UNIVERZA V LJUBLJANI
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DOKTORSKA DISERTACIJA

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NANOPARTICLE TOXICITY ASSESSMENT IN A MODEL PROTOZOAN *Tetrahymena thermophila*

DOCTORAL DISSERTATION

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Delo je bilo opravljeno v laboratorijih Katedre za mikrobiologijo in mikrobno biotehnologijo Biotehniške fakultete Univerze v Ljubljani. Del raziskav vezanih na ugotavljanje oksidativnega stresa je bilo opravljenih v laboratorijih Katedre za zoologijo v sodelovanju s Skupino za nanobiologijo in nanotoksikologijo. Proteomske študije so bile opravljene v laboratorijih Oddelka za biokemijo in biofiziko Univerze v Stockholmu.

Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa Senata Univerze z dne 28. septembra 2009 je bilo potrjeno, da kandidatka izpolnjuje pogoje za neposreden prehod na Podiplomski študij bioloških in biotehniških znanosti ter opravljanje doktorata znanosti s področja biotehnologija. Za mentorico je bila imenovana prof. dr. Romana Marinšek Logar.

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Delo je rezultat lastnega raziskovalnega dela. Podpisana se strinjam z objavo svojega dela na spletni strani Digitalne knjižnice Biotehniške fakultete. Izjavljam, da je delo, ki sem ga oddala v elektronski obliki, identično tiskani verziji.

Katarina Rajapakse

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AI Z evkariontskim mikroorganizmom *Tetrahymena thermophila* smo proučevali interakcije med pogosto uporabljanimi nanodelci TiO_2 in celico. Pri eni od študij smo uporabili tudi kvasovko *Saccharomyces cerevisiae* in nekatere druge pogosto uporabljane nanodelce (ZnO, CuO, Ag, ogljikove nanocevke). Klasični dejavniki strupenosti so pokazali, da testirani nanodelci niso strupeni za modelni organizem in se razen v primeru odziva proteoma ne razlikujejo od delcev TiO_2, večjih od 100 nm. Delci TiO_2 povzročijo spremembe v profilih dolgoverižnih maščobnih kislin, ki nakazujejo povečanje rigidnosti membrane, nismo pa ugotovili sprememb v koncentraciji ATP, nastanka reaktivnih kisikovih zvrsti ali lipidne peroksidacije. Spremembe v membranah kažejo na aklimacijo na neugodne okoljske razmere in ne na toksični odziv. Uporabljena testa genotoksičnosti sta dala nasprotujoče si rezultate in v tem delu razlagamo možnosti za to razhajanje. V primeru kometnega testa smo eksperimentalno dokazali možnost lažnih pozitivnih rezultatov. V celicah *T. thermophila* se kopičijo nanodelci TiO_2 v prebavnih vakuolah, kar je odvisno od njihove velikosti, koncentracije in trajanja izpostavitve. Analiza citosolnega proteoma po izpostavitvi delcem TiO_2 je pokazala spremembe fiziološkega stanja, stres in nanospecifični odziv. Spremembe v izražanju proteinov so povezane z metabolizmom maščobnih kislin, energetskim metabolizmom in ionskim ravnovesjem. S pridobljenimi rezultati v doktorskem delu smo zaključili, da delci TiO_2 modificirajo membrane, spremenijo citosolni proteom modelnega mikroorganizma in povzročajo prelome DNA ob neposrednem stiku. Delci TiO_2 v modelnem organizmu povzročijo biološke odzive, ki še ne kažejo na strupenost, vendar niso biološko inertni.

