^[136-141], viruses^[142,143], cyanobacterial toxins^[144-146]. Also natural organic matter is known to interact with CNT^[147-150]. The success of CNT as an adsorbent media for the removal of biological contaminants, i.e. especially pathogens, is mainly attributed to their unique physical, cytotoxic and surface functionalizing properties^[127].

1.4.3 Influence of functionalization

Carbon nanotubes are nearly insoluble in any solvent (including water) because they form bundles. Functionalization is the process whereby CNT are separated and coated with certain molecules and is a way of improving CNT purity^[151]. Many commercially available CNT are therefore functionalized before being used in a biological environment^[152]. The functional groups on the NP surfaces are also very important to the nano–bio interactions because they decide the potential specific interactions (like hydrogen bonding) and the active sites on the nano–bio interfaces; they may also change the surface charges of the NP and influence the electrostatic interactions^[153]. Surface coating by surfactants or polymers would change the surface properties of the NP, and thereupon change the environmental behaviors of the NP and the nano–bio interactions^[47].

In general, it has been showed that CNT will interact in the biological milieu with proteins and interfere with their structure, possibly causing cell death^[14]. It has been established by various researchers that functionalization (i.e. polyethylene glycol, taurine, carboxylic, nitric, and sulfuric acid) can significantly improve the dispersibility and biocompatibility, even as reducing

the toxicity of CNT^[14,154-157]. Thus, functional groups can change the wettability of CNT surfaces and make them more hydrophilic and suitable for the adsorption of relatively low molecular weight and polar compounds, while making them less affine to adsorption of nonpolar hydrocarbons because of the reduced hydrophobicity^[110]. On the other hand, functional groups may increase diffusional resistance and reduce the accessibility and affinity of CNT surfaces for organic chemicals. Furthermore, Castillejos et al.^[158] hypothesized that an increasing number of oxygen groups on CNT can increase the electron density at their surfaces, favoring π - π stacking interactions, hydrogen bonds and electron-accepting interactions.

As a result in the environment, natural coatings by e.g. organic matter will increase the pristine CNT dispersability in aquatic solutions by covering their hydrophobic surface. This reduces CNT agglomeration, prolongs residence time in the water column, increases CNT mobility and thus intensifies risk of aqueous phase exposure and toxicity^[41,115,147,159-161]. Depending on length, diameter, entanglement, surface modification and environmental conditions, CNT may have a very different behavior under natural conditions and thus environmental fate.

1.4.4 Influence of dispersion

Ultrasonication is a common procedure used to purify and obtain homogeneous dispersions of CNT as well as to mix them with other components for further processing into composites. It has been discovered that metal impurities is one of the main factors determining CNT toxicity and might influence their bioavailability^[162], resulting in cell death through various mechanisms including mitochondrial destruction and oxidative stress^[163]. One study demonstrated that even ultrasonication of CNT for 5 min can significantly reduce their toxicity. It has been suggested that one reason why ultrasonication has this effect is because ultrasonication promotes the release of a metallic impurities into solution^[162]. Until now, only little is known and studied about what happens to the bioavailability of metallic impurities during ultrasonication.

1.5 Triclocarban

Triclocarban (TCC; Figure 4), a biphenyl ether, is a synthetic biocide which is often used as an additive to personal care products (especially soaps) and solvents due to its antimicrobial properties^[164]. TCC is able to adsorb on the cell membrane and to destroy its semi permeable character, leading to cell death^[165]. Another mode of action which has been hypothesized by McDonnell^[166] is a hydroxylation of proteins or the membrane phospholipid bilayer, which could disrupt the proton motive force across the bacterial surface as well as interrupt active transport and energy metabolism^[167]. The polychlorinated aromatic antimicrobial TCC is applied since 1957^[164] and the high vapor pressure of 3.6×10^{-9} mm Hg (at 25°C) suggests low volatility. Its log K_{ow} of 4.9 suggests high solubility in nonpolar solvents, whereas the water solubility of TCC is poorly with 0.65 mg/L^[168].

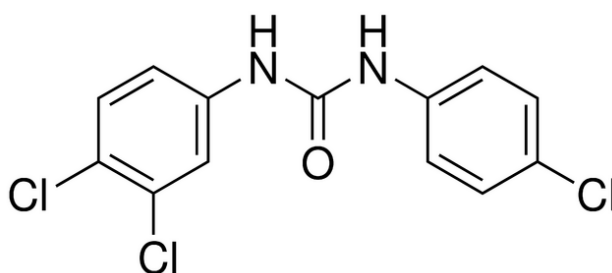


Figure 4. Structural formula of 3,4,4'-trichlorocarbanilide, known as triclocarban (TCC).

The environmental ubiquity of TCC is now detectable in house dust worldwide, in ocean water, and locations as remote as the water loop of spacecraft^[169]. Therefore, the concentrations in the environment are high, and risk assessment concerning TCC is from urgent necessity, especially as TCC ranks within the top 10 of the most commonly detected organic wastewater compounds, as well for frequency as for concentrations^[164]. Moreover, waste water treatment plants cannot remove triclocarban completely from the water, so it is randomly found in surface waters^[170]. According to Miller et al.^[171] less than 3 % enters those waters, which measure about 6.75 µg TCC/L. Nevertheless, high accumulation is observed in aquatic organisms and sediment, actually even in mg/kg concentrations^[171].

Some research have been performed dealing with the question to which extent TCC is able to affect aquatic organisms. Acute toxicity tests have been performed with *Daphnia magna* (48 h EC₅₀: up to 12 µg TCC/L), *Pseudokirchneriella subcapitata* (72 h EC₅₀: up to 35 µg TCC/L) and *Oryzias latipes* (96 h LC₅₀: up to 100 µg TCC/L)^[172]. Considering that the measured average concentration in the U.S. surface waters (1.2 µg/L)^[173] and the median effective concentration for *Daphnia magna* (10 µg/L) are not far apart and a high risk of TCC for the environment can be concluded^[172]. Therefore, research concerning TCC and its interactions with other substances must be of high priority, to conduct a meaningful risk assessment.

1.6 Overview of cellular stress responses

Nanoparticles interacting with proteins, membranes, cells, DNA and organelles establish a series of nanoparticle/ biological interfaces that depend on kinetics and thermodynamic exchanges between NM surfaces and the surfaces of biological components, as well as dynamic biophysicochemical interactions. These interactions lead to the formation of protein coronas, particle wrapping, intracellular uptake and biocatalytic processes that could have biocompatible or bioadverse outcomes^[174] In the special case of cellular stress, cells respond in a variety of ways ranging from activation of pathways that promote survival towards eliciting programmed cell death that eliminates damaged cells. The cell's initial response to a stressful stimulus is geared towards helping the cell to defend against and recover from insult^[175].

During tissue homeostasis there is an equilibrium between the net growth rate and the net rate of cell death^[176]. Depending on the type of cellular stress and its severity, the cell's response can be various. If the stress stimulus does not exceed a certain threshold, the cell can deal with it by rising an appropriate protective cellular response, which ensures the cell's survival. On the contrary, the failure to activate or maintain a protective response, e.g. if the stressful agent is too strong, results in activation of stress signaling cascades that eventually lead into cell death pathways^[177,178]. Two main types of stress are explained hereinafter because they are key endpoints of the present thesis.

1.6.1 The heat shock responses

One of the main prosurvival activities of cells is the heat shock response (i.e., elevations in temperature of 3–5 °C above normal)^[179,180]. Since, it has been recognized that many stimuli can activate this response, including oxidative stress and heavy metals. One of the main cellular consequences of these stresses is protein damage leading to the aggregation of unfolded proteins. During initiation of the heat shock response general protein transcription and translation is halted, presumably to alleviate the burden of misfolded proteins in the cell^[175]. However, transcription factors that enhance expression of a specific subset of protective genes are selectively activated under these conditions (Figure 5); these are the heat shock factors (HSF)^[181]. Vertebrate cells have three different HSF: HSF1 is essential for the heat shock response and is also required for developmental processes, HSF2 and HSF4 are important for differentiation and development, while HSF3 is only found in avian cells and is probably redundant with HSF1^[182,183].

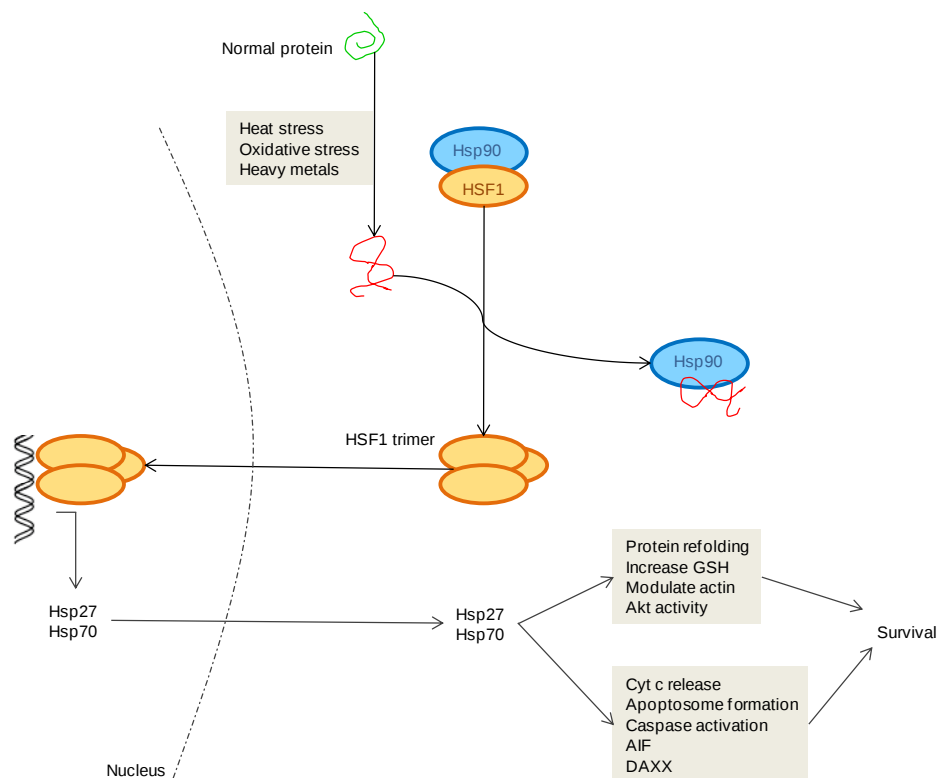


Figure 5. Induction of heat shock proteins inhibits apoptosis and promotes cell survival (based on Fulda et al.^[175])

HSF bind to upstream sequences (heat shock elements) in the promoters of target genes, leading to the expression of heat shock proteins (Hsp). Hsp are a set of evolutionary conserved proteins that are grouped into subfamilies with different molecular weights, e.g. 70 kDa^[181,184]. Particularly Hsp70 are usually expressed at low basal levels and increase in response to environmental and physiological stressors, and as such they are termed inducible Hsp and are part of the heat shock response^[184]. Hsp70 has been shown to protect cells against the induction of cell death by a variety of stresses and by different modes of cell death, including apoptosis^[185] and necrosis^[186-188]. They achieve these effects directly, through inhibition of cell death pathways, and indirectly, through general prosurvival activities. Overall, Hsp can be activated or induced by a number of stresses and they act to protect the cell by influencing a variety of cellular processes which determine cellular fate. Hsp are, in general, prosurvival and anti-apoptotic molecules.

1.6.2 The response to oxidative stress

Oxidative stress occurs when there is an oxidant/ antioxidant imbalance caused by a greater number of oxidants than antioxidants present within the cell^[189] and it has been implicated in several biological and pathological processes^[190]. Although most oxidative insults can be overcome by the cell's natural defenses, sustained perturbation of this balance may result in either apoptotic or necrotic cell death^[190-194]. Reactive oxygen species can originate from intracellular or extracellular sources. Reactive products of oxygen are amongst the most potent and omnipresent threats faced by cells^[175]. These include ROS such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), singlet oxygen, hydroxyl radical ($OH^{\cdot}$), peroxy radical, as well as the second messenger nitric oxide ($NO^{\cdot}$) which can react with $O_2^{\cdot-}$ to form peroxynitrite ($ONOO^-$).

ROS can be produced directly due to nanoparticles' large proportional surface area^[195], or nanoparticles can mechanically disrupt cell structures such as mitochondria^[196]. Alternatively, nanoparticles can become coated with a 'corona' of proteins^[197,198] perhaps providing routes of passage through biomembranes (e.g., via receptors or endocytosis) with potential for effects

on cell signaling pathways^[199,200]. A high level of ROS can damage cells by altering protein structure, disrupting DNA, interfering with signaling functions, and modulating gene transcription^[14]. This may result in cancer, renal disease, neurodegeneration, cardiovascular or pulmonary disease at higher organisms.

1.7 Objectives of the thesis

In general, the present study deals with several ecotoxicological endpoints on different trophic levels towards MWCNT exposure, once within the risk assessment of NP, as well as the bioavailability and toxicology of TCC. Thereby, *in vivo* and *in vitro* studies were conducted to elucidate the toxicology and bioavailability of both substances, also in combination. Furthermore, this study was projected as a part of the investigation of OECD-guidelines and Standard Operating Procedures for the testing of nanomaterial, which were to be examined regarding potentially relevant endpoints and parameters not included in the test protocols.

Whether CNT are inherently toxic or is it a wide array of external factors such as length, surface modification, degree of dispersion and the presence of metal impurities playing a major role in CNT-induced toxicity is still a subject of intense investigations. Because CNT are known to be biopersistent^[23], knowledge on their toxic potential is still required.

The high surface-area-to-volume ratio of CNT enables interactions with substances such as adsorption on the CNT surface, interior and interstitial areas^[110]. Especially the interaction of MWCNT and TCC poses a major question in the present study, if one of them is more or less toxic when cells or organisms are exposed to mixtures of both.

The polychlorinated aromatic antimicrobial triclocarban, a high-production-volume chemical, is widely used for killing microorganisms thoughtless, rapidly, and by nonspecific action. More than a decade into the accelerated use of TCC, there now are instantly recognizable signs of this chemical taking a toll on the health of the environment^[167,201]. This situation has drawn an increased inquiry of fact by agencies in the U.S., Canada and Europe, including the U.S.

Environmental Protection Agency (EPA)^[202,203], Food and Drug Administration^[204], as well as the Centers for Disease Control and Prevention^[205], and the European Union^[206]. Analogous to the detection of environmental pollution and new health risks of antimicrobials^[207], concerns about the appearance of microbial pathogens resistant to multiple groups of antibiotics of medical importance^[208] have triggered the need for reevaluation the present status of antimicrobial usage^[209]. Because CNT are known to interact with hydrophobic organic compounds, triclocarban was selected as a model substance to examine mixture toxicity in this study.

Three different cell lines were exposed to MWCNT, TCC as well as the mixture of both substances to get more information on their cytotoxicity, endocrine disruption, and the intracellular generation of reactive oxygen species (**Chapter 2**). Furthermore, it was suggested that the toxicity of carbon nanomaterials may also be caused by sorption of toxic substances to their surface. Therefore, knowledge of toxic compound adsorption by carbon nanomaterials is critical and useful for risk assessment of these nanomaterials because in the environment both, nanomaterials and chemical pollutants, are present as complex mixtures.

So far, only little is known about the chronic effects of CNT to aquatic organisms. Hence, long-term exposure of MWCNT to *Daphnia magna* was performed by using the population test over a period of 93 days (**Chapter 3**). Additionally, three short-term exposure scenarios with TCC were conducted during this period. Furthermore, the effects of TCC on population level were specified and compared to the output of an individual-based *Daphnia magna* population model (IDamP). The use of this modeling approach offers a tool to extrapolate from effects derived from laboratory experiments to effects on the population level and can improve the ecological risk assessment of chemicals.

Danio rerio is a representative indicator species for aquatic organisms of a high trophic level. In the present study, the toxic potential, robust stress response, and the passage through the chorion of MWCNT as well as the toxicity of TCC to zebrafish embryos was to be investigated (**Chapter 4**). The extended fish embryo toxicity test (FET) was chosen as test system and the

test organisms were a wild type zebrafish and a transgenic heat shock reporter zebrafish. Especially the question whether MWCNT and TCC interact and are more or less toxic when zebrafish embryos are exposed to mixtures of both was addressed in this chapter.

Finally, a general conclusion will be drawn combining the main points of the study to an entire picture.

1.8 References

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Chapter 2

Effects of multiwalled carbon nanotubes and triclocarban on several eukaryotic cell lines: Elucidating cytotoxicity, endocrine disruption, and reactive oxygen species (ROS) generation

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2.1 Introduction

The annual worldwide production of carbon nanotubes (CNT) surpassed the multi metric ton level and is expected to further increase^[1]. Their structure gives them exceptional properties, which makes this material suitable for the use in composite materials, sensors, drug delivery, hydrogen-storage fuel cells, and various environmental applications^[2-6]. The probability of occupational and public exposure to CNT has significantly increased^[7]. With this nanophase invasion of new materials and products into many aspects of life comes the need for increasing safety measures for exposure risks^[8]. In October 2011, the European Union defined nanomaterials as natural, incidental or manufactured materials containing particles, in an unbound state or as an aggregate or agglomerate, where 50 % or more of the particles exhibited one or more external dimension in the size range of 1–100 nm^[9].

Carbon nanotubes represent one of the most promising nanomaterials for various applications^[10]. However, public concerns on the widespread use of these materials increase due to their close similarity to other toxic fibers regarding their high aspect ratio, reactivity and biopersistence. Multiwalled carbon nanotubes (MWCNT) used in this study were the most highly produced CNT materials until 2012^[10]. A pilot plant with an annual capacity of 60 tons is since 2007 in operation in southern Germany. Thus, knowledge on the toxic potential of MWCNT is required also regarding the very different nature of various types differing in flexibility or stiffness, varying in length and aspect ratio as well as having different contents of metal catalysts and surface properties.

All MWCNT have a tubular structure with a high aspect ratio and between two and 30 concentric cylinders with outer diameters commonly between 30 and 50 nm. The small size and the high surface area define the chemical reactivity of CNT and induce changes in permeability or conductivity of biological membranes^[11]. Therefore, engineered CNT may pose health risks because of their ability to reach every part of the organs and tissues and their interaction with cellular functions. The primary risk of these materials may come from

their ability to enter cells, which may cause damage to plants, animals, and humans^[12-15]. Important characteristics are the surface chemistry and purity of CNT. For MWCNT synthesized using a metal catalyst, the toxicity may be the combined effect of the MWCNT themselves and an oxidative stress response to the residual metal catalyst^[16] typically amounting to less than about 5 weight percent. This complicates clear determination of pure MWCNT toxicity. Despite these concerns, very few studies have been simultaneously conducted with various human cell lines to assess the health effects of different CNT. At present, there is no global agreement about the risk of CNT on human health^[17].

Previous researchers have explored the toxicity of carbon nanomaterials to animal and human cells^[18-24]. It was suggested that the toxicity of carbon nanomaterials may also be caused by sorption of toxic substances to their surface^[25-27]. Therefore, knowledge of toxic compound adsorption by carbon nanomaterials is critical and useful for risk assessment of these nanomaterials because in the environment both, nanomaterials and chemical pollutants, are present as complex mixtures.

CNT are carbonaceous adsorbents with hydrophobic surfaces that exhibit strong adsorption affinities to organic compounds^[28-37]. Thereby, a combination of chemical, and physical interactions play a major role for adsorption processes. CNT have uniform structural units but are prone to aggregate, forming bundles of randomly tangled agglomerates because of the strong van der Waals forces along the length axis^[38]. The outermost surface, inner cavities, interstitial channels, and peripheral grooves of CNT constitute four possible sorption sites for organic compounds^[37]. Nanotechnology has initiated different types of nanomaterials to be used in water technology in recent years that can have promising outcomes.