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AB We studied the bioactivity of TiO_2 nanoparticles in a model eucaryotic microorganism *Tetrahymena thermophila* (*Protozoa*). We also investigated genotoxicity on yeast *Saccharomyces cerevisiae* (*Ascomycetes*), testing some other nano- and bulk- particles, that were also chosen based on widespread use (TiO_2, ZnO, CuO, Ag and SWCNT). We used different methods at distinct levels of biological organization to study the biological activity of nanomaterials. As the first interaction of nanoparticles with any living organism is the cell membrane, this was our first research scope. We provide experimental evidence that changes in the membrane fatty acid profile of *T. thermophila* incubated with nano- or bulk-TiO_2 particles are not accompanied by ROS generation or lipid peroxidation. We interpreted these changes as acclimation to unfavorable conditions and not as toxic effects. We also observed reversible filling of food vacuoles, but this was different in case of nano- or bulk-TiO_2 exposure. At the DNA level, we employed a Comet assay. Exposure to bulk- or nano-TiO_2 of free *T. thermophila* cells, cells embedded in gel or nuclei embedded in gel, all resulted in a positive Comet assay result but this outcome could not be confirmed by cytotoxicity measures such as lipid peroxidation, elevated reactive oxygen species or cell membrane composition. The genotoxicity was studied also on *S. cerevisiae.* Two genotoxicity assays, GreenScreen and the Comet assay were employed for comparisson reasons. The assays produced different genotoxicity results and we discuss the reasons for this discrepancy. Based on previous results, we created a study design to investigate the early response of *T. thermophila* to nano-TiO_2 or bulk-TiO_2 particles at subtoxic concentrations (0.1 and 100 µg TiO_2 /ml) using proteomic analyses of cytosolic cell fraction. The results of our work showed that during early response of *T. thermophila* to TiO_2 particles in suspension, alteration of lipid and fatty acid metabolism, energetic metabolism and ion regulation already at low exposure concentrations occur. TiO_2 nanoparticles could have nano-specific effects and can also cause nano-specific responses in a model organism. Such responses arguably cannot be referred to as toxic, but they clearly showed that once present inside an organism, the tested nanoparticles are not biologically inert.

KAZALO VSEBINE

KAZALO SLIK

str.

KAZALO PRILOG

OKRAJŠAVE IN SIMBOLI

ATP - adenozin trifosfat
DNA - deoksiribonukleinska kislina (ang. deoxiribonucleic acid)
ICP-MS - Inductively-coupled plasma mass spectrometry ali masna emisijska spektrometrija z induktivno sklopljeno plazmo
nanodelci TiO_2 - nanodelci titanovega oksida
nanodelci ZnO - nanodelci cinkovega oksida
nanodelci CuO - nanodelci bakrovega oksida
SWCNT - ogljikove nanocevke
MK - maščobne kisline
TEM - transmisijska elektronska mikroskopija/transmisijski elektronski mikroskop

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

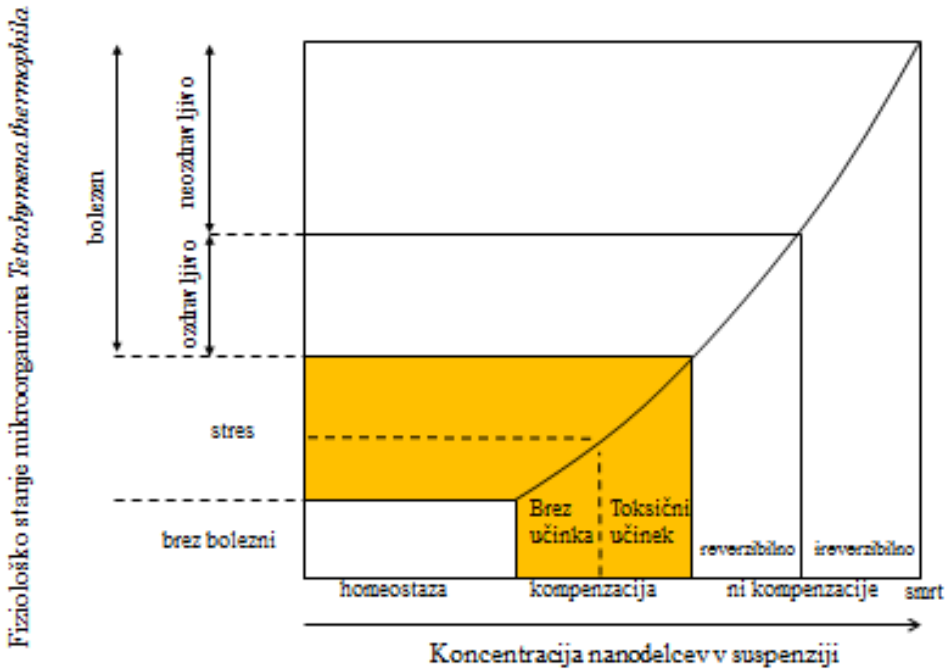
Zaradi svojstvenih fizikalnih in kemijskih lastnosti zahteva študij strupenosti (ali dokazovanje nestrupenosti) nanomaterialov intedisciplinarni pristop, ki mora vključevati vidike kemije, fizike, biologije in medicine (Novak, 2013). Delci nanometrskih velikosti lahko predstavljajo nevarnost za človekovo zdravje, imajo pa velik potencial kot diagnostično orodje ter kot sistem za prenašanje učinkovin na tarčna mesta v telesu.

1.1 INTERAKCIJE NANODELCEV S CELICO

Mehanizme interakcij nanodelcev z biološkimi sistemi razdelimo na kemijske in fizikalne (Nel in sod., 2009). Posledice obojih so procesi, ki tvorijo biološki odziv (Slika 1). Do biološkega odziva lahko pride pred ali po vstopu nanodelcev v celice. V naši študiji smo se omejili na preučevanje zgodnjega odziva dveh modelnih mikroorganizmov: praživali *Tetrahymena thermophila* in kvasovke *Saccharomyces cerevisiae* na nanodelce TiO_2 in delce TiO_2 večje od 100 nm (in v primeru kvasovke tudi na nanodelce (ZnO, CuO, Ag, SWCNT) in delce večje od 100 nm (ZnO, CuO, Ag)). Zgodnji odziv sodi v fazo kompenzacije, kot to prikazuje Slika 1.

Kemijski mehanizmi vključujejo nastanek reaktivnih kisikovih zvrsti (ROS) (Nel in sod., 2009), raztapljanje in sproščanje toksičnih ionov (Xia in sod., 2008), motnje aktivnosti membranskega transporta ionov (Auffan in sod., 2008), oksidativne poškodbe preko katalize (Foley in sod., 2002) in lipidno peroksidacijo (Kamat in sod., 2000).

Fizikalni mehanizmi so v večini primerov posledica velikosti delcev in njihovih površinskih lastnosti (Walczyk in sod., 2010). Fizikalne lastnosti nanodelcev so ključne za poškodbe celičnih membran (Leroueil in sod., 2008; Hussain in sod., 2005), spremembe membranskih aktivnosti (Navarro in sod., 2008), za kvarni vpliv na transportne procese (Ovrevik in sod., 2004), poškodbe zgradbe in zvijanja proteinov (Hauck in sod., 2008) (Billsten in sod., 1997) in agregacijo ali fibrilacijo proteinov (Chen in von Mikecz, 2005).



Slika 1: Shematski prikaz biološkega odziva organizma izpostavljenega stresorju, ki je lahko kemikalija, nanodelec, sevanje, itd (povzeto po Sokolova in sod., 2012).
Fig. 1: Representation of biological response to stress (Sokolova et al., 2001)

1.1.1 Interakcije nanodelcev s celično membrano

Zunanja celična membrana razmejuje notranje okolje od zunanjega okolja celice in omogoča selektiven transport ionov, molekul in tudi nanodelcev (Elsaesser in Howard, 2012). Ne glede na tip izpostavitve ali na vrsto organizma, je membrana mesto, kjer pride do prvega stika z nanodelci (Banaszak, 2009). V naših raziskavah smo učinek TiO_2 nanodelcev na membrano preučevali z ugotavljanjem sprememb v profilih dolgoverižnih maščobnih kislin.

Znano je, da molekule nanometrskih velikosti in supramolekularni združki v celico vstopajo z endocitozo (Conner in Schmid, 2003). Stopnja in mehanizem privzemanja nanodelcev sta odvisna od celičnega tipa in variirata glede na lastnosti nanodelcev; tip, obliko, velikost, naboj (Chithrani in Chan, 2007; Chithrani in sod., 2006; Maysinger in sod., 2007; Mailander in Landfester, 2009; Verma in Stellacci, 2010; Hillaireau in Couvreur, 2009; Delehanty in sod., 2009; Hild in sod., 2008). Pri praživali *T. thermophila* o endocitozi in kopičenju različnih nanodelcev v prebavnih vakuolah (ZnO, CuO, TiO_2)

poročajo različni avtorji (Mortimer in sod., 2010; Ghafari in sod., 2008; Rajapakse in sod., 2012).