Nanosorbents such as CNT have exceptional adsorption properties and can be applied for removal of heavy metals, organics, and biological impurities^[33,39]. CNT, as adsorbent media, are able to remove heavy metals such as Cr^{3+} ^[40], Pb^{2+} ^[41], and Zn^{2+} ^[42], metalloids such as arsenic compounds^[43], organics such as polycyclic aromatic organic compounds (PAH)^[28,34,44], pesticides^[45], and a range of biological contaminants including bacteria^[46-51],

viruses^[52,53], cyanobacterial toxins^[54-56] as well as natural organic matter (NOM)^[57-60]. The success of CNT as an adsorbent media in the removal of biological contaminants, especially pathogens is mainly attributed to their unique physical, cytotoxic and surface functionalizing properties^[33].

To date, many studies on the safety of different CNT materials have been conducted but the results are often controversial and depending of the species of the applied CNT. A wide range of results from *in vitro* studies, dealing with MWCNT, has been reported. On the one hand MWCNT decreased cell viability and induced apoptosis^[61,62], whereas minimal to no decrease of cell viability was observed^[63]. One explanation of this controversy is the type of cells used. Additional explanations are that MWCNT are produced by different processes, tested with varying dispersion methods, and that their life cycle may confer changes in their surface characteristics and reactivity. For example, in some studies, the presence of metal trace impurities explains demonstrated toxicity and reactive oxygen species (ROS) production^[63], whereas in other cases no such effects were reported^[64]. Nevertheless, it is recognized that nanoparticles produce ROS^[63,65] inside and outside the cell, which has to be considered as one of the key factors for toxicological effects^[8]. Hence, further evaluation and characterization of their toxic potential and other effects on cells like cytotoxicity, endocrine disruption, and the production of ROS, which can result in cell damage, is of highest concern.

Relatively little research has been conducted examining biocidal components of personal care products, as for example Triclocarban (TCC), although such products are continually released into the aquatic environment and are biologically active and some of them persistent^[66]. Therefore, they are detected often and in rather high concentrations in the environment^[66]. TCC is a high-production-volume chemical^[67] that is widely used as an antimicrobial compound^[66,68]. It is able to adsorb on the cell membrane and to destroy its semi-permeable character, leading to cell death^[69]. In the U.S., the annual production of TCC in 2002 added up to 500 metric tons^[70,71]. The primary route for the polychlorinated aromatic antimicrobial to enter the environment is through discharge of effluent from wastewater

treatment plants and disposal of solid residuals on land^[68,71,72]. Due to its lipophilicity (log K_{ow} 4.9^[73]) TCC has an affinity to adsorb to organic matter^[74], therefore over 70 % of the initial mass has been found to be adsorbed to sludge^[75,76]. TCC has been detected at microgram per liter levels in waterways in the United States and Switzerland, indicating extensive contamination of aquatic ecosystems^[67,77-79]. TCC was chosen in this study for its widespread use, toxicity^[71], bioaccumulation potential^[80-82], environmental persistence and endocrine effects^[83].

As TCC is used since 1957 in huge amounts^[66], and MWCNT is supposed to reach the amount of a large scale production, both substances might involuntarily occur together in the environment.

This study aimed to provide new information on toxicity of TCC and nanotoxicity of MWCNT as well as the mixture of both substances by using three different eukaryotic cell lines. Key questions were to get more information about the cytotoxicity of MWCNT and the estrogenic potential of TCC as well as the potential of MWCNT to generate ROS in cell lines. Especially the interaction of MWCNT and TCC poses a major question in the present study, if one of them is more or less toxic when cells are exposed to mixtures of both.

As many studies already showed that CNT are toxic for different cell lines^[7,11], we investigated cells by determination of cytotoxicity in the Neutral Red retention (NR) assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay^[84] to verify whether MWCNT showed a toxic potential for the used cells, namely RTL-W1, T47Dluc, and H295R. A combination of cytotoxicity assays, particularly the NR and MTT assay, was preferred in many studies^[85-88], as this would increase the reliability of the results obtained.

Furthermore, mechanism specific endpoints, such as estrogenic effects and alterations of the steroid synthesis were analyzed by using the Estrogen Receptor mediated Chemical Activated Luciferase gene eXpression (ER-Calux) assay^[89] and the H295R Steroidogenesis assay (H295R)^[90,91], respectively. The evaluation of the endocrine activity in waste-water

samples could already been proven by using these assays^[92-95]. As previously reviewed by Hecker and Hollert^[96] results of several studies indicated that a combined use of receptor-mediated and non-receptor-mediated-methods is necessary to enable objective assessment of endocrine potential in complex samples. Additionally, Grund et al.^[97] demonstrated that the combination of receptor-mediated and non-receptor-mediated assays such as the ER Calux and the H295R was appropriate for a holistic evaluation of potential endocrine activity of complex environmental samples.

The measurement of cellular reactive oxygen species was investigated by using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) assay^[98].

2.2 Material and methods

2.2.1 Chemicals

The test substance 3,4,4'-trichlorocarbanilide was purchased from Sigma Aldrich and had a purity of 99 % (CAS:101-20-2). Multiwalled carbon nanotubes (Baytubes C150P, >95 % purity) were provided from Bayer MaterialScience (Bayer AG, Leverkusen).

2.2.2 Cell cultures

RTL-W1 cells. The rainbow trout liver cell line (RTL-W1)^[99] was grown in L15-Leibovitz medium (Sigma-Aldrich) supplemented with 9 % fetal bovine serum (FBS, Biowest) and penicillin/ streptomycin (10,000 E/mL; 10,000 µg/mL in 0.9 % NaCl, Sigma-Aldrich) in 75 cm² flasks (Techno Plastic Products; TPP) at 20 °C in darkness according to the protocol detailed in Klee et al.^[100].

T47Dluc cells. The human T47Dluc breast adenocarcinoma cells were obtained from BioDetection Systems BV (Amsterdam) and were cultured in Dulbecco's modified Eagle medium / Nutrient mixture F-12 (DMEM/F12) with phenol red (Gibco) supplemented with sodium bicarbonate (Sigma-Aldrich), MEM 100x (Gibco), penicillin / streptomycin solutions

(Gibco) and 7.5 % fetal bovine serum (FBS) according to the methods details in Maletz et al.^[101]. T47Dluc cells were cultured at 37°C, 7.5 % CO₂ and maximum humidity.

H295R cells. The human adrenocarcinoma cells (H295R) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and were grown in 75 cm² flasks with 8 mL supplemented medium at 37°C with a 5 % CO₂ atmosphere as described previously^[90,102].

2.2.3 Nanoparticles suspension

Test suspensions of 1 to 100 mg/L of MWCNT were prepared by ultrasonication of the raw material with a microtip (70 W, 0.2" pulse and 0.8" pause; Bandelin) in distilled water for 10 minutes. Transmission electron microscopy (TEM) images showed the presence of small agglomerates and individual nanotubes in the medium (data not shown).

2.2.4 Cytotoxicity assays

For determining the effect of particles on cell viability, different assays were used. Potential interferences of MWCNT and the fluorescence measurement were prevented by using black microtiter plates.

a) Neutral Red retention assay

The Neutral Red retention (NR) assay was performed according to Borenfreund & Puerner^[103] with slight modifications as detailed in Heger et al.^[104] by using RTL-W1 cells. Briefly, 4 x 10⁵ cells were seeded into each well (except for the blanks) of a 96-well microtiter plate (Nunc) and directly treated in triplicates with the particle suspensions. To guarantee optimal culture conditions, cells were exposed in a 1:1 mixture of MWCNT suspension or TCC solution and double-concentrated L15-Leibovitz medium, resulting in final MWCNT-concentrations of 3.13 to 50 mg CNT/L and TCC concentrations of 7.8 to 10x10³ mg/L. After incubation for 48 h at 20 °C in the dark, the sample solution was discarded, and each well was rinsed with 100 µL phosphate buffered saline (PBS) to remove any excess medium. 100 µL of a 0.005 % Neutral Red solution (2-methyl-3-amino-7-dimethylaminophenazine;

Sigma-Aldrich) was added to each well except for the blanks. After an incubation time of 3 h at 20 °C in darkness, the amount of extracted NR was determined by absorption measurement at 540 nm and a reference wavelength of 690 nm using a microtiter plate reader (Infinite M200, Tecan Instruments). Thereafter, concentrations resulting in cell vitality of 80 % were calculated and identified as NR₈₀-values according to Heger et al.^[104]. For detection of significant differences, the *t*-test following square root transformation was performed using SigmaPlot 12. Results are given as relative values to the untreated control in percent.

b) MTT assay

The Cell viability was evaluated by the reduction of water soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Aldrich) to water-insoluble formazan crystals by mitochondrial dehydrogenase^[105]. The amount of the formed blue formazan is proportional to the amount of viable cells^[106], and the absorbance was measured at 492 nm using a microtiter plate reader (Tecan).

H295R cells

The exposure of H295R cells was conducted according to the methods of Hecker et al.^[90,91]. In brief, one mL of cell suspension, at a concentration of 2.5×10^5 H295R cells/mL, was added to each well of a 24-well microtiter plate and cells were allowed to attach for 24 h. Cells were treated in triplicate with a 1:1 mixture of the MWCNT suspension and/or TCC solution and double-concentrated medium, resulting in final concentrations of 3.13 to 50 mg CNT/L and 31.25 to 500 µg TCC/L for 48 h as well as the two reference substances forskolin and prochloraz (quality control plate). The plates were checked for cytotoxicity and contamination after 24 h of exposure. The culture supernatants were removed and frozen at -80 °C for later analysis of alterations in steroid synthesis in the Enzyme Linked Immunosorbent Assay (ELISA)-assay. Cells were rinsed with 600 µl PBS per well. Then, 400 µL of a freshly prepared MTT (Thiazolyl Blue Tetrazolium Bromide, ≥ 97.5 % TLC) solution at 500 µg/mL was added to each well and incubated for 30 min at 37 °C and 5 %

CO₂ in air atmosphere. The MTT solution was discarded and 800 µl DMSO was added to each well in order to lyse the cells. Plates were finally placed on a horizontal shaker for 15 min before measuring the absorbance. Results are given as relative values to the solvent control in percent.

T47Dluc cells

The MTT assay was performed according to Mosmann et al.^[107]. In brief, T47Dluc cells were seeded into a 96-well microtiter plate (TPP) at a density of 1×10^4 cells per well. After 24 h of pre-incubation, the old medium was removed and cells were treated with a 1:1 mixture of the MWCNT suspension and/or TCC solution and double-concentrated medium. A serial dilution resulted in five concentrations of the MWCNT suspension and TCC solution and a solvent control were applied to each plate. For each concentration, three wells were foreseen. The exposure medium was removed and the absorbance was measured after adding the freshly prepared MTT solution (500 µg/mL, Sigma-Aldrich) with a luminescence counter (Tecan) at 492 nm.

For both cell lines (H295R and T47Dluc), concentration-response curves were fitted with a nonlinear 'log(agonist) vs. response - variable slope' regression using GraphPad Prism 5 as detailed in Heger et al.^[104].

2.2.5 ER Calux

The ER Calux assay with stably transfected T47Dluc human breast cancer cells was developed by Legler et al.^[89] and was conducted in this study according to the detailed protocol given in Maletz et al.^[101]. 10×10^4 T47Dluc cells/mL, resulting in a density of 1×10^4 cells per well, were plated into 96-well microtiter plates in medium (DMEM/F12 free of phenol red supplemented with sodium bicarbonate, MEM 100x and fetal calf serum) and incubated for 24 h at 37°C (7.5 % CO₂, 100 % humidity). After this time, the assay medium was renewed, and the cells were incubated for another 24 h. Then, a 1:1 mixture of the MWCNT suspension and/or TCC solution and double-concentrated medium replaced the medium by

using a serial dilution resulting in five concentrations. All concentrations of the test compound and the positive control (E2) as well as blanks (DMSO) and solvent control (EtOH) were introduced to each plate in triplicate. After 24 h of exposure, the plates were checked for cytotoxicity and contamination and the medium was removed. Following addition of a mixture of 1:1 of PBS and steady light solution (Perkin Elmer Inc.), the plates were incubated on an orbital shaker in darkness for 15 minutes. Luminescence was measured using a plate reader (Tecan). The luciferase activity per well was measured as relative light units (RLU). The mean RLU of blank wells was subtracted from all values to correct for the background signal. The relative response of all wells was calculated as the percentage of the maximal luciferase induction determined for 17 β -estradiol (E2)^[108]. Only suspensions that did not cause cytotoxicity were used for quantification of the response.

2.2.6 Enzyme Linked Immunosorbent Assay

For quantification of hormone production by H295R cells the protocol given by Hecker et al.^[90,91] was used. To ensure that modulations in hormone synthesis were not a result of cytotoxic effects, viability of the cells was assessed with the MTT bioassay^[107] before initiation of exposure experiments. Only non-cytotoxic concentrations (>80 % viable cells per well) were evaluated regarding their potential to affect steroid genesis^[97]. In brief, H295R cells were exposed as described above. The frozen medium was thawed and extracted using liquid extraction with diethylether as described previously in Maletz et al.^[101]. The amount of E2 was determined in an Enzyme Linked Immunosorbent Assay (ELISA)-assay (Cayman Chemicals)^[97].

2.2.7 Measurement of cellular reactive oxygen species (ROS)

The production of reactive oxygen species in RTL-W1, T47Dluc and H295R cells was measured using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) as previously described^[63,98,109-112]. This dye is a stable cell-permeant indicator which becomes fluorescent when cleaved by intracellular esterases and oxidized by intracellular hydroxyl radical, peroxynitrite, and nitric oxide^[109]. The intensity of fluorescence is therefore

proportional to the amount of reactive oxygen species produced in cells. RTL-W1, T47Dluc and H295R cells were charged as explained above, except for that H295R cells were seeded in 96-well plates as well. After an exposure time of 24 h or 48 h, the medium was discarded, cells were washed three times with PBS because black particles strongly reduced the fluorescence signal, and 100 μ L of H₂DCF-DA (final concentration of 5 μ M in PBS) was added to each well. Subsequently, the plates were incubated for 45 min at room temperature on a horizontal shaker in darkness. Fluorescence at excitation and emission wavelengths of 485 nm and 530 nm, respectively, was measured with a microtiter plate reader (Tecan).

2.2.8 Statistical methods

Statistical analysis were carried out with SigmaPlot 12. Results are presented as mean $\pm$ standard deviation (SD). To enhance the comparability of the assays results were normalized to the average value of the solvent controls (SC) and are expressed as percent change or fold change relative to the SC. Prior to conducting statistical analyses, all data were checked for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene's test. A one way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used to determine treatments that differed significantly from the SC for data fulfilling the parametric assumptions.. Otherwise, the non-parametric Kruskal-Wallis test followed by Dunn's post hoc test was used. For detection of significant differences in cytotoxicity assays, the *t*-test following square root transformation was performed. Differences were considered significant at $p < 0.05$.

2.3 Results

2.3.1 Cytotoxicity

a) Neutral red retention assay

An NR₈₀-value (concentrations resulting in 80 % viability of the RTL-W1 cells) of 2.1 mg/L was obtained for the biocide TCC (Figure 6). The exposure of cells to MWCNT at concentrations ranging between 0.78 and 50 mg/L, and to the mixture of CNT and TCC

(0.39-25 mg CNT/L +1 % TCC; percentage relative to CNT concentration) did not result in cytotoxicity.

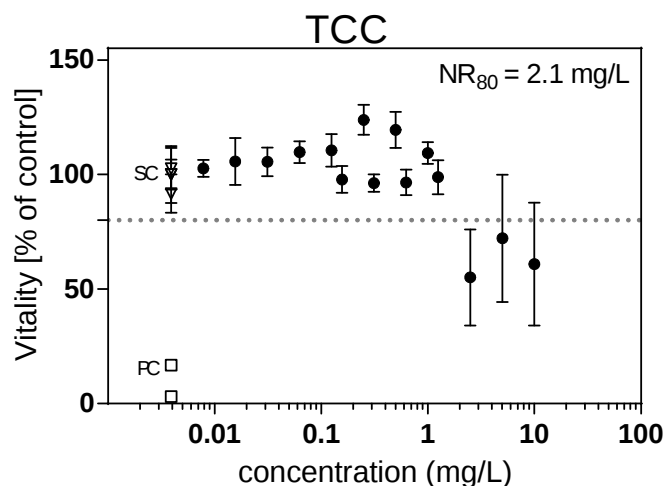


Figure 6. Cytotoxicity of TCC assessed in the Neutral Red retention assay with RTL-W1 cells. Dots represent the mean of three independent exposure experiments with three internal replicates, and are given in % of the viability of the control. The whiskers show the standard deviation of the mean; PC: positive control (3,5-dichlorophenol); SC: solvent control (EtOH); the dashed line marks the threshold of 80 %.

Concentrations of TCC in the subsequently ROS assay were kept below 0.5 mg/L, i.e., below the NR_{80} -value of 2.1 mg/L.

b) MTT assay

Additionally to the testing of RTL-W1 cells, cytotoxicity was assessed for T47Dluc cells and H295R cells in the MTT assay.

All concentrations of MWCNT (0.5-50 mg/L), TCC (31.25-500 μ g/L) and mixtures of both (1.56 mg CNT/L+15.6 μ g TCC/L - 25 mg CNT/L+250 μ g TCC/L, i.e. CNT + 1 % TCC) did not result in cytotoxicity in T47Dluc cells (data not shown).

Results of the MTT cell viability assay with H295R cells are presented in Figure 7. The percentage of viable cells relative to the ethanol (EtOH) control is plotted against the respective sample concentration.

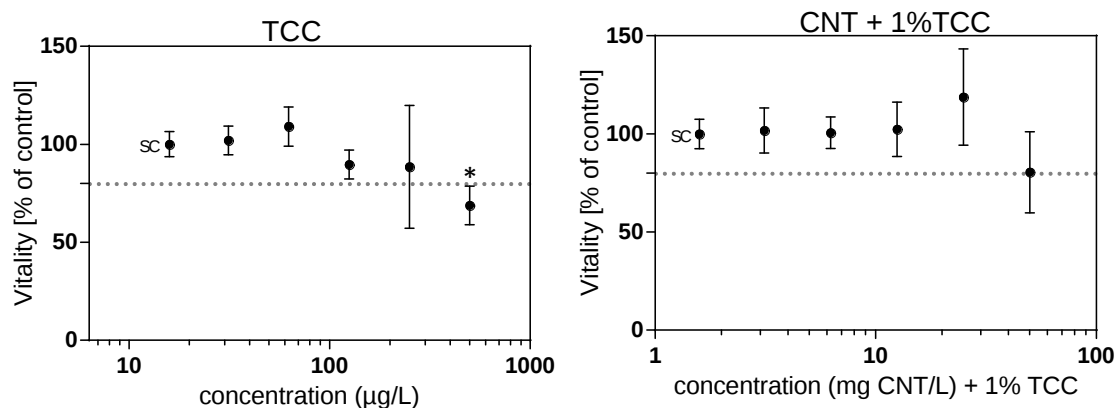


Figure 7. Cytotoxicity of TCC and a mixture of CNT with 1 % TCC (percentage relative to CNT concentration) as assessed in the MTT cell viability assay with H295R cells. Percent of viable cells after 48 h of exposure are given compared to the solvent control. Dots represent the mean of four independent exposure experiments with three internal replicates each. Error bars: standard deviation; SC: solvent control. The dashed line marks the threshold of 80 %.

The highest concentration of TCC (500 µg/L) revealed cytotoxicity after the exposure to H295R cells. In combination with CNT, lower cytotoxicity of the biocide was observed although the same concentration of TCC was applied to the cells (Figure 7). The lower cytotoxicity of the mixture testing was not significantly different from the exposure to TCC alone. MWCNT treated cells showed no cytotoxicity after exposure to concentrations between 3.13 and 50 mg CNT/L (data not shown).

2.3.2 ER Calux assay

Estrogenic activities were determined in CNT suspensions, TCC dilutions and mixture of both substances using the ER Calux assay. Figure 8 shows that CNT had no estrogenic effect in the range of 3.13 to 50 mg CNT/L. Interestingly, a decrease of luciferase activity by high concentrations of the biocide TCC can be seen in Figure 8 on the right side. Cytotoxicity could be excluded for the concentrations used as shown in the MTT assay with T47Dluc cells. The anti-estrogenic potential of TCC was reduced when cells were exposed to the mixture of CNT and 0.5 % TCC. This effect was not observed after application of CNT including 1 %TCC.

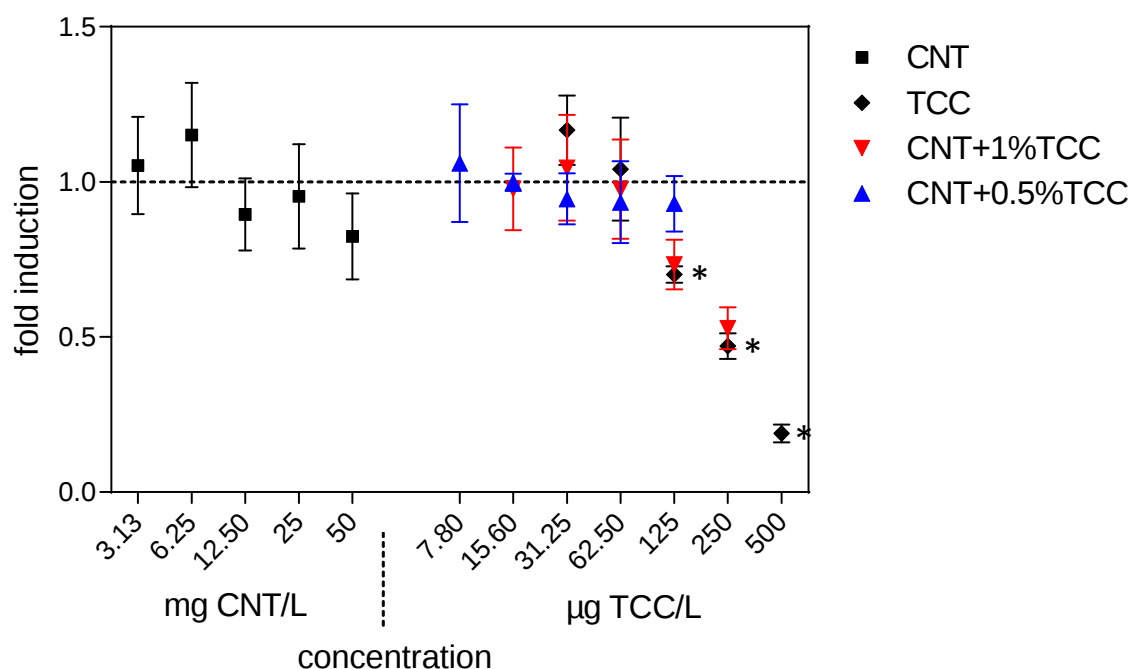


Figure 8. Estrogenic activity given as luciferase induction relative to solvent control in the ER Calux assay plated in 96 well plates. T47Dluc cells were treated with CNT, TCC and mixture of both (**CNT+1% TCC:** 1.56 mg CNT/L+15.60 µg TCC/L - 25 mg CNT/L+250 µg TCC/L; **CNT+0.5% TCC:** 1.56 mg CNT/L+7.80 µg TCC/L - 25 mg CNT/L+125 µg TCC/L). Dots represent means of two independent exposure experiments with three internal replicates each. Error bars: standard deviation; *: Statistically significant from the EtOH control in Repeated Measures ANOVA on Ranks with Dunn's post-hoc and $p < 0.05$.

2.3.3 Alterations of steroid synthesis in H295R cells

CNT did not have a pronounced effect on hormone production of 17β -estradiol (E2) in H295R cells. E2 levels were all in the range of the negative control. Also after exposure to TCC concentrations, the hormones were at the level of the EtOH control. Mixture of CNT and TCC did not significantly alter production of E2 in H295R cells in the range of 1.56 mg CNT/L+15.6 µg TCC/L to 25 mg CNT/L+250 µg TCC/L.

2.3.4 Measurement of cellular ROS

Effects of MWCNT and TCC on radical formation were assessed by measuring intracellular ROS in RTL-W1, T47Dluc, and H295R cells. Compared to the EtOH control, no significant difference in the ROS generation by TCC, and the combination of MWCNT and TCC in all three cell lines was observed. In MWCNT treated cells, however, a much higher ROS

production than in the controls was measured. The ROS content was 1.8, 2.9, and 4.7 times higher compared to the control levels in RTL-W1 cells, 1.5, 1.9, and 3.2 times higher than in T47Dluc cells, and 1.2, 1.4, and 2.2 times higher than in H295R cells following incubation with CNT at 12.50 mg/L, 25 mg/L, and 50 mg/L, respectively (Figure 9). The lowest observed effect concentration (LOEC) was 12.50 mg/L for RTL-W1 and T47Dluc cells, with a no observed effect concentration (NOEC) of 6.25 mg/L. For H295R cells higher LOEC and NOEC were determined amounting to 25 and 12.5 mg CNT/L, respectively.

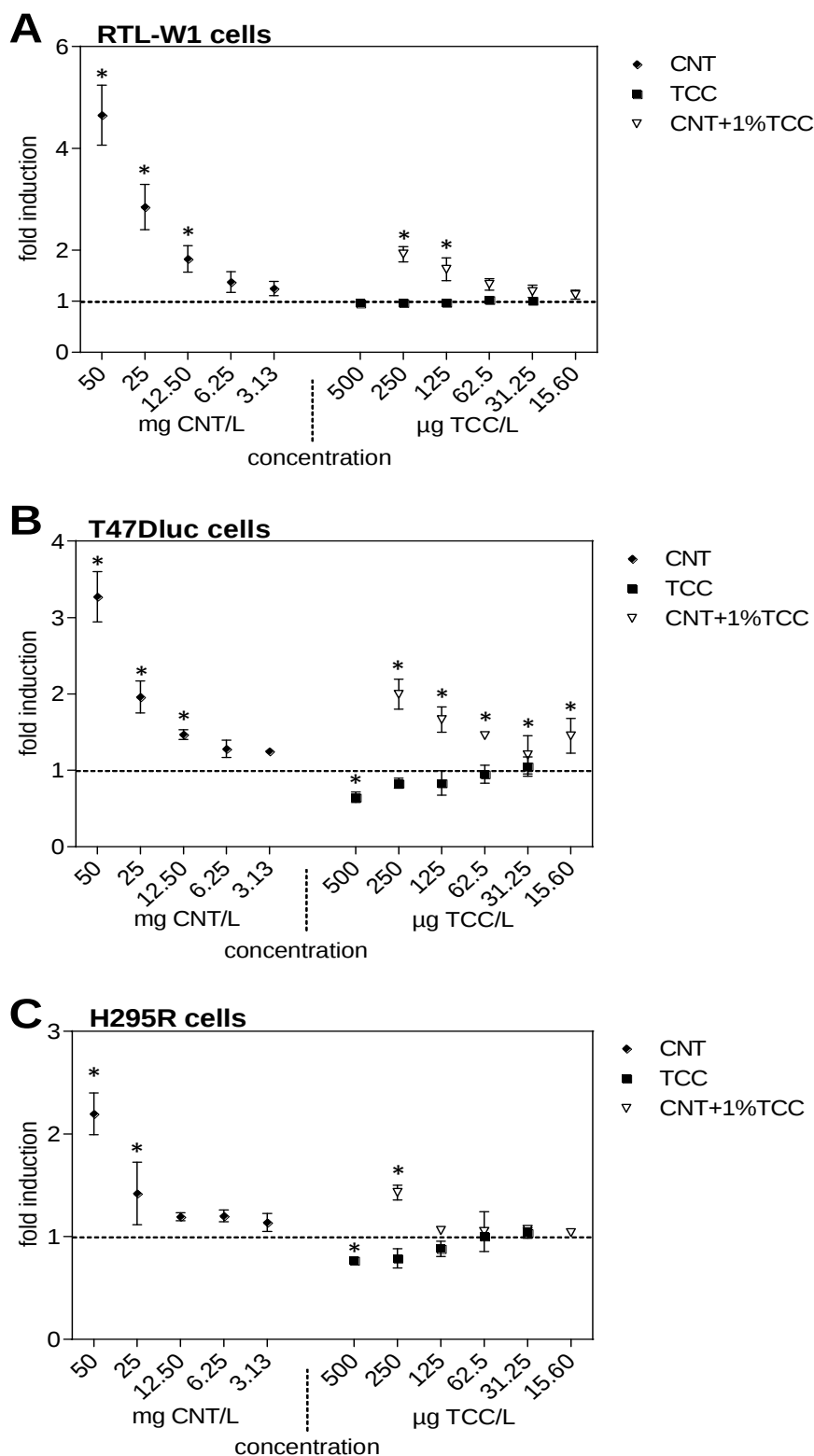


Figure 9. ROS generated in RTL-W1 (A), T47Dluc (B) and H295R (C) cells exposed to MWCNT, TCC and mixture of both substances (1% TCC: with respect to the concentration of CNT). The intensity of H₂DCF-DA was measured in cell lysates, and normalized to negative/solvent control (= 1, dashed line). Data are expressed as mean $\pm$ standard deviation of 3 independent exposure experiments with three internal replicates each. *: Statistically significant from the negative control in Repeated Measures ANOVA on Ranks with Dunn's post-hoc and $p < 0.05$.

2.4 Discussion

2.4.1 Multiwalled carbon nanotubes

In the case of long and stiff CNT, it has been argued that analogous mechanisms to those of other fibrous particles such as asbestos exist^[113,114], which may penetrate the lung and persist in the tissue. The bio-persistence, large aspect-ratio and fibrogenic character of CNT are important features that may cause adverse health effects. Other mechanisms include hydrophobic contact, through which nanoparticles may interrupt cell membranes, disturbing surface protein receptors^[115]. Uptake of nanofibers by human macrophages sized smaller than the length of the nanotubes - a process defined as frustrated phagocytosis - has been shown by backscatter scanning electron microscopy^[15]. Overall, nanomaterial size and composition plays a distinct role in the cellular response. In addition, this response is variable between cell types and is likely related to the physiological function of the cell types^[112].

However, in our study flexible multiwalled CNT were investigated for which less concern of their toxic potential has been expressed^[116].

Cytotoxicity

Exposure of RTL-W1, T47Dluc and H295R cells to 50 mg CNT/L for 24 h or 48 h did not induce acute cell toxicity. Several authors have shown that CNT were cytotoxic to different lung epithelial cell lines^[117-121], to human astrocyte D384 cells^[117], to skin keratinocyte cells, lung cells, T4 lymphocytes^[122], and human epidermal keratinocytes^[21]. However, in a recent study, Thurnherr et al.^[10] also showed that the same type of industrially produced MWCNT had no effect to another cell line. Contradiction to different effects observed in this study and in many other publications might be explained by differences in the CNT-material used (metal contaminants, structural defects, size, stiffness, MWCNT vs. SWCNT) and by cell-line dependency^[10,109]. More likely, positive results are often only due to very high concentrations, which already elicit cytotoxic responses^[123,124] or might interfere with the test systems used^[125]. The hydrophobic nature of CNT is a general problem when working with these

materials not only concerning the generation of stable suspensions that can be applied to the cultures but also for potential interference with the assay due to their high propensity to stick to various molecules or cells^[126,127]. For this reason, we used no detergents to prevent MWCNT aggregation during the experiments. The exclusion of such interference with the test systems as well as thorough material characterization is therefore a prerequisite for each study to allow the comparison of results obtained from different researchers^[128].

ROS generation

Main effects of CNT seem to be due to oxidative stress, which triggers inflammation via the activation of oxidative stress-responsive transcription factors^[129].

The highest intracellular ROS production could be observed in MWCNT treated RTL-W1 cells, which was up to 5 times higher than control levels. A LOEC of 12.5 mg CNT/L was determined. They were followed by MWCNT treated T47Dluc cells, in which up to three times more ROS was produced compared to the control. The lowest generation of ROS was observed in H295R cells with up to 2 times higher ROS levels compared to the control level with a LOEC of 25 mg/L.

ROS production can be partially inhibited by metal chelators, indicating that metal components (nickel, iron, yttrium) of CNT are able to contribute to the oxidant response observed^[124]. CNT can contain relatively high concentrations of metals as impurities (e.g. 30 %), which can contribute to their toxicity. In contrast, purified carbon nanotubes with no bioavailable metals were shown to decrease local oxidative stress development^[130], suggesting that similar to fullerenes, ROS may be “grafted” at the surface of CNT via radical addition due to their high electron affinity^[129]. Barillet and coworkers came also to the conclusion that CNT induced the same level of ROS whatever their length and purity was^[109]. They suggested that intracellular ROS production induced by CNT exposure refers to more complex mechanisms than simple redox reactions if we consider the fact that CNT are less accumulated than metal oxide nanoparticles^[109].

Ye et al.^[121] suggested that ROS and the activation of the redox-sensitive transcription factor NF-kappaB were involved in upregulation of interleukin-8 in A549 cells exposed to MWCNT. Yang et al.^[131] found CNT induced significant glutathione depletion, malondialdehyde increase and ROS generation in a dose-dependent manner. Pulskamp et al.^[63] failed to observe any acute toxicity using the WST-1 assay in cultured rat NR8383 macrophages and A549 cells on viability and inflammation upon incubation with CNT. But they indicated a dose-dependent decrease of the mitochondrial enzyme activity (MTT assay) after 24 h of exposure, similar to results seen before in other published studies^[18,19,132], and detected a dose- and time-dependent increase of intracellular ROS^[133]. ROS induction was also observed by exposure to carbon black^[134]. Some doubt on the evaluation of MTT toxicity assays were expressed by Worle-Knirsch et al.^[135] because they demonstrated that MTT-formazan interacts with CNT interfering with the basic principle of the assay. The authors strongly suggest verifying cytotoxicity data with an independent test system as we did by using different test systems.

A key finding in our study was that ROS generation in three cell lines (RTL-W1, T47Dluc and H295R) went up in 45 minutes even in a low dose of incubation group (3.13 mg/L), which was 1.2 times higher than in the controls. Chen et al.^[133] assumed that ROS generation came out much earlier than other phenotypes including oxidative stress and cytotoxicity. This might be the reason why other studies in which ROS was measured after more than 4 h exposure to CNT showed inconsistent results^[63,136-139]. Several studies^[131,140] concluded that cytotoxicity can be attributed to oxidative stress. Interestingly, no cytotoxic effect was found in this study in three different MWCNT cells, although generation of ROS was observed in all cell lines used.

Similar experiments to determine the ROS generation in RTL-W1 cells were performed using multilayer graphene flakes (synthesized by thermal reduction of graphitic oxide at the Federal Institute for Materials and Research and Testing BAM, Berlin) as non-nanomaterial (data not shown). Thereby same increases of ROS generation were observed up to concentrations of

12.5 mg/L. Whereas, 1.5 times lower increases could be observed for both 25 mg/L and 50 mg/L compared to the MWCNT treatment. This lead us to the conclusion that the impurities of metal catalysts (cobalt) are not responsible for the increased production of ROS and such effects may be due to the nanostructure of this materials. Our findings are in accordance with other studies where intracellular ROS generation could be determined by using pristine graphene treated murine RAW 264.7 macrophages^[141], few-layer graphene (3-5 layers) treated PC12 cells^[142], and graphene oxide treated human lung epithelial cells^[143] in a time- and dose-dependent manner. However, Creighton et al.^[144] showed that graphene-based materials have significant potential to interfere with in vitro toxicity testing methods, such as the H₂DCF-DA assay, through optical and adsorptive effects at toxicologically relevant doses (less than 10-100 mg/L). They could also show that the removal of the nanomaterial by washing can remove optical interferences. Depending on the graphene material the washing step can lead to accurate data (e.g. for graphene oxide) or to underreporting of ROS as few-layer graphene (3-5 layers) adsorbs and quenches the H₂DCF-DA dye in a manner that depends on surface area^[144]. Optical interferences can be excluded for the present study because the cell lines were washed accurately with PBS, but the adsorptive effect is still unclear and may lead to underestimate the production of ROS generation.

2.4.2 Triclocarban

Cytotoxicity

There is very limited information concerning the cytotoxic actions of TCC in mammalian cells, although these actions have been examined, to some extent, in aquatic and terrestrial organisms^[145-147]. Morita et al.^[146] showed no cell lethality after the incubation of rat thymocytes with TCC at concentrations ranging from 30 nM to 500 nM for 1 h. The incubation with TCC at concentrations ranging from 10 nM to 1 μ M for 1 h did not affect the viability of rat thymocytes^[148]. Another study by Kanbara et al.^[149] showed an increase in cell lethality when rat thymocytes were incubated with 10 μ M TCC. In the present study, a

cytotoxic effect to treated RTL-W1 cells was already observed at concentrations above 4 μM TCC. Both human cell lines (T47Dluc, H295R) showed no cell lethality when exposed up to 1.6 μM TCC. These results are in agreement with the open literature^[148,149].

Estrogenic activity

As shown in Figure 8, a decrease of luciferase activity in the ER Calux assay was determined after exposure to high TCC concentrations (1.6 μM). Down-regulation of estrogen receptors (ER) or other mechanisms of negative feedback may cause this decrease^[150]. TCC did not significantly alter the production of E2 in H295R cells up to a concentration of 1.6 μM determined in the ELISA assay.

Ahn et al.^[67] observed weak ER activity of TCC at concentrations of 1 and 10 μM . They also found that in presence of estrogen or testosterone (T), TCC enhanced the actions of these hormones. A cell-based androgen receptor-mediated bioassay with TCC was investigated by Chen et al.^[83]. Neither cytotoxicity nor the competition between TCC and testosterone for binding sites could be observed in their studies. However, TCC did amplify testosterone induced transcriptional activity both in a time and dose dependent manner^[83]. Altogether, the results suggest that the effects seen with TCC in luciferase-based transactivation assays are due to interference with firefly luciferase, rather than due to causing of the ER α or the androgen receptor (AR)^[151]. Similar false positives have been reported in previous high-throughput screens^[152]. A recent screen of the NIH Molecular Libraries Small Molecule Repository identified 12 % of the 360,864 molecules to be inhibitors of firefly luciferase^[153]. In some cases inhibition paradoxically resulted in an increase of the luminescence signal, probably because of enzyme stabilization^[154]. Such a mode of action is also supported by the PubChem Bioassay Database (<http://pubchem.ncbi.nlm.nih.gov>), which quotes a preliminary EC₅₀ value of 8.9 μM TCC for the inhibition of luciferase.