Pri celicah KB (humana epidermalna rakasta celična linija) je bilo ugotovljeno, da lahko poleg endocitotskega prehoda v celico, nanodelci skozi lipidni dvosloj prehajajo tudi s penetracijo celične membrane (Chen in sod., 2009). Med sintetiziranimi nanomateriali lahko skozi celično membrano na ta način vdrejo le kationski nanodelci (Leroueil in sod., 2008) oziroma nanodelci namensko oblikovani za prodor v celice kot so nanodelci za vnos zdravil ter nanodelci, ki se uporabljajo za diagnostično vizualizacijo (Tkachenko in sod., 2003; Chen in von Mikecz, 2005; Nativo in sod., 2008). Neposreden način prehoda nanodelcev pa je za celico lahko toksičen, saj pri prehodu ustvarijo pore v celičnih membranah in s tem porušijo občutljivo znotrajcelično koncentracijsko ravnovesje ionov, proteinov in drugih makromolekul, ki uravnavajo delovanje celice (Verma in Stellacci, 2010).

V naši študiji nas je zanimalo ali suspenzija nanodelcev TiO_2 vpliva na strukturo membrane pri evkariontskem mikroorganizmu *T. thermophila*. Izpostavitev nanodelcem smo izvedli v temi. Na ta način smo preprečili fotokatalitsko aktivnost TiO_2 in s tem možen nastanek reaktivnih kisikovih zvrsti. Zanimalo nas je tudi ali gre pri tem za toksični odziv ali za aklimacijo na stresne (neugodne) okoljske razmere (Slika 1).

Številne študije navajajo funkcionalne spremembe membran ob neugodnih spremembah okolja, kot je sprememba temperature in prisotnost kemikalij. Bearden in sod. (1999) in Shultz in sod. (Schultz in sod., 2002), so poročali o spremembi profilov maščobnih kislin pri praživali *Tetrahymena sp.* zaradi vpliva pentaklorofenola in 1-oktanola. Shug in sod. (1969) poročajo o vplivu železovih ionov na desaturacijo maščobnih kislin v membranah *T. thermophila*. Ugotovljene so bile tudi spremembe v profilih dolgoverižnih maščobnih kislin po izpostavitvi kultur *T. thermophila* metil-živemu srebru (Marinšek Logar, 2008).

Spremembe v profilih maščobnih kislin so ugotovili tudi ob izpostavitvi talne mikrobne združbe vodni suspenziji fulerena (C60), zabeležene spremembe pa so bile odvisne od koncentracije omenjenih nanodelcev (Tong in sod., 2007). Mortimer in sod. (2011) so ugotovili spremembe v profilih dolgoverižnih maščobnih kislin pri praživali *T. thermophila* po izpostavitvi nano-CuO delcem. Spremembe v membranskih maščobnih profilih nekateri avtorji interpretirajo kot fiziološko adaptacijo na ekstremne pogoje imenovano aklimacija (Bearden in sod., 1999). Funkcionalne spremembe na celičnih membranah torej lahko razložimo kot aklimacijo na neugodne spremembe v neposrednem okolju organizma. Vendar pa aklimacija ni vezana le na membrane, osnovna definicija je širša in sicer: »Aklimacija obsega kratkotrajne spremembe fenotipa, ki omogoča preživetje v sub-optimalnih okoljskih razmerah, vključno z onesnaženjem« (Bearden in sod., 1999; Kameyama in sod., 1984; Bearden in sod., 1997). V shematskem prikazu biološkega

odziva organizma izpostavljenega stresorju aklimacija torej ustreza fazi kompenzacije (Slika 1).

Ob nastopu sub-optimalnih razmer v okolju organizma, zaradi kemikalij, primarni stresni odziv organizma kompenzira kvarne učinke na celice. V primeru povečane koncentracije in daljšega časa izpostavitve organizma kemikalijam, pa neizogibno pride do toksičnih učinkov (Slika 1).

Povezavo med spremembo v profilu dolgoverižnih maščobnih kislin in citotoksičnostjo so omenili že pri človeških celicah dojk (Clarke in sod., 1990) in pri praživali *T. thermophila* (Mortimer in sod., 2011). Ugotovljeno je bilo, da se pri mikroorganizmu *T. pyriformis* (sorodna vrsta *T. thermophila*) ob spremembi temperature okolja spremeni profil dolgoverižnih maščobnih kislin (Kameyama in sod., 1984). Enak tip odziva *T. pyriformis* je bil ugotovljen ob prisotnosti nepolarnih organskih topil v mediju (Bearden in sod., 1997; Bearden in sod., 1999). Mortimer in sod. (Mortimer in sod., 2011) je poročala o zmanjšani fluidnosti membrane, ki so jo interpretirali kot