The focus of the present study was to get more information about the biocide in cell based assays as well as about interactions of TCC and MWCNT. Our results on the activity of TCC in the ER-responsive cells provide an explanation for the mechanism how chemicals

enhance the endocrine-disrupting activity of chemicals^[67]. Chemicals acting as endocrine-disrupting compounds (EDC) affect the ER receptor and lead to activation/inhibition of hormone-dependent gene expression^[67]. However, EDC may also alter hormone receptor function simply by changing phosphorylation of the receptor (activating him) without the responsible chemical or natural ligand ever binding to the receptor^[155].

Clearly, further examinations are required especially the confirmation of our findings *in vivo*.

Triclocarban at concentrations up to 1.6 μ M showed no generation of ROS in three cell lines. Two similar studies suggested the production of reactive oxygen species in rat thymocytes after an incubation time of 1 h to 300 nM or higher concentrations of TCC^[146,149]. On the contrary, Fukunaga and coworkers^[148] supposed that the same cells recovered the initial loss of cellular glutathione as a biomarker of oxidative stress in the continued presence of 300 nM TCC. Thus, the ability of TCC to generate ROS in human cell lines is still under discussion and further research is required.

2.4.3 Interaction of MWCNT and TCC

Most reported studies have illustrated that the CNT surface area is an adsorbent for organic chemicals, such as polycyclic aromatic hydrocarbons, phenolic compounds, and endocrine disrupting chemicals^[34,156,157]. In the present study, we determined lower cell toxicity in MWCNT and TCC treated H295R cells compared to the cytotoxic potential of TCC alone. Even the anti-estrogenic potential of TCC in the ER Calux assay with T47Dluc cells was reduced in the presence of MWCNT compared to the absence of the nanotubes in the whole experimental design. The antimicrobial agent TCC seems to interact with MWCNT resulting in a lower available concentration of TCC in the test medium. This could be proven in the ER Calux assay (Figure 3). Treatment of the cells with higher levels of CNT combined with a lower TCC concentration (0.5 % of the nanotubes) did not result in a decrease of luciferase activity compared to same concentrations of the antimicrobial biocide and the mixture of MWCNT and TCC (concentration 1 % of that of CNT).

Only few studies have been conducted to understand the adsorption of organic contaminants by CNT^[30-32,34,158-163]. A common observation from these studies was that CNT are very strong adsorbents for hydrophobic organic compounds. Possible adsorption mechanisms are the hydrophobic interactions between TCC and CNT or non-covalent π - π electron-donor-acceptor (EDA) interactions^[164]. With a log K_{OW} of 4.9 for TCC^[73] and considering the strong hydrophobicity and high surface area of carbon nanotubes^[165], the hydrophobic effect might be the dominant factor for the adsorption of TCC on the MWCNT. Chen et al.^[165] reported that the strong adsorption of polar nitroaromatics, compared to apolar compounds, was due to π - π EDA interactions between the nitroaromatics (π acceptor) and the graphene sheets (π donors) of CNT. An important implication from several of the studies is that electronic polarizability of the aromatic rings on the surface of CNT might considerably enhance adsorption of the organic compounds^[30,158,160,163]. As concluded by Chen and coworkers^[165], no studies have been conducted to systematically compare adsorptive interactions between carbon nanotubes and organic compounds with significantly different physical-chemical properties (e.g., polarity, functional groups, etc.). In addition, engineered carbon nanomaterials can vary significantly in shape, size and morphology, and impurity, e.g., metal, amorphous carbon and O-containing groups, which can further complicate the adsorptive properties of these materials for organic contaminants^[165].

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Chapter 3

Long-term exposure of multiwalled carbon nanotubes: Population test with *Daphnia magna* over 93 days and additional short-term exposure to triclocarban

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3.1 Introduction

Carbon nanotubes (CNT) are currently synthesized and produced on a substantial scale worldwide^[1]. Because of their desirable combination of physical and chemical properties, multiwalled carbon nanotubes (MWCNT) are being produced at increasing rates and are among the most widely used types of engineered nanomaterial^[2,3]. MWCNT are thin and long hollow fiber-like nanomaterials composed of multiple layers of rolled graphene^[4]. With an increasing amount of industrial applications for carbon nanomaterials, such as MWCNT, large quantities of nanoparticles (NP) may reach the environment intentionally or accidentally^[5,6]. Thus, proactive research on the potential environmental and health impacts of NP is crucial to protect the environment and to ensure a sustainable nanotechnology industry^[7-9]. Results of previous toxicology studies indicate that major physical and chemical properties, such as nanoparticle type, crystalline phase, size, shape, surface, functional groups and surface charge, could control the toxicity of nanomaterials^[10,11].

The manufacturing methods, pre-treatment processes, surface coating and preparation of nanoparticles suspension may alter the morphology, chemical composition and surface chemistry of the nanomaterials^[12]. Thus the responses of aquatic organisms to NP might vary^[13]. Moreover, any change and the presence of surface functional groups on the engineered nanoparticles (ENP) might affect the interactions between ENP and organisms^[10,13-19]. Thus far, no clear guidelines have been established to assess the ecotoxicological and environmental effects of direct and indirect exposure to NP^[20].

Environmental contamination by personal care products (PCP) has recently gained widespread public attention as a pervasive problem^[21]. PCP are important and indispensable elements of modern life^[22,23]. To date, more than 70 different pharmaceuticals and PCP such as prescription and over the counter drugs have been detected in surface water and ground water in the United States^[24-29]. However, information on their transport and fate is still limited. The maximum acceptable concentrations of PCP in the environment are still

undefined due to the lack of knowledge regarding the long-term effects of continuous exposure to these compounds and their metabolites^[23]. Triclocarban (TCC, 3,4,4'-trichlorocarbanilide) has been used as a PCP since 1957^[30] and is a high-production-volume chemical^[31] that is widely used as an antimicrobial compound^[32,33]. It has been shown to be highly hydrophobic ($\log K_{OW} = 4.9$), to have a low solubility in water ($S_W = 0.65 \text{ mg/L}$)^[34], and to be relatively persistent in soils and aquatic sediments^[30,35,36]. Due to their hydrophobic nature, TCC is likely to sorb to sediments with a high organic carbon content^[35,37].

Only few studies dealt with the toxic potential of triclocarban, but algae growth inhibiting effects^[38,39], amplification effects of endocrine disrupters in *D. rerio*^[31] and acute toxicity to *D. magna* were observed^[38]. A study of Tamura et al.^[38] concerned chronic and acute toxic effects of triclocarban to algae, daphnids and fish. Acute tests resulted in an EC_{50} -value of $29 \text{ } \mu\text{g TCC/L}$ for the green algae *Pseudokirchneriella subcapitata*, an EC_{50} -value of $10 \text{ } \mu\text{g TCC/L}$ for *D. magna* and an EC_{50} -value of $85 \text{ } \mu\text{g TCC/L}$ for the test fish *Oryzias latipes*. Those results are in this respect alarming as in surface waters TCC concentrations of $6.75 \text{ } \mu\text{g TCC/L}$ were measured^[40]. Interactions with other substances, which might be of biological or synthetic origin, are still poorly investigated and cannot be predicted. Additionally, chemical contaminants can affect natural *Daphnia* populations either by direct toxin uptake or by indirect ingestion of contaminated algae^[41]. Therefore, research concerning TCC and its interactions with other substances must be of high priority to conduct a meaningful risk assessment.

The filter feeder *Daphnia magna* represents an important standard test organism in toxicity testing because of its ease of handling, short generation time, and high sensitivity towards various chemicals^[42-45]. The most likely route of uptake of nanoparticles by *Daphnia magna* is through ingestion, including active selection by the feeding apparatus^[46,47], as well as passive diffusion or uptake alongside larger particles^[48,49]. The maximum diameter of particles that can be ingested is determined by the size of the animal^[50]. For a fully grown *D. magna*, the largest ingestible particles can be approximately $70 \text{ } \mu\text{m}$. The appearance of ingested matter

in the gut is reported to be very rapid after feeding. For example, under optimal conditions, the gut has been reported to fill within 30 min of exposure to a food source^[51]. Several studies dealt with the effects of different substances on individual daphnids, but very little is available concerning the effects at the population level.

Today it is recognized that ecological risk assessment can markedly improve its biological relevance by considering responses to contaminant exposure at the population level rather than at the organism level^[52]. From this prospective, population models are particularly helpful tools that allow assessment of multiple toxic effects observed in laboratory experiments. Organism survival and fecundity can be combined into one population-level endpoint, such as the asymptotic population growth rate. Priority has recently been given to the use of matrix population models^[53] for their prospective potential in modeling population health, including the effects of toxic compounds on the different age or development stages of organisms within populations^[54-57].

In the present study, the influence of MWCNT and/or TCC on population level was investigated. Therefore, different treatments were used to detect differences in MWCNT treated populations as of day 0 or day 14 compared to the control. Furthermore, three short-term exposures with different concentrations of TCC were performed to help address the continued need to better understand the ecotoxicological relevance of this antimicrobial agent. Finally, a model approach was carried out to compare the laboratory data set for TCC with the prediction of an individual-based population model for *Daphnia magna*, named IDamP^[58], linked to a toxicokinetic-toxicodynamic model (TK/TD model) simulate the time-course of toxic effects on survival of the individuals.

3.2 Material and methods

3.2.1 Chemicals

The test substance 3,4,4'-trichlorocarbanilide was purchased from Sigma Aldrich with a purity of 99 % (CAS:101-20-2). Multiwalled carbon nanotubes (Baytubes C150P, >95 % purity) were provided from Bayer MaterialScience (Bayer AG, Leverkusen).

3.2.2 Nanoparticles suspension

Test suspensions of 1 mg CNT/L were prepared by ultrasonication with a microtip (70 W, 0.2'' pulse and 0.8'' pause; Bandelin) for 10 minutes. Transmission electron microscopy (TEM) images showed the presence of small agglomerates and individual nanotubes in the medium (data not shown).

3.2.3 Test organisms

Daphnia magna Straus (Clone 5) were taken from the culture of the Institute for Environmental Research at the RWTH Aachen University, originally obtained from stock cultures at the University of Sheffield^[59]. Daphnids were cultured individually in M4 medium^[60] at a constant temperature of $20 \pm 1^\circ\text{C}$, and a photoperiod of 16 h light and 8 h dark. M4 medium was renewed once a week and daphnids were fed with the green algae *Desmodesmus subspicatus* three times a week (0.2 mg C / daphnid per feeding).

3.2.4 *Daphnia* immobilization test

Daphnia magna were used for the immobilization test conducted in conformity to the OECD test guideline for the testing of chemicals^[60] with slight modifications. In brief, four replicates of five neonates (20 mL), four replicates of five juveniles (50 mL), and five replicates of four adults (80 mL) were prepared in glass beakers for at least four concentrations of TCC (between 15 and 60 $\mu\text{g TCC/L}$) and the solvent control (acetone) at $20 \pm 1^\circ\text{C}$ and a 16/8 h light/dark photoperiod. The static system was used for the test. Dissolved oxygen and pH of the solution were monitored at the beginning and the end of the test (96 h). Concentrations of

the test substance TCC were analyzed using a liquid chromatography-tandem mass spectrometry (LC/MS-MS). The median effect concentration (EC_{50}) was determined based on the measured concentration using ToxRat[®] Professional.

3.2.5 Population experiment

The population test was carried out as a batch approach for 93 d at $20 \pm 1^\circ\text{C}$ and with a 16/8 h light/dark photoperiod, as previously described^[61]. Populations were initiated with five neonate (age < 24 h), one juvenile (2.5 weeks old) and two adult (5 weeks old) daphnids, and kept in 800 mL M4 medium. The daphnids were daily fed with 0.5 mg total organic carbon of the green algae *D. subspicatus* per population. Three replicates were provided for each treatment. The medium renewal took place every Monday, Wednesday, and Friday. At the same time, a picture of each population was taken using an incident light scanner. Considering the medium preparation (ultra-sonication with microtip), the vitamins were added after the scanning process. The experimental setup consisted of 6 treatments:

- a) control (ultrasonicated M4 medium without vitamins)
- b) solvent control (acetone)
- c) 1 mg CNT/L from the beginning ("CNTd0")
- d) 1 mg CNT/L as of day 14 ("CNTd14")
- e) treatment with 25 μg TCC/L, 41 μg TCC/L, and 61 μg TCC/L on day 14, 54, and 68, respectively ("TCC")
- f) 1 mg CNT/L from the beginning and treatment with 25 μg TCC/L, 41 μg TCC/L, and 61 μg TCC/L on day 14, 54, and 68, respectively ("CNT+TCC")

The exposure to MWCNT lasted the complete experimental duration and was renewed during the change of media with freshly sonicated suspensions. The three short-term exposures with TCC to higher concentrations over time remained for 2 days in the treatments each. After this time, the exposure medium was replaced by M4 medium or MWCNT suspension. The TCC exposure gave an acetone volume of 16.25 μL /L for each spiking. This concentration was also used to spike the solvent control. TCC was rapidly metabolized in

appearance of algae (unpublished recent results from the laboratory of the Institute of Environmental Research, RWTH Aachen University), and therefore, it was expected that TCC mostly disappeared within the renewal interval of the test medium. Concentrations of the tested TCC were analyzed using LC/MS-MS.

3.2.6 Carapax length measurement

As described by Agatz et. al.^[59], scans of each population were made three times a week. The whole population was transferred to a Petri dish and fixed by removing the media until a minimal movement of the organisms was observed. The Petri dish was then scanned using an incident light scanner (Canon, CanoScan 8800F) at a resolution of 1200 dpi. The images were analyzed with purposely designed software (T.G. Preuss, RWTH Aachen University, unpublished data), which counted the number of daphnids and measured the length of each individual. The carapax length is defined as the distance from the top of the eye to the base of the tail spine. The daphnids were classified into three groups by body size: Size class 1, <0.9 mm (neonate); size class 2, ≥ 0.9 mm and ≤ 2.6 mm (juvenile); and size class 3, > 2.6 mm (adult).

3.2.7 Endpoints

The endpoints are listed in Table 1 and defined as follows.

(i) Maximal abundance: the day where the average abundance of a population reached the maximum. (ii) Equilibrium: the first day beyond the day of maximum abundance where the number of organisms reached the defined equilibrium of each treatment. (iii) Abundance in equilibrium and subdivision per size class: the average of the abundances between day 21 and day 93. (iv) Variance per size class: the average of the variances over time given in percent; the variance of each sampling time was determined by dividing the standard deviation of the population abundance per size class by its average. (v) Overall minimal and maximal body length: the average of the smallest and biggest daphnids measured at each time point. (vi) Factor juveniles to adults of each sampling time was determined by dividing the average number of neonates and juveniles by the number of adults.

3.2.8 Modeling approach

The modeling approach was performed using a multimodel approach IDamP as an individual-based population model for *Daphnia magna*^[58] and the general unified threshold model of survival (GUTS)^[62] to simulate the effects of TCC. GUTS unifies existing TK/TD models of survival that can be derived from two main specific assumptions, stochastic death (SD) or individual tolerance (IT), and different dose metrics. Due to the lack of toxicokinetic data to estimate internal TCC concentrations the scaled internal concentration was selected as dose metric. The calibrations were performed by maximizing the likelihood function^[62] using the Simplex-Algorithm^[63]. Calibrations were run with random start values and those with the best likelihood were selected for parameter estimation. To generate 95 % confidence intervals of survival rates over time, the concept of profiling the likelihood function^[64] was applied.

First, GUTS were calibrated for the toxicity effects of TCC on survival. The resulting parameters are listed in Table 1. As described by Gabsi et al.^[65], within the *Daphnia* population model IDamP, predictions of the population dynamics of *D. magna* based on individual life cycles, including the feeding on algae, developing, growing, aging, reproducing and surviving were given. The key responsibilities of these processes are the food terms and the density of the population (via crowding effects). The model is applicable at a laboratory scale by feeding the daphnids with *D. subspicatus* only. Predictions of the full model regarding population size and structure were successfully validated against population tests with different food supplies including hunger, crowding conditions, different feeding scenarios (flow-through or semi-static) or initial population sizes and structures^[58]. Additionally, the potential of IDamP to extrapolate toxicity effects was demonstrated for 3,4-dichloroaniline^[66], nonylphenol^[67], and dispersogen A^[65]. A full model description following the ODD protocol (Overview, Design concepts, Details)^[68] was made available in Preuss et al.^[58].

Table 1. Parameters, given at means with the lower and upper 95 % confidence interval within parentheses, used from the GUTS model to calibrate the toxic potential of TCC within the model IDamP.

Parameters	Mean
Killing rate	0.01 (0.008-0.013) L/(mol·d)
Elimination rate constant	0.0036 (0.003-0.004) /d
Background hazard rate	0 (0-0) /d
Threshold for effects	0.288 ((-1)-0.650) mol/L

3.2.9 Statistical analysis

The statistical analyses were conducted using SigmaPlot 12. First of all, normal distribution and variance homogeneity were tested by performing Shapiro–Wilk test and Levene's test. Afterwards, a One Way Repeated Measures Analysis of Variance on Ranks following the Holm–Sidak method or Dunn's method was performed for comparison of the endpoints given in Table 3. Endpoints tested were the total population abundance, the population structure subdivided into each size class within equilibrium or per sampling day, the minimal and maximal body length of individuals within the population, and the factor juveniles to adults per sampling day. Treatments were always tested against the solvent control population.

3.3 Results

3.3.1 *Daphnia* immobilization test

The results of the acute toxicity test indicate an size- or age-dependent mortality, whereby juvenile daphnids (2.5 weeks old) were the less sensitive individuals, followed by adult daphnids (5 week old), and neonate daphnids (age < 24h) were the most sensitive individuals (Table 2). 96 h EC₅₀-values ranged from 13 (Neonates), to 26 (Adults), and up to 33 (Juveniles) µg TCC/L.

Table 2. 96 h EC₅₀-values of the *Daphnia* immobilization test per age class.

Age class	96 h EC ₅₀ (µg/L)
Neonates	12.65 (n.d.)
Juveniles	33.16 (31.00-35.32)
Adults	25.71 (n.d.)

n.d.: not determined

3.3.2 Development of the Population

Within the experiment, numbers of mainly small individuals initially overshoot (growth phase), subsequently were reduced (phase of negative population growth), and then reached the final level (equilibrium phase) after a maximum of 21 days (Table 3, Figure 10). This was maintained in the control until the end of the experiment at day 93. A peak density of 157 ± 3 individuals per population could be observed at day 16 for the untreated population. Thereafter, the population decreased significantly, recording oscillating values between 70 and 105 individuals, and finally maintained its equilibrium of 83 ± 4 individuals (Figure 10).

Table 3. Average $\pm$ standard deviation of key data for a *Daphnia magna* population test with CNT and/or additional short-term exposures

Endpoints	Control	MWCNT day 0	MWCNT day 14	TCC	MWCNT and TCC
Maximal abundance	157 $\pm$ 3	140 $\pm$ 10	162 $\pm$ 25	201 $\pm$ 47	200 $\pm$ 71
Day of reaching the equilibrium	21	19	21	19	21
Abundance in equilibrium (ind)	83 $\pm$ 4	89 $\pm$ 4	85 $\pm$ 1	82 $\pm$ 9	75 $\pm$ 15
Abundance in equilibrium: neonates	22 $\pm$ 3	37 $\pm$ 3*	35 $\pm$ 1*	37 $\pm$ 7*	40 $\pm$ 14*
Abundance in equilibrium: juveniles	43 $\pm$ 4	37 $\pm$ 3*	35 $\pm$ 1*	32 $\pm$ 6*	25 $\pm$ 4*
Abundance in equilibrium: adults	19 $\pm$ 2	15 $\pm$ 2*	14 $\pm$ 1*	13 $\pm$ 2*	11 $\pm$ 1*
Variance in equilibrium: neonates (%)	28 $\pm$ 16	33 $\pm$ 17	43 $\pm$ 18	45 $\pm$ 32	60 $\pm$ 37
Variance in equilibrium: juveniles (%)	16 $\pm$ 8	18 $\pm$ 11	26 $\pm$ 16	32 $\pm$ 29	38 $\pm$ 35
Variance in equilibrium: adults (%)	17 $\pm$ 8	23 $\pm$ 11	18 $\pm$ 16	41 $\pm$ 46	44 $\pm$ 47
Minimal body length	0.87 $\pm$ 0.16	0.75 $\pm$ 0.18*	0.78 $\pm$ 0.19	n.d.	n.d.
Maximal body length	3.72 $\pm$ 0.44	3.52 $\pm$ 0.32	3.60 $\pm$ 0.49	n.d.	n.d.
Factor juveniles to adults	3 $\pm$ 1	6 $\pm$ 1*	6 $\pm$ 1*	10 $\pm$ 4*	8 $\pm$ 3*

n.d.: not determined; *: statistically significant from the control population, $p < 0.05$; ind = individual

When the population was exposed to 1 mg CNT/L during the entire time (CNTd0), the total abundance reached 126 individuals at day 14 and finally reached its equilibrium of 89 ± 4 individuals at day 19. A continuous presence of MWCNT as of day 14 (CNTd14) showed a peak density of 160 individuals at day 12 and maintained its equilibrium of 85 ± 1 individuals at day 21. MWCNT seemed not to markedly affect the population dynamics.

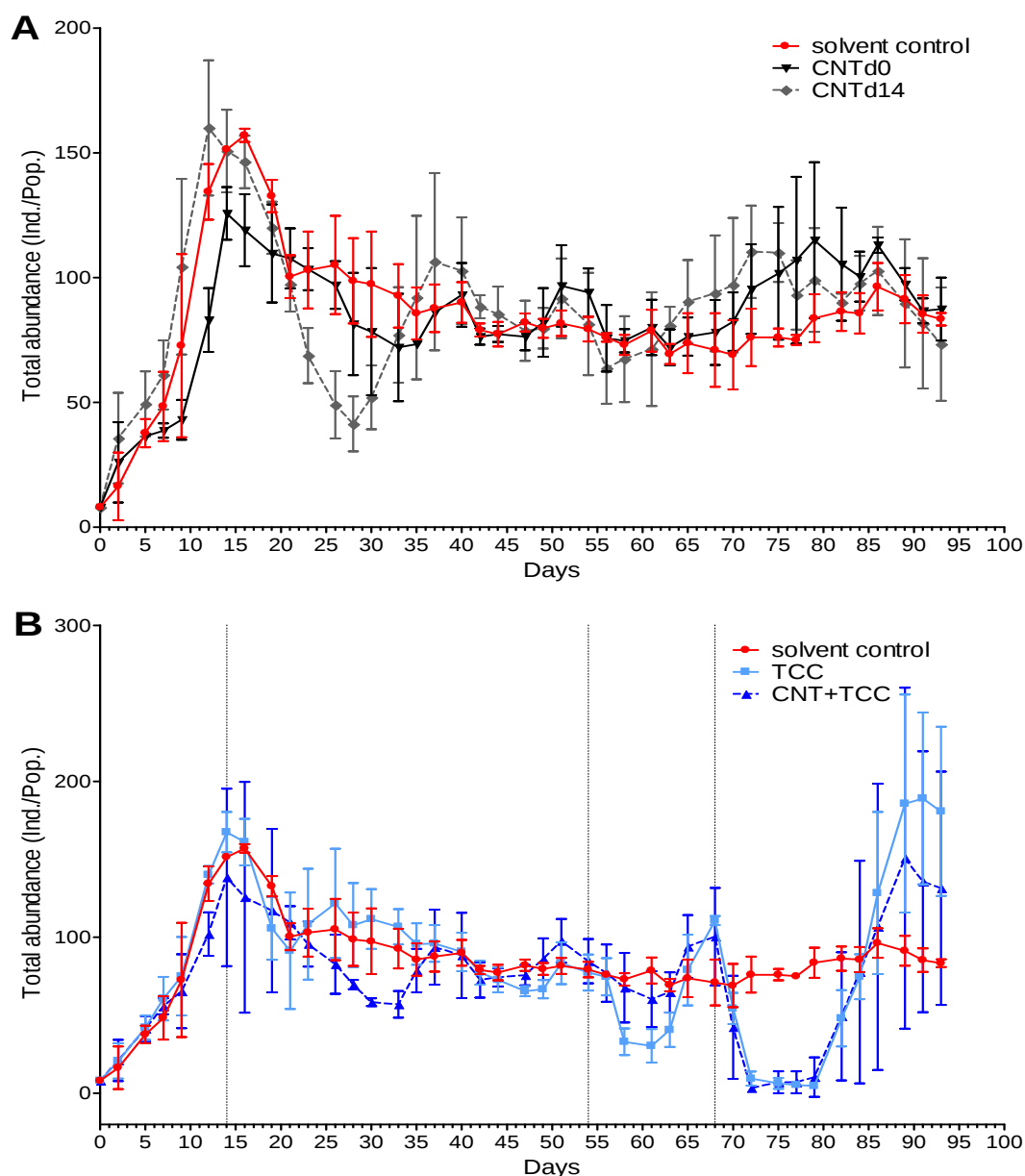


Figure 10. Total abundance of *Daphnia magna* populations over time (A) with a continuously exposure to 1 mg CNT/L and (B) three short-term exposures with triclocarban (d14: 25 μ g TCC/L; d54: 41 μ g TCC/L; d68: 61 μ g TCC/L). Dotted lines marks the day of the short-term exposure to TCC. Whiskers represent the standard deviation of three independent replicates. Ind./Pop. = individuals per population.

Looking more in detail, a higher fluctuation in abundance could be observed for each size class after treated to MWCNT compared to the control (Figure 11, A-C). Additionally, a significant increase in size class 1, and a significant decrease in size class 2 and 3 could be determined for CNTd0 as well as for CNTd14. The abundance of neonate daphnids continuously exposed to MWCNT (CNTd0) fluctuated over time with a maximum of 1.25 times higher than the control at day 21. Treatment CNTd14 first showed a decrease in the

abundance of neonates after the addition of MWCNT at day 14, which was 6 times lower compared to the control (day 26), followed by an increase resulting in a neonate abundance 4 times higher than the control at day 37 (Figure 11 A). The same fluctuations within size class 1 could be observed in CNTd0 and CNTd14 as of day 44 until the end of the exposure. The abundance of juvenile daphnids in treatment CNTd0 decreased significantly and was 9 times lower compared to the control at day 14 (Figure 11 B). The total amount of juveniles remained fewer until day 70 and reached thereafter nearly the same level as the control population. This significant increase of the abundance in size class 2 could be observed in CNTd14 14 days later than in the treatment CNTd0. This indicates the relationship between size class development and the exposure to MWCNT. The abundance of adult daphnids in treatment CNTd0 was 1.8 times higher than the control at day 12 and reached the minimum, which was 2 times smaller compared to the control, at day 30 (Figure 11 C). In contrast, the total amount of adults in CNTd14 was continuously lower during the entire population test in comparison to the control group with a minimum of 2.4 times fewer than the control at day 58.

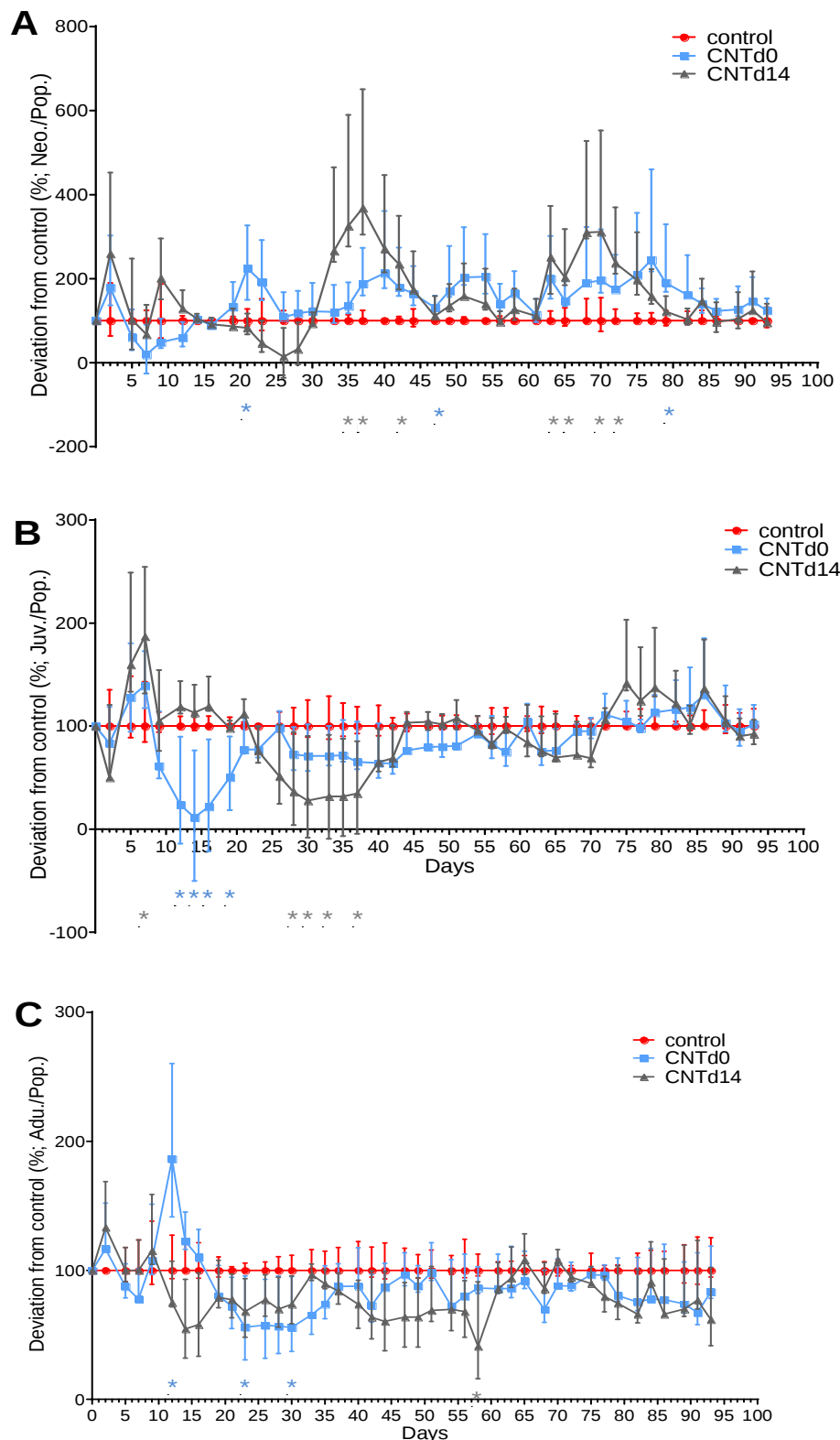


Figure 11. Abundance per size class of *Daphnia magna* populations over time with a continuously exposure to 1 mg CNT/L as of day 0 ("CNTd0") and day 14 ("CNTd14"). A: expressed as the percentage of neonates per population (Neo./Pop.) to the control group. B: expressed as the percentage of juveniles per population (Juv./Pop.) to the control group. C: expressed as the percentage of adults per population (Adu./Pop.) to the control group. *Statistically significant from the control population, $p < 0.05$.

The different development in age classes of MWCNT treated populations and the control was quantified by dividing the number of neonate and juvenile daphnids by the number of adult daphnids (factor juveniles to adults; Figure 12). Over the period of 93 days, more fluctuation of this factor can be seen for populations exposed directly to 1 mg CNT/L (CNTd0) as well as for populations exposed to MWCNT as of day 14 compared to the control. This change demonstrates a significant difference in the population structure of *Daphnia magna* after a continuous exposure to MWCNT.

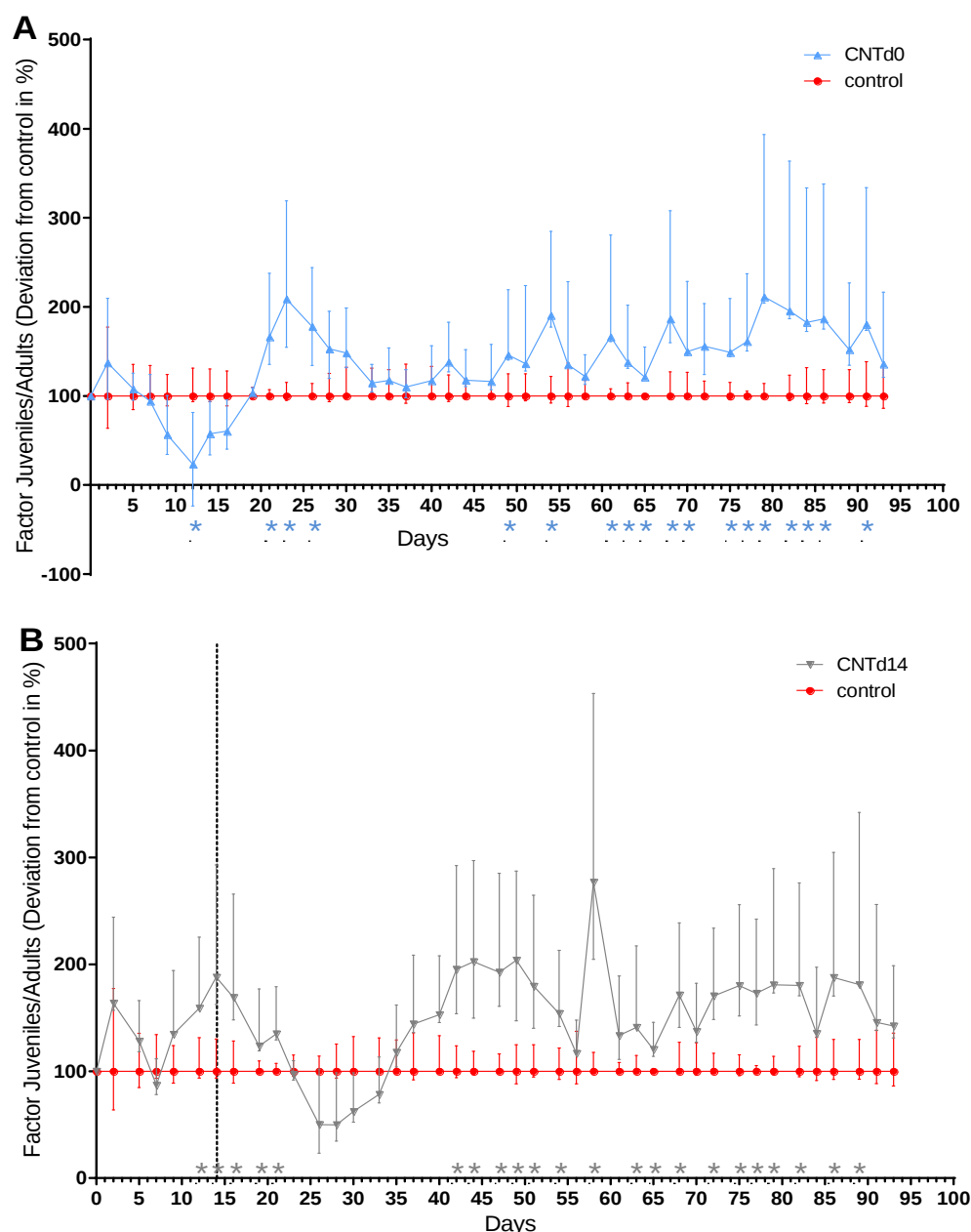


Figure 12. Factor juveniles (neonates and juveniles) to adults of *Daphnia magna* populations over time with a continuous exposure to 1 mg CNT/L as of day 0 (A) and day 14 (B) expressed as the percentage relative to the control population. Dotted line marks the start of MWCNT exposure at day 14. Whiskers represent the minimum and maximum of three independent replicates. *Statistically significant from the control population, $p < 0.05$. No significant differences from the control

treatment were observed on day 14 prior to the first TCC pulse exposure (Figure 13). Even after this short exposure with 25 µg TCC/L, no significant changes on population abundance were observed within 2 days. The maximal abundances reached 167 and 138 organisms before equilibrium on day 19 and 21 for the TCC and CNT+TCC treatment, respectively. The maximal abundances reached 201 ± 47 and 200 ± 71 individuals and finally equilibrium was maintained at 82 ± 9 individuals at day 19 (TCC) and 75 ± 15 organisms at day 21 (CNT+TCC). The exposure to a higher concentration of TCC (40 µg/L) reduced the population density by 55 % within 7 days (day 54-61). A reduction of the total abundance by 12 % was observed for treatments also continuously exposed to 1 mg CNT/L (CNT+TCC). The higher abundance in CNT+TCC was not significant compared to TCC. However, populations only exposed to TCC showed a significantly decreased population abundance compared to the control (Figure 13).

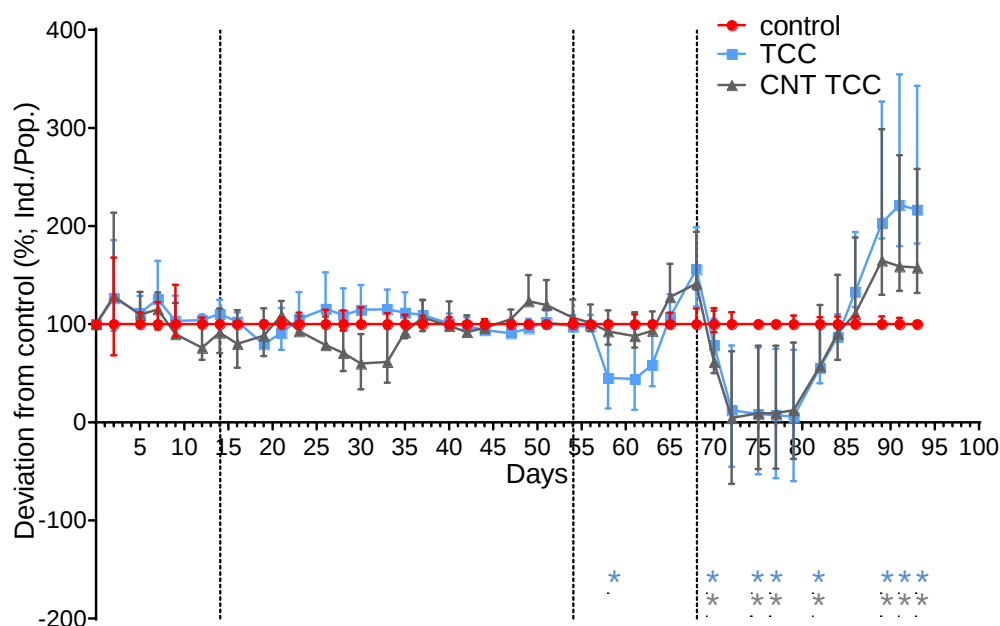


Figure 13. Total abundance of *Daphnia magna* populations over time with three short-term exposure with triclocarban (d14: 25 µg TCC/L; d54: 41 µg TCC/L; d68: 61 µg TCC/L). Dotted lines marks the day of the short-term exposure to TCC. Whiskers represent the minimum and maximum of three independent replicates. Ind./Pop. = individuals per population. *Statistically significant from the control population, $p < 0.05$.

The highest exposure with TCC (60 µg/L) was made during the growth phase of the populations at day 68 and resulted in an almost complete extinction of the populations. The population density in the TCC treatment was reduced by 94 % to a total abundance of 5 ± 1 organisms within 9 days after medium renewal. A reduction of the total abundance by 96 % could be determined for CNT+TCC treatment already within 2 days to a total abundance of 3 ± 1 individuals.

3.3.3 Population structure

The size distribution of individuals in the control population at equilibrium consisted of mostly juvenile daphnids, with only 21 % of neonates and 25 % of adults (Figure 14). A higher variability in the population structure over time was found for populations continuously exposed to MWCNT. After reaching the equilibrium, the abundance of neonates in the control treatments fluctuated between 16 and 37 %. Fluctuations between 23 and 54 % and 8 and 67 % were observed for CNTd0 and CNTd14 treatments, respectively. On day 26, the abundance of neonates in the CNTd14 treatment significantly decreased (leading to 4 individuals) compared to CNTd0. Thereby the abundance of adult daphnids reached a maximum of 36 %, with 7 % neonates and 56 % juveniles. Continuous exposure of daphnids to 1 mg CNT/L reduced daphnid size by 13 % for neonates and 6 % for adults (Figure 14). Daphnid size was also significantly smaller when exposed to 1 mg CNT/L as of day 14. Reduction of 10 % for size class 1, and 3 % reduction for size class 3 could be observed.

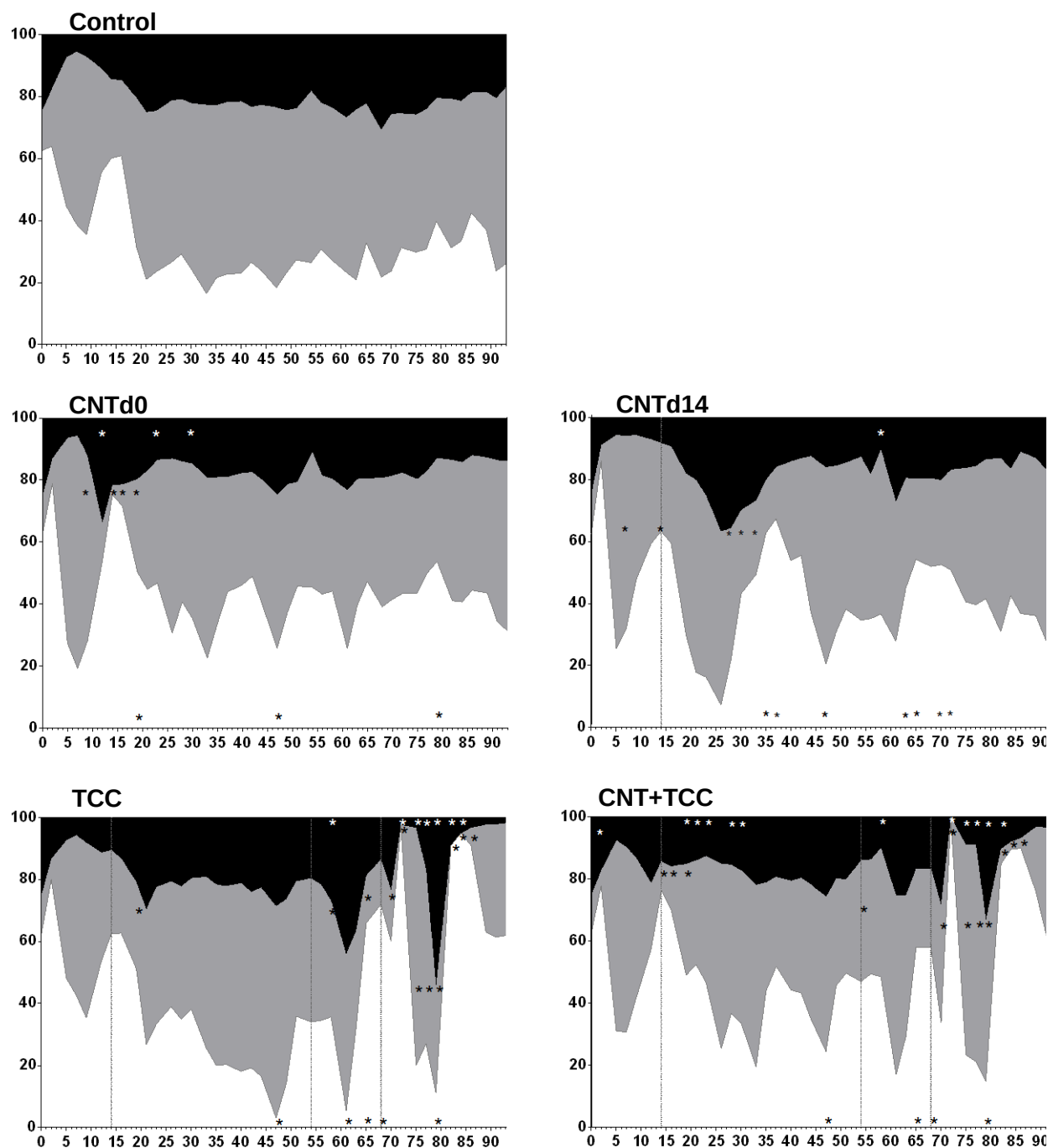


Figure 14. Population structure of *Daphnia magna* populations as a function of time as percentage composition out of neonates < 9 mm (white), juveniles > 9 mm ≤ 2.6 mm (gray) and adults > 2.6 mm (black). (A) control population. (B) With a continuous exposure to 1 mg CNT/L as of the beginning of the experiment. (C) With a continuous exposure to 1 mg CNT/L as of day 14. (D) With three short-term exposures with TCC: 25 µg TCC/L at day 14, 41 µg TCC/L at day 54, and 61 µg TCC/L at day 68. (E) With a continuous exposure to 1 mg CNT/L and three short-term exposures with TCC: 25 µg TCC/L at day 14, 41 µg TCC/L at day 54, and 61 µg TCC/L at day 68. Dotted lines marks the day of the short-term exposure with TCC or the application of MWCNT in the treatment CNTd14. *Statistically significant from the control population, $p < 0.05$.

The pulse of 25 µg TCC/L at day 14 did not result in significant differences in the population structure compared to the control, whereas the short-term exposure to 41 µg TCC/L at day 54 revealed a significant decrease in the abundance of size class 1 and an increase within size class 3. A reduction from 83 ± 13 individuals to 30 ± 11 individuals after exposure to 41 µg TCC/L was observed. However, the adult animals continued reproduction even immediately after the chemical application, and therefore, the proportion of neonates soon increased. This significant decrease in the total abundance in the TCC treatment could not be observed for the CNT+TCC treatment, as shown in Figure 13. The toxicity of TCC was thus reduced in appearance of 1 mg CNT/L. A reduction from 98 ± 14 individuals to 60 ± 18 individuals for an exposure with 41 µg TCC/L under MWCNT influence was observed.

More extensive changes in population structure were observed in both treatments, TCC and CNT+TCC after the highest pulse of TCC with a concentration of 61 µg TCC/L. Within the group of neonates and juveniles, reductions from 96 ± 4 individuals to 3 ± 2 individuals was found for TCC treatment, leading to 97 % effect. TCC reduced the population abundance by 90 % under MWCNT influence. A reduction from 84 ± 20 individuals to 8 ± 5 individuals for an exposure with 61 µg TCC/L was observed.

3.3.4 Modeling approach

At the start of the experiment, deviations between model predictions and the measured population structure were observed (Figure 15). The model showed a maximum in abundance 6 days earlier as observed in the laboratory experiment. Furthermore, a smaller total population abundance was predicted with a deviation of 26 % at day 14. The first short-term exposure with 25 µg TCC/L indicated a lower reduction in population size than predicted by the IDamP model. The recovery of the population size was captured well with only 15 % to 29 % deviation from the measured data until day 54. Moreover, the two additional short-term exposures of 41 µg TCC/L and 61 µg TCC/L were captured well. The model predictions are more exact when the population has already reached the equilibrium.

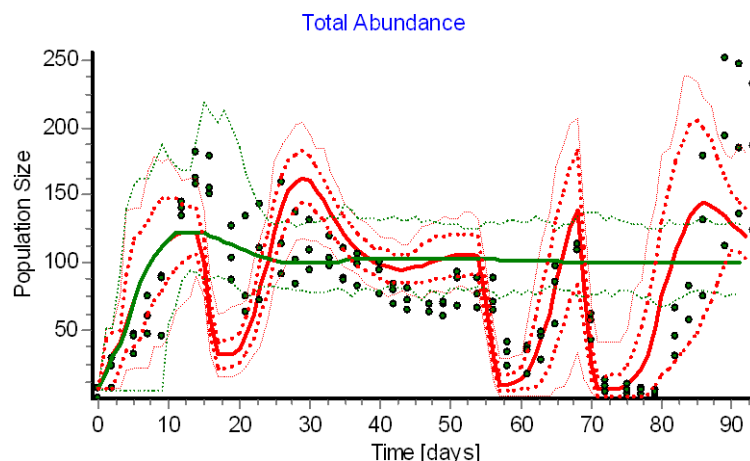


Figure 15. Comparison of predicted and measured population dynamics for different concentrations of TCC: 25 μg TCC/L at day 14, 41 μg TCC/L at day 54, and 61 μg TCC/L at day 68. Dots represent each value determined in laboratory experiments. The solid line shows the average of 1000 Monte-Carlo simulations (red = short-term exposure to TCC, green = control group), dashed lines the 95 % confidence interval, dotted lines the 68th percentiles.

3.4 Discussion

3.4.1 Multiwalled carbon nanotubes

In this study, a more complex and environmentally relevant system was investigated using the population test with *Daphnia magna*. Until now, chronic exposure data are limited and cover exclusively a period of maximum 21 days, whereas many researchers occupied the acute toxicity of CNT. Populations exposed to 1 mg CNT/L reached the equilibrium after 19 or 21 days. More fluctuations in the number of daphnids were observed for all three size classes over the period time of 93 days compared to the control populations. Thereby, a significant increase in the number of neonates, and a significant decrease in the number of juveniles and adults indicate the relationship between size class development and the exposure to MWCNT. This leads to a higher variability in the population structure over time when daphnids were continuously exposed to MWCNT. Finally, the continuously exposure of daphnids to 1 mg CNT/L as of day 0 significantly reduced daphnid size.

Exposure of daphnids to carbon-based nanoparticles has been associated with a number of harmful effects. These effects have often been linked to the chemical nature of the nanoparticles but sometimes also to the preparation method for the nanoparticles^[69].

Rosenkranz et al.^[70] found the translocation of 20 nm polystyrene nanoparticles from the digestive tract into other parts of the daphnids. They forewarned that even if nanoparticles are not digested by the daphnids it is possible that a small fraction might be taken up. Olasagasti and coworkers^[71] exposed *Daphnia* to MWCNT at concentrations of 3 to 16 mg CNT/L and found mortalities up to 95 %. An exposure to 6 mg CNT/L resulted in 10 % mortality, meanwhile smaller tested concentrations and negative controls did not exhibit effects. Previous studies at the Institute for Environmental Research at the RWTH Aachen University (data not published) showed neither mortality in acute tests up to 50 mg CNT/L, nor effects on the reproduction in chronic tests (21 d). Therefore, the concentration of 1 mg CNT/L was chosen to expose the daphnids over a period of 93 days.

A study by Lovern et al.^[72] showed significant changes in *D. magna* behavior when exposed to the lowest observable effect concentration of 260 µg/L of fullerenes. Their observations included repeated collisions with the glass beakers, swimming in circles at the water surface, changes in the number of hops, appendage movement, and increased heart rate. However, the reasons for these behavioral changes are not clear and may be caused by other factors than particle adhesion^[73]. These observations could not be noted in the present study. The behavior of daphnids in treated and untreated populations was normal.

Another study by Petersen and coworkers^[74] showed that *Daphnia magna* were not able to fully purge CNT from their guts after a depuration period of one day. Even when *Daphnia* were fed algae during the depuration period, they were unable to purge CNT completely from their systems within 48 h. The results largely accord with those previously found qualitatively for *Ceriodaphnia dubia*, which showed that feeding with algae was necessary for gut clearance of nanotubes^[75]. Gillis and coworkers^[49] found no apparent depuration of sediment ingested by *D. magna* after 48 h in clean water, but substantial removal after 24 h when the *Daphnia* were fed algae. This is in accordance with the observations in the present study. On each sampling day, a picture of the whole population was made and the guts of the MWCNT treated daphnids were conspicuously stuffed with the nanoparticles.

The impeded CNT depuration and the observed physiological effects on growth are reasons for concern about potential CNT food chain transfer to organisms at higher trophic levels, an effect that might be exacerbated by decreased *Daphnia* health and a consequent increased susceptibility to predation^[74,76]. Therefore, the fact that the investigated MWCNT affected daphnid size is an important result. On the other hand, TEM examination provided no evidence of absorption of MWCNT by *D. magna* in a separate study^[77]. Furthermore, in a study in which CNT were suspended using several methods, a greater degree of CNT aggregation was related to higher toxicity in *Ceriodaphnia dubia* suggesting that the greater degree of clumping of CNT within the gut may have been related to toxicity^[78].

3.4.2 Triclocarban

Effects of short-term exposure with TCC

The present study showed that a short-term exposure of *Daphnia* populations with TCC caused size-dependent mortality. We found EC₅₀-values of 13 µg TCC/L for neonates, 26 µg TCC/L for juveniles, and 33 µg TCC/L for juveniles. Previously, acute toxicity threshold values in crustacea were determined to range from 1.9 to 40 µg TCC/L, and chronic toxicity was observed at levels as low as 0.06-4.7 µg TCC/L^[79].

Size- or age-dependent sensitivity is a general property found in most species^[66]. In general, early life stages are the most sensitive^[80-82]. This also applies for daphnids and the fact that neonate daphnids are more sensitive than adults is described for different substances^[83-85]. It is assumed that these differences in sensitivity are related to toxicokinetics. The elimination and uptake rate of a substance depend on the volume to surface ratio, which is related to the size of the individual^[86,87]. Adult daphnids are up to 5 times larger than neonates and the toxicokinetics in daphnids seem to be proportional to their size or weight^[88], which can explain the lower sensitivity of adult daphnids to chemical stress^[66]. After each single pulse with TCC, a recovery of the populations was observed along with an overshooting effect in population abundance. The higher abundance might lead to lower food availability for each

individual, and could increase the extinction risk of the population as shown by Preuss et al.^[66].

Effects on a population level

The first TCC pulse exposure with 25 µg TCC/L revealed in no significant differences compared to the control. Whereby, the exposure to a higher concentration of TCC (41 µg/L) reduced the population density significantly by 55 %. This decrease in population density was not observed in presence of 1 mg CNT/L, with a reduction of the total abundance by 12 %. Furthermore, the highest exposure with 60 µg TCC/L resulted in an almost complete extinction of the populations. To our knowledge, this is the first study to gain information of TCC exposure on the population level of *Daphnia magna*.

Modeling approach

The IDamP model was able to predict the effects of TCC on the population level after equilibrium was reached. A perfect match of the experimental data could not be predicted for the exposure to 25 µg TCC/L. Chemical analysis with LC-MS/MS resulted in a half-life value of 1.4 days for TCC whereby the model was calibrated. The deviation found between the model prediction and the experimental data after the first pulse can originate from the underestimation of the population abundance at the beginning of the experiment. In fact, there were more neonate daphnids at the beginning of the laboratory study than predicted by the model that cannot be explained. Therefore, more daphnids are present and a lower amount of TCC was available for each individual compared to in the model prediction. Once equilibrium was reached, the model could predict the effect of TCC very well. Therefore, the results demonstrate that the model is able to predict population dynamics of *D. magna* exposed to TCC.

3.4.3 Interaction of MWCNT and TCC

It could be shown that the toxicity of TCC (41 μg TCC/L) could be reduced in presence of 1 mg CNT/L. CNT are known to interact with organic molecules via non-covalent forces, such as hydrogen bonding, π - π bonding, electrostatic forces, Van der Waals forces and hydrophobic interactions^[89,90]. The log K_{OW} describes the hydrophilicity or hydrophobicity of an organic chemical and allows evaluation of possible hydrophobic interactions with other compounds. With a log K_{OW} of 4.9 for TCC^[34], the hydrophobic effect might be the dominant factor for the adsorption of TCC to MWCNT. Chen et al.^[91] also reported that the strong adsorption of polar nitroaromatics, compared to apolar compounds, was due to π - π electron-donor-acceptor (EDA) interactions between the nitroaromatics (π acceptor) and the graphene sheets (π donors) of CNT. Limited numbers of studies have been conducted to understand the adsorption of organic contaminants by CNT^[92-101]. A common observation from these studies was that CNT are very strong adsorbents for hydrophobic organic compounds. This is understandable considering the strong hydrophobicity and high surface area of carbon nanotubes^[91]. An important implication is that electronic polarizability of the aromatic rings on the surface of CNT might considerably enhance adsorption of organic compounds to CNT^[92,94,95,101]. As concluded by Chen and coworkers^[91], no studies have been conducted to systematically compare adsorptive interactions between carbon nanotubes and organic compounds with significantly different physical-chemical properties (e.g., polarity, functional groups, etc.).

To our knowledge, no studies are available dealing with the interactions of MWCNT and TCC. However, Long et al.^[95] found CNT to be a superior sorbent for Dioxin compared to black carbon. Furthermore, CNT were observed to adsorb trihalomethanes^[96], methyl ethyl ketones and 1,2-dichlorobenzene^[98], and polycyclic aromatic compounds like phenanthrene^[99]. Moreover, Baun et al.^[73] observed a 1.9 times higher decrease in toxicity for pentachlorophenol in the presence of CNT.

In addition, adsorptive interactions between CNT and organic substances depend on the properties of the CNT (there under geometric structure, wall number, functionalization, hydrophobicity) as well as environmental conditions like the pH of the surrounding medium^[102,103]. Additionally, the dispersion of CNT is an important factor for the adsorption of organic chemicals for several reasons. Dispersed CNT have a larger surface area that is available for adsorption when compared to agglomerated CNT, since their mobility is increased^[103], and CNT surfaces which are not accessible for adsorbates in CNT agglomerates are accessible in dispersion.

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Chapter 4

Assessment of uptake and toxicity of multiwalled carbon nanotubes and triclocarban in zebrafish (*Danio rerio*) early life stages

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Article is currently in preparation

4.1 Introduction

The variety of nanomaterials (NM) rapidly increased in the last few decades in the areas of biotechnology, electronics, medicinal drug delivery, cosmetics, material science and aerospace engineering^[1]. One of the key members in this nanomaterial family are carbon nanotubes (CNT). They are known for their unique chemical, physical and electronic properties, which make them potentially useful in many applications in nanotechnology, electronics, optics, and other fields of material science, and various environmental applications^[2-9]. Since CNT are so attractive in both basic science and applied technology, uncontrolled accidental exposure of CNT to human beings and ecosystems will unavoidably increase through direct or indirect routes^[10-13]. Environmental impacts due to engineered nanoparticles (ENP) occur both from releases of the NM themselves as well as from their synthesis^[14].

Single or multiple graphene sheets can be folded into cylindrical structures to give multiwalled carbon nanotubes (MWCNT), which take on unique and novel properties^[15]. CNT can be many microns in length, and are typically termed 'long' if their length exceeds 15 μm ^[16]. Any toxicity that results from interactions between CNT and biological systems will depend on physicochemical and electronic properties, including nanotube purity (residual metal and amorphous carbon content), diameter, length, surface charge, functionalization, chirality, and aggregation state^[17-19]. At higher trophic levels, toxicity exhibits strong dependence on uptake pathways, biotransformation, distribution, and removal of nanomaterials through physiological mechanisms specific to the organism^[14]. In recent years, limited information on risk assessment of CNT has been published. While some studies dealt with the human toxicological assessment^[20,21], there is still a lack of information concerning the environmental hazard identification and effects or exposure assessment of CNT^[22]. However, experience with NM in ecotoxicological laboratories is improving and recommendations for systematic and comparable evaluations are emerging^[6,23,24].

Whether CNT are inherently toxic or due to external factors such as length, surface modification, degree of dispersion and the presence of metal impurities is still a subject of intense investigations^[1].

CNT are carbonaceous adsorbents with hydrophobic surfaces that exhibit strong adsorption affinities to organic compounds^[25-37]. Thereby, a combination of chemical, electrostatic, and physical interactions play a major role for adsorption processes. The outermost surface, inner cavities, interstitial channels, and peripheral grooves of CNT constitute four possible sorption sites for organic compounds^[37]. Nanotechnology has initiated different types of NM to water technology in recent years that can have promising outcomes. CNT in particular received special attention for its exceptional water treatment capabilities and proved to work effective against both chemical and biological contaminants^[33].

Environmental contamination by personal care products (PCP) has recently increased widespread public attention as an ubiquitous problem^[38]. PCP are important and essential elements of modern life^[39,40]. Antimicrobial agents of such PCP, e.g. triclocarban (TCC)^[41,42], are used for a wide variety of purposes in foods, cosmetics, pharmaceuticals, clothes and plastic products^[43]. TCC has been detected at microgram per liter levels in waterways in the United States and Switzerland, indicating extensive contamination of aquatic ecosystems^[44-47]. In surface waters, TCC concentrations of up to 6.75 µg/L were measured^[48]. Interactions with other substances, which might be of biological or synthetic origin, are still poorly investigated and cannot be predicted.

TCC has been shown to be highly hydrophobic ($\log K_{ow} = 4.9$), has a low solubility in water ($S_w = 0.65 \text{ mg/L}$)^[49], and is generally found to be relatively persistent in soils and aquatic sediments^[47,50,51]. Due to its lipophilicity, TCC has an affinity to adsorb to organic matter and to accumulate in organisms^[50,52,53].

Due to the results of an evaluation of available toxicity data for fish, aquatic invertebrates, and aquatic plants by the U.S. EPA, TCC has been classified as a high priority chemical^[54].

Only minimal aquatic toxicity data exists for TCC and threshold values for acute toxicity in fish have been determined to range from 49 to 180 µg/L, whereas chronic effect thresholds were in the range of 5 µg/L^[55]. As was mentioned above, such TCC levels were detected in the environment in the past. TCC was thus chosen for this study because of its widespread use, toxicity^[56], bioaccumulation potential^[57-59], and environmental persistence^[60]. As TCC is used since 1957 in huge amounts^[42], and MWCNT is prognosticated to reach the amount of a large scale production, both substances will involuntarily occur together in the environment.

Embryos of the zebrafish (*Danio rerio*) were selected as a vertebrate-based test system in this study because it is a well-established model organism for toxicological^[61,62] as well as ecotoxicological research^[63-66]. The zebrafish embryo has several advantages as a model in developmental toxicology including low cost maintenance, high fecundity and rapid generation time. The fertilization is external and the embryos are transparent, allowing for a continuous monitoring of every stage of development^[67-69]. Additionally, the fish embryo toxicity test (FET) is an excellent alternative to the acute fish test since it is more sensitive^[63,70-72]. We further investigated a transgenic heat shock reporter zebrafish^[73], which is ideal to test toxicity, teratogenicity and up-regulation of defense pathways in a complete vertebrate as previously reported by Pan and coworkers^[74]. Other studies also reported the activation of heat shock protein (Hsp) expression in the presence of toxic compounds^[75-81].

This study aimed at providing new information on the toxicity of MWCNT and TCC as well as the mixture of both substances by using embryos of the zebrafish *Danio rerio*. The toxic potential, robust stress response, and the passage through the chorion of MWCNT as well as the toxicity of TCC to zebrafish embryos were investigated. Especially the question whether MWCNT and TCC interact and are more or less toxic when zebrafish embryos are exposed to mixtures of both was addressed in this study.

4.2 Material and methods

4.2.1 Chemicals

3,4,4'-trichlorocarbanilide and 3,4-dichloroaniline were purchased from Sigma Aldrich with a purity of 99 % or 98 %. Multiwalled carbon nanotubes (Baytubes C150P, >95 % purity) were provided from Bayer MaterialScience (Bayer AG, Leverkusen). Radiolabeled multiwalled CNT (^{14}C -CNT) were synthesized by means of catalytic chemical vapor deposition in cooperation with Bayer Technology Services GmbH (BTS, Leverkusen). In brief, ^{14}C -labeled benzene ($^{14}\text{C}_6\text{H}_6$; 10 mCi/mmol) was first diluted with unlabeled benzene (1:1), and vaporized in H_2 at 700 °C. Cobalt was used as catalyst and N_2 functioned as the carrier gas in this process. The resulting radiolabeled nanotube aggregates were finally washed with a 12.5 wt.-% hydrochloric acid in order to remove excess catalyst. The ^{14}C -CNT had a specific radioactivity of 1.3 MBq/mg.

4.2.2 Nanoparticles suspension

Test suspensions of 0.1-100 mg/L of MWCNT were prepared by ultrasonication with a microtip (70 W, 0.2" pulse and 0.8" pause; Bandelin) for 10 minutes. Transmission electron microscopy images showed the presence of small agglomerates and individual nanotubes in distilled water (Figure 16).

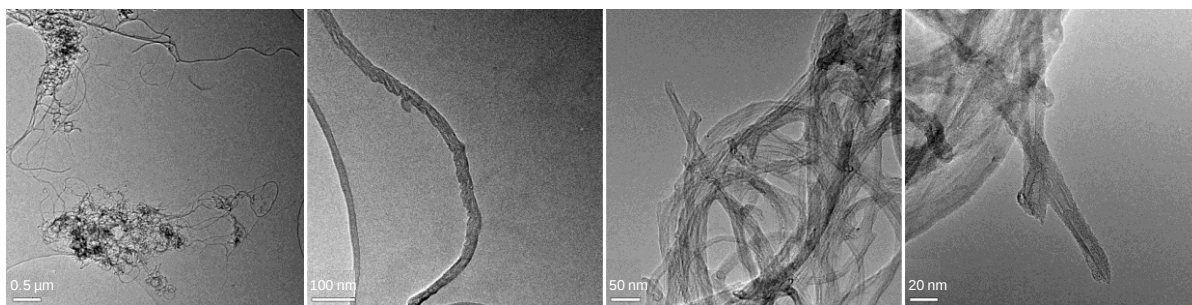


Figure 16. Agglomerates (A), single nanotubes (B), and tubes sticking out of the agglomerates (C,D) visualized by transmission electron micrographs of sonicated MWCNT in distilled water.

4.2.3 Fish embryo toxicity test (FET)

a) Zebrafish maintenance

Wild type zebrafish

Wild type zebrafish were raised and maintained at 26 ± 1 °C and a 14/10 h light:dark rhythm according to standard laboratory conditions^[82] and Peddinghaus et al.^[83].

Transgenic zebrafish

Tg(Hsp70: GFP) transgenic zebrafish^[73] were maintained at 26.5 °C and a 14 h/10 h light dark cycle. Embryos were obtained from individual fish by pairwise breeding as previously described by Pan et al.^[74].

Test design

The assay was carried out according to the German version EN ISO 15088:2008 and the OECD-draft^[84,85] for the FET with zebrafish (*Danio rerio*) with slight modifications. The FET was extended up to 120 h and carried out in glass vessels (20 ml, Ø 40 mm, VWR). All experiments were conducted in accordance with the Animal Welfare Act and with permission of the federal authorities (Landesamt für Natur, Umwelt und Verbraucherschutz NRW, Germany), registration number 84-02.04.2012.A015.

After the light was turned on for photo-induced spawning, the embryos were collected within 30 minutes. The developmental age of the embryos was measured (hours post fertilization, hpf) and staged according to the method described by Kimmel et al.^[68]. Fertilized eggs, not older than 3 hpf (128-cell stage), were identified by using a binocular with a minimum magnification of 25 x or a dissection microscope for the transgenic zebrafish. Only those embryos that developed normally were selected for subsequent experiments.

5 embryos exposed to 10 ml of test medium were provided per concentration and each concentration was tested six times. The embryos in each replicate were investigated using an inverted microscope at magnifications of 40 x and 100 x each 24 h, and the number of

surviving and hatching embryos as well as sublethal effects (edema, no bloodstream, no pigmentation, spine malformation, and general developmental aberrations) were recorded. MWCNT were tested in a maximum of 5 concentrations, separately prepared and ultrasonicated in artificial water^[86]. After determining the EC₅₀-value for TCC, the chemical was tested in 3 concentrations (37 µg/L, 66 µg/L, and 111 µg/L) as well as in mixtures with 0.1, 1, and 10 mg CNT/L medium, respectively. Negative controls (NC) were conducted with artificial water only (mortality ≤ 10 %), whereas positive controls (PC) contained a concentration of 3.7 mg/L 3,4-dichloroaniline (DCA, mortality > 10 %). The results were valid according to DIN EN ISO 15088:2008 and OECD-draft criteria^[84,85]. The induction of the heat shock protein in Tg(Hsp70: GFP) zebrafish was used as a process control. Therefore, one embryo (72 hpf) was seeded into each well of a 96-well microtiter plate. The microtiter plate was incubated at 38 °C for one hour to induce heat shock as described previously^[74]. Following the induction, the embryos were further cultured at 26.5 °C. GFP expression was continuously monitored by time-lapse video microscopy using an inverted microscope (Leica DMI 6000B).

b) Additional investigation with ¹⁴C-CNT

Zebrafish embryos were exposed to 3 mg/L radiolabeled MWCNT (¹⁴C-CNT) to determine the distribution of CNT in the test set up, and to answer the question whether CNT are able to pass the chorion and reach the embryo. This test design was conducted according to DIN EN ISO 15088:2008^[84] as a semi-static exposure with a daily MWCNT suspension renewal. Therefore, embryos were manually dechorionated at each time point (24 h, 48 h, 72 h, 96 h, and 120 h) except for those embryos that already hatched. No additional narcotics were used for the dechorionation. The radioactivity in the water phase, the chorion, and the embryo was separately measured by using a liquid scintillation counter (LSC; LS 5000 TD). The chorions and the embryos were carefully washed several times with distilled water to ensure that no slightly adhered ¹⁴C-CNT remained available on the tissues.

4.2.4 Statistical analysis

Statistical evaluation was accomplished by use of SigmaPlot 12. First of all, normal distribution and equal variance were tested by performing a Shapiro–Wilk test and Levene's test. Additionally, a One Way Repeated Measures Analysis of Variance on Ranks followed by the Holm–Sidak method was performed. Treatments were always tested against the negative control or in the case of the mixture testing against the TCC concentrations. The significance level used throughout all comparisons was $p = 0.05$.

4.3 Results

4.3.1 Fish embryo toxicity assay

Wild type zebrafish

The survival of zebrafish embryos to different MWCNT concentrations were determined at specified time points. The mortality did not achieved the 10 % threshold up to 100 mg CNT/L after 96 hpf (data not shown). No malformations in developing zebrafish embryos could be observed. The greater the concentration, the more adhesion of MWCNT to the chorion or the integument of zebrafish embryos was observed (Figure 17). The results showed that embryos exposed to MWCNT could develop normally and no significantly delayed hatching compared to the control group was determined. Hence, the no observed effect concentration (NOEC) was 100 mg CNT/L.

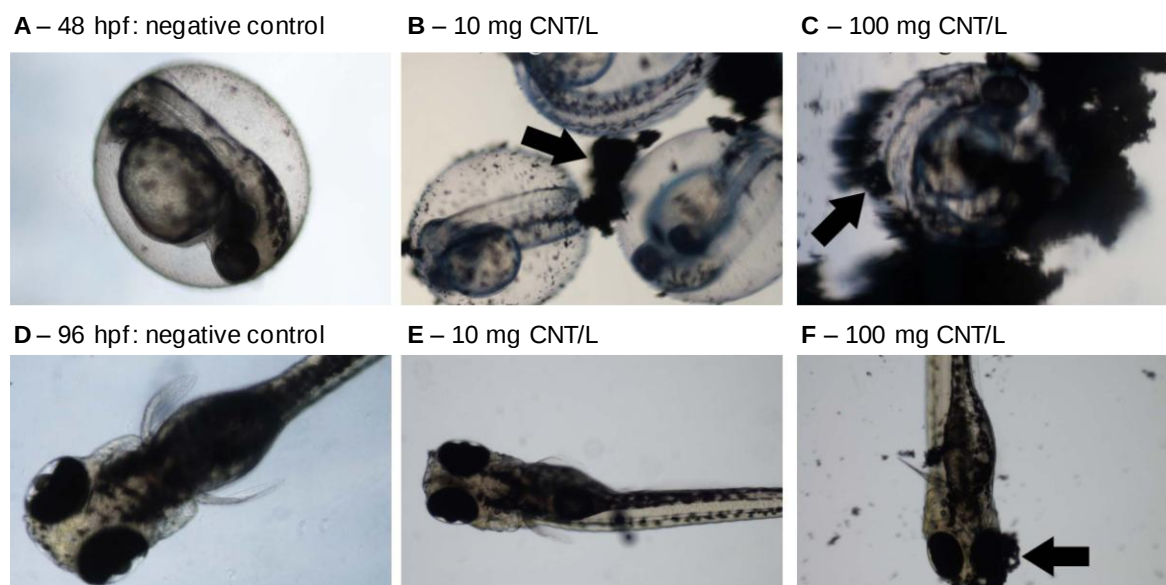


Figure 17. Adhesion of MWCNT agglomerates to the chorion or the integument of zebrafish embryos after 48 hpf (A-C) or 96 hpf (D-F) for the negative control (A,D), and concentrations of 10 mg CNT/L (B,E), and 100 mg CNT/L (C,F). Black arrows show the adhesion to the organisms.

Transgenic zebrafish

As described by Blechinger et al.^[76], all fish with unspecific fluorescence in the eyes were considered as transgenic. And beyond this, an unspecific fluorescence was also observed in some muscles of the tail. An unspecific fluorescence in the whole body of the larvae was observed after the non lethal heat shock for 60 minutes in positive control embryos. MWCNT treated embryos did except for the unspecific fluorescence not show additional fluorescence after 96 hpf and therefore no proteotoxic stress was induced (data not shown). Additionally, no significant malformation, delayed hatching or mortality was observed compared to the control group.

4.3.2 Distribution of ^{14}C -CNT in the test system

Most of the ^{14}C -CNT was detected in the water phase (Figure 18). After 72 h and up to 120 h of exposure, about 10 % of the radioactivity was connected to the chorion. It should be mentioned that after hatching of the embryo both the chorion and the ^{14}C -CNT agglomerated together at the test vessels bottom. No statistically relevant amount of radioactivity was found in the embryos, i.e. less than 1 % of the initial concentration of 3 mg CNT/L. The recovery rate of ^{14}C -CNT ranged from 84 % to 106 % (data not shown).

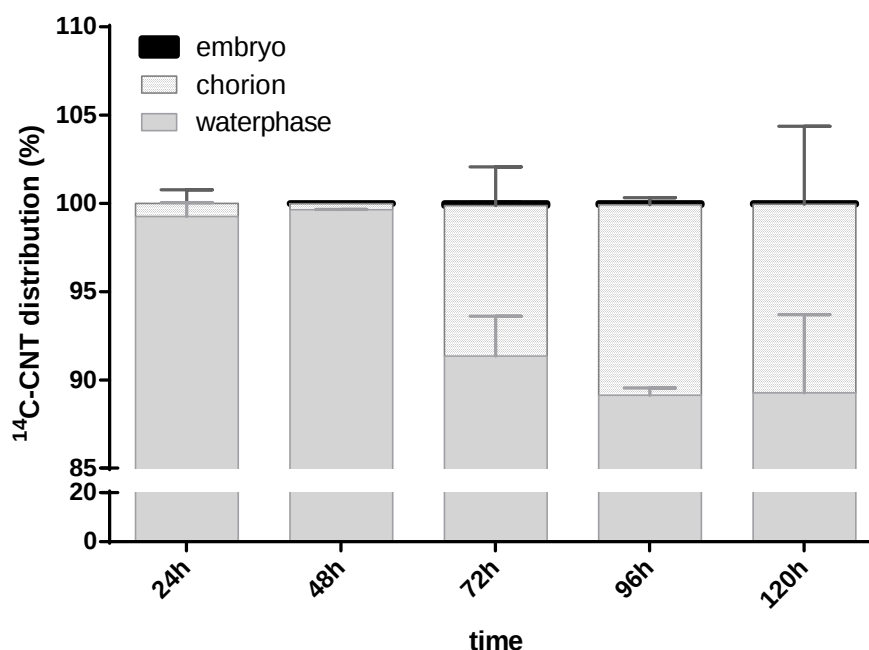


Figure 18. Distribution in percentage of the total amount of ^{14}C -CNT in the system to the water phase, the chorion, and the embryo over time.

4.3.3 TCC and mixture of TCC and MWCNT

Mortality of the embryos did not exceed 10% after exposure to 37 and 66 μg TCC/L (Figure 19 A-B). Mean mortality $\pm$ standard deviation of 111 μg TCC/L treated embryos was 96.7 ± 10.3 %, which was significantly higher as the mortality in the control group (Figure 19 C). The NOEC was 66 μg TCC/L and the LOEC 111 μg TCC/L.

Combined treatment of zebrafish embryos with MWCNT (0.1, 1, and 10 mg CNT/L) and TCC in the two lowest concentrations of 37 and 66 μg /L did not result in increased mortality after 96 hpf compared to the control group and the single TCC concentration (Figure 19 A-B). At the highest TCC concentration of 111 μg /L and in the mixtures with 0.1 and 1 mg CNT/L mortality was significantly higher than in the control group. The mixture toxicity of 111 μg TCC/L and MWCNT resulted in mortalities of 70.0 ± 27.6 %, 83.3 ± 19.7 %, and 10.0 ± 11.0 % after 96 hpf with MWCNT concentrations of 0.1, 1, and 10 mg CNT/L, respectively (Figure 19 A-C). The addition of 10 mg CNT/L to 111 μg TCC/L resulted thereby in a significantly lower mortality as observed for treatment groups containing 111 μg TCC/L alone (Figure 19 C).

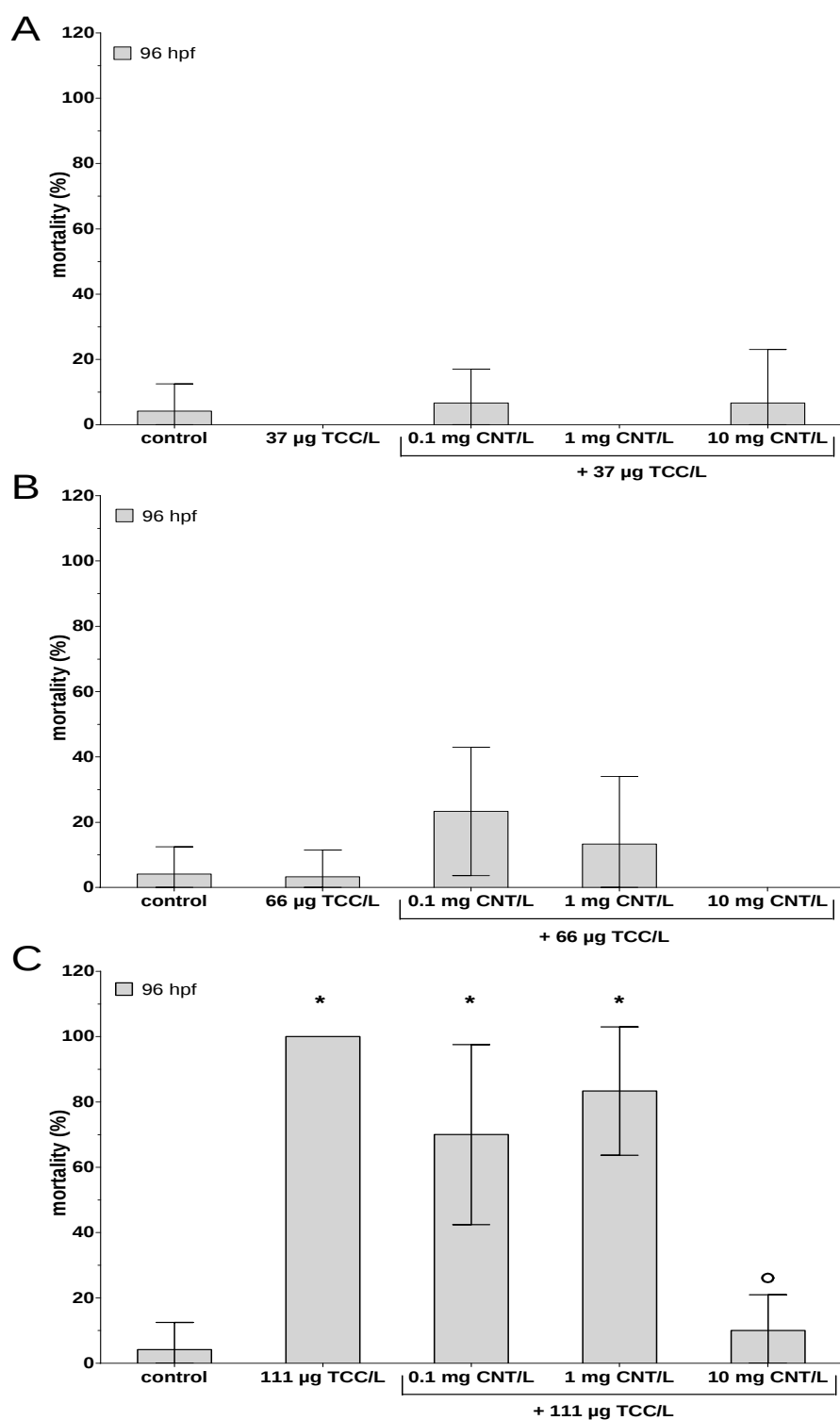


Figure 19. Mortality of fish embryos after exposure to triclocarban and the mixture of TCC and MWCNT after 96 hpf. A: 37 µg TCC/L and mixtures with 0.1, 1, and 10 mg CNT/L. B: 66 µg TCC/L and mixtures with 0.1, 1, and 10 mg CNT/L. C: 111 µg TCC/L and mixtures with 0.1, 1, and 10 mg CNT/L. Bars represent means, error bars give standard deviation, n = 6, *: statistically significant different from the control group, o: statistically different from 111 µg TCC/L.

4.4 Discussion

4.4.1 Nanoparticle toxicology

Non functionalized MWCNT were used to investigate the toxic potential, induction of proteotoxic stress, distribution in the test system, and interactions of these nanotubes with an organic chemical. Several studies dealt with the toxic potential of different CNT to zebrafish embryos as well as the interactions of organic compounds and CNT. But only little to no literature is available concerning the activation of Hsp expression in the presence of nanoparticles, i.e. CNT and in particular, the transgenic zebrafish expressing green fluorescent protein (GFP) under the control of the heat shock protein 70 kDa (Hsp70) promoter Tg(Hsp70: GFP).

Wild type zebrafish

Up to 100 mg CNT/L neither toxicity in wild type zebrafish nor developmental abnormalities or delayed hatching success were observed. Other studies found no toxicity to slight increases in mortality. Pan et al.^[87] determined increased dose-dependent mortality of zebrafish embryos after exposure to up to 10 mg amide-functionalized SWCNT/L. Olasagasti and coworkers^[88] also showed an increased mortality (up to 45%) with increasing concentrations of carboxyl-functionalized MWCNT (3, 6, and 12 mg CNT/L). However, they used a medium (Tween 20) that already caused more than 10 % mortality, which indicates that the fish did not die due to CNT alone. An earlier study by Cheng et al.^[89] showed that SWCNT induce significant hatching delay in zebrafish embryos between 52 and 72 hpf at concentrations above 120 mg CNT/L. However, 99 % of the exposed embryos hatched at 75 hpf. Double-walled CNT also induced a hatching delay at concentrations above 240 mg CNT/L. This hatching delay was likely caused by the Ni and Co catalysts used in the production of SWCNT that remained available at trace concentrations after purification^[16]. Adengua et al.^[90] concluded that seven different types of water soluble CNT (SWCNT, DWCNT, and MWCNT) were not toxic to zebrafish embryos; no significant adverse effects on development were observed. This is in agreement with our findings.

Transgenic zebrafish

No induction of the Hsp promoter by MWCNT in Tg(Hsp70: GFP) zebrafish as well as no toxicity and no adverse effects on development could be determined up to 100 mg CNT/L.

One study by Pan et al.^[74] used the same transgenic zebrafish to test the toxicity of gold nanoparticles (AuNP). They could demonstrate the induction of Hsp promoter by AuNP in Tg(Hsp70: GFP) zebrafish. This indicates that the transgenic zebrafish is able to activate the Hsp expression after nanoparticle exposure. To our knowledge, no other studies are available dealing with transgenic zebrafish and CNT. Our results suggest that the chorion plays a protecting role against CNT and that the agglomerates of MWCNT had no toxic influence on the larvae after hatching. Therefore, it was assumed that the bioavailability of MWCNT for larvae was infinitesimal.

Hu and coworkers^[78] showed the toxic potential of ZnO and chitosan NP to zebrafish embryos and the higher expression of Hsp70 protein in the nanoparticles treated group using Western Blot analysis. Another study by Lin et al.^[79] concluded that the stimulation of Hsp70 gene expression by metal oxide NP (CuO, ZnO, and NiO) could be attributed to nanoparticle dissolution and shedding of transition metals. However, the best characterized heat shock protein is Hsp70, a very sensitive stress-inducible member of this family that is often expressed in a tissue-specific fashion in a number of vertebrate species^[91,92]. Hsp70 is conserved throughout evolution and has been shown to protect cells against induction of cell death by a variety of stresses and different modes of cell death^[93]. Heat shock proteins could be induced by a variety of stimuli, and are potentially damaging to the cell. They are associated with many cellular processes including protein synthesis, folding and translocation, and assembly of larger protein complexes, all of which can be impaired by stress^[94].

4.4.2 Distribution experiment - chorion as a barrier?

The radioactive distribution experiment pointed out that more than 90 % of the initial amount of MWCNT inserted to the medium remained in the water phase, whereas up to 10 % was found to agglomerate to the chorion after 72 hpf, when the embryo was hatched. Furthermore, no significant adhesion of MWCNT to zebrafish embryos was observed.

Few studies could demonstrate the passage of nanoparticles with particle sizes ≤ 45 nm, e.g. silver and gold NP, through the chorion and entrance to the embryo^[95-97]. Larger silica NP on the other hand have been shown to not be able to pass the chorion, and could be found on the chorion surface^[98]. MWCNT have particle sizes ranging from 5 - 100 nm, but only reach these small scales in one dimension.

The zebrafish chorion, composed of two major types of glycoproteins, acts as a barrier that protects the embryo against noxious physical and chemical stimuli^[99]. As described in detail by Rawson et al.^[100], the chorion consists of three layers. The outer and innermost layers, 0.2-0.3 μm and 1.0-1.6 μm in thickness, are separated by a 0.3 to 0.6 μm -thick, low contrast, middle layer. The outermost layer of the chorion is covered with an extremely osmophilic layer, which is uneven and consists of granular material that obfuscates openings to the pore canals located in the inner and middle layers of the chorion. The pores are cone-shaped with a larger diameter at the inner surface and the diameter of the outer opening of the pore canals was measured to be 0.5-0.7 μm ^[96,100]. It seems to be that the osmophilic layer of the outermost layer hinders the MWCNT in the present study to reach the chorion pore canals. Furthermore, typical lengths of MWCNT lie in the μm range. Similar observations were made by Cheng et al.^[89] after the exposure of zebrafish embryos to SWCNT. They went on to demonstrate that concentrations of up to 360 mg CNT/L, micro scaled or larger, agglomerates were unable to compromise the nanoscale pores of the protective embryo chorion after 96 hpf, indicating that the chorion is an effective protective barrier to SWCNT agglomerates.

4.4.3 Mixture of multiwalled carbon nanotubes and triclocarban in the FET

The key finding in the present study was the reduced toxic potential of the biocide TCC caused due to the presence of MWCNT. The mean mortality of 10 mg CNT/L and 111 µg TCC/L was 90 % lower compared to the mortality of embryos exposed to 111 µg TCC/L alone.

The antimicrobial agent TCC seems to interact with MWCNT resulting in a lower bioavailability of TCC to zebrafish embryos. CNT, as adsorbent media, are able to remove heavy metals such as Cr^{3+} ^[101], Pb^{2+} ^[102], and Zn^{2+} ^[103], metalloids such as arsenic compounds^[104], organics such as polycyclic aromatic organic compounds^[25,34,105], pesticides^[106], and a range of biological contaminants including bacteria^[107-112], viruses^[113,114], cyanobacterial toxins^[115-117] as well as natural organic matter^[118-121]. The success of CNT as an adsorbent media in the removal of biological contaminants, especially pathogens is mainly attributed to their unique physical, cytotoxic and surface functionalizing properties^[33].

The adsorption kinetics of organic compounds by CNT has only been investigated in recent years^[26,122-126]. Possible adsorption mechanisms between CNT and TCC are non-covalent forces, such as hydrogen bonding, π - π bonding, electrostatic forces, Van der Waals forces and hydrophobic interactions^[127]. With a log K_{OW} of 4.9 for TCC^[49] and considering the strong hydrophobicity and high surface area of carbon nanotubes^[128], the hydrophobic effect might be the dominant factor for the adsorption of TCC on the MWCNT.

Moreover, TCC is an aromatic compound, and aromaticity of molecules contributes to their sorption to CNT due to π - π interactions as demonstrated by Lin and coworkers^[129]. They observed that sorption of several phenolic organic compounds to MWCNT increased with an increasing number of aromatic rings. Chen et al.^[128] reported that the strong adsorption of polar nitroaromatics, compared to apolar compounds, was due to π - π electron-donor-acceptor (EDA) interactions between the nitroaromatics (π acceptor) and the graphene sheets (π donors) of CNT. An important implication from several of the studies is that

electronic polarizability of the aromatic rings on the surface of CNT might considerably enhance adsorption of the organic compounds^[30,130-132].

Another important aspect for the reduced toxicity of TCC in the mixture with CNT might be the agglomeration potential of CNT. CNT are known to form highly entangled agglomerates and the size of these agglomerates is drastically larger than that of single MWCNT, which could interact with TCC. The Van der Waals forces increase with the agglomerate sizes of MWCNT. Still, the Van der Waals forces are not sufficient for the bundling and clustering morphologies that MWCNT exhibit, since the tubes would then be aligned preferentially parallel to each other, which is a more favorable condition from an energetic point of view^[133]. Raw MWCNT usually exist in clusters with a crossed mesh configuration. The intermolecular forces of a clustered network of MWCNT are increased by each contact of MWCNT with another MWCNT along these meshes, physically fixing the network^[133]. In the present study, increasing intermolecular forces with increasing agglomerate sizes could promote the adsorption of TCC to MWCNT. However, engineered carbon nanomaterials can vary significantly in shape, size, morphology, and impurity, e.g., metal, amorphous carbon and O-containing groups, which can further complicate the adsorptive properties of these materials for organic contaminants^[128].

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Chapter 5

General Conclusion

As described in Chapter 1 the toxicological potential of carbon nanotubes depends on their length, shape, surface modification, purity, and their propensity to form agglomerates and aggregates. In the present study no modification of the nanoparticles was used and the multi walled CNT were prepared by ultrasonication. To avoid false positive results occurring from metal impurities, the negative control was also prepared by sonication of the test medium only. TEM analyses revealed the presence of well dispersed, isolated nanotubes as well as aggregated clusters in our assays. Within the following chapter, the cytotoxicity and the endocrine potential of unfunctionalized, flexible MWCNT and their capability to enhance the production of intracellular reactive oxygen species was investigated. The tested CNT are not toxic to RTL-W1, T47Dluc and H295R cells. As assumed, no significant change in luciferase activity in the ER Calux assay with T47Dluc cells nor a significant alteration of E2 production in H295R cells after treatment with MWCNT was found. Consistent with other studies, Chapter 2 also shows the generation of ROS by MWCNT. 1.6 μ M concentrations of the biocide triclocarban decreased the luciferase activity in ER Calux assays but did not affect the production of E2 in H295R cells in ELISA assays. In mixtures of MWCNT and TCC the anti-estrogen potential of TCC in T47Dluc cells was reduced because the lipophilic biocide adsorbed to the nanotubes resulting in a lower available concentration of triclocarban in the test medium.

The population test with *Daphnia magna* as a long-term exposure scenario for 1 mg CNT/L over a period of 93 days was described in Chapter 3. Changes in population structures were observed leading to significantly more neonate daphnids (< 0.9 mm) and lower amounts of juvenile and adult daphnids compared to the control population as well as significantly smaller daphnids when exposed to 1 mg CNT/L over 93 days. Additionally, short-term exposures of *D. magna* to TCC were investigated, leading to a decrease in the total number of daphnids followed by recovery of the population each time. Chapter 3 also highlighted that the individual-based population model, IDamP, was able to predict the effects on the population level under constant laboratory conditions. The use of validated population models in combination with laboratory population experiments is a powerful tool to examine

toxicity effects. Furthermore, this approach will help to compare the effects measured in the laboratory with effects found in the field and to improve the current risk assessment of chemicals. Finally, the toxicity of triclocarban (up to 41 µg TCC/L) could be reduced in appearance of 1 mg CNT/L.

Chapter 4 reports on the toxicity and the stress response of multi walled carbon nanotubes to zebrafish (*Danio rerio*) embryos and their distribution in the test system consisting of the water phase, the embryo, and the chorion. CNT were shown to be not toxic to wild type and transgenic zebrafish. In addition, neither malformations in development nor delayed hatching was observed. Most of the MWCNT remained in the water phase and were not able to pass the chorion canal and to reach the embryo. Even after hatching, MWCNT were not ingested or accumulated by zebrafish larvae. On the other hand, the low observed effect concentration of the antimicrobial agent triclocarban were determined amounting to 111 µg TCC/L, which is in accordance with the open literature. In mixtures of MWCNT and triclocarban, adsorption of TCC to the nanotubes results in reduced toxicity and lower available concentration of TCC to wild type zebrafish embryos.

Many efforts have been made to carefully investigate the *in vitro* and *in vivo* toxicity of CNT but researchers still fail to reach consensus on the toxicity of CNT and the mechanism, as the core of nanotoxicology, remains out of reach. There is still a need to define scenarios of exposure. The assessment of carbon nanotubes effect on the cells, organ, or entire organism should be standardized systematically so that nanotoxicity mechanisms can be revealed and the safe use of CNT can succeed. Exposure scenarios should also consider the fate and behavior of CNT in the aquatic environment, with or without the presence of natural and anthropogenic substances and conditions that may influence the agglomeration. Another critical point is the chemical characterization of the nanosized test material. Some controversies and inconsistencies between different researchers originated from some specific properties of nanomaterials. Preliminary toxicological data and predicted concentrations suggesting that CNT are not acute toxicants call for a warning to possible sublethal and long-term effects, as shown in Chapter 3, and possible synergistic effects with

other toxic pollutants present in the same environmental compartments. More research is still needed to better understand the molecular interactions of carbon nanotubes and organic contaminants. In such experiments the properties of both contaminants, CNT and pollutants, should be systematically varied.

Chapter 6

Curriculum vitae and scientific contributions

Curriculum vitae

Name	Anne Simon
Date of Birth	February 7th, 1985
Place of Birth	Eupen
Nationality	Belgian
2011-2014	PhD student at the Institute for Environmental Research, RWTH Aachen University, Germany
2010	Diploma in Biology
2003-2010	Studies of Biology at the RWTH Aachen University, Germany
2003	Abitur
1997-2003	Pater Damian Secondary School in Eupen; Belgium
1991-1997	Primary school in Eynatten; Belgium

Scientific contributions

Research articles to be submitted to international peer-reviewed journals

- Simon, A., Maletz, S.X., Hollert, H., Schäffer, A. and Maes, H.M. (2014) *Effects of multiwalled carbon nanotubes and triclocarban on several eukaryotic cell lines: Elucidating cytotoxicity, endocrine disruption, and reactive oxygen species (ROS) generation*. Submitted to Nanoscale Research Letters
- Simon, A., Preuss, T.H., Schäffer, A., Maes, H.M. and Hollert, H. (2014) *Long-term exposure of multiwalled carbon nanotubes: Population test with Daphnia magna over 93 days and additional short-term exposure to triclocarban*. To be submitted to Ecotoxicology
- Simon, A., Maes, H.M., Hollert, H. and Schäffer, A., (2014) *Assessment of uptake and toxicity of multiwalled carbon nanotubes and triclocarban in zebrafish (Danio rerio) early life stages*. Manuscript in preparation

Platform presentations

- Simon, A., Hotz, S., Carpentier, D., Maes, H. M., Hollert, H. and Schäffer, A. (2012). Effect of multi-walled carbon nanotubes by means of different cell line assays. Proceedings, SETAC German Language Branch Annual Meeting, Leipzig, Germany
- Maes, H.M., Rhiem, S., Stibany, F., Daniels, B., Deutschmann, B., Simon, A., Gieffers, S., Kurth, M., Riding, M., Pan, Y., Semple, K.T., Jones, K.C., Martin, F.L., Baumgartner, W., Jahnen-Dechent, W., Hollert, H. and Schaeffer, A. (2011). Bioavailability of CNT for organisms of different trophic levels! Consequences of CNT – cell interactions to vital functions??? ICCE 2011, 13th EuCheMS International Conference on Chemistry and the Environment, Zurich, Switzerland
- Simon, A., Zielke, H., Schmidt, B., Seiler, T.-B., Schäffer, A., Kukkonen, J.V.K., Hollert, H.(2011). Chemo- and bioanalyses on the partitioning of radiolabelled organic chemicals in sediment-water-organisms-systems. Proceedings, 2nd SETAC Yes Meeting, Aachen, Germany
- Simon, A., Zielke, H., Schmidt, B., Seiler, T.-B., Schäffer, A., Kukkonen, J.V.K., Hollert, H.(2010) Bioanalytische und radioaktive Untersuchungsmethoden zum Verteilungsverhalten von Monosubstanzen im Sediment-Wasser-Organismus-System. Proceedings, SETAC German Language Branch Annual Meeting, Dessau, Germany

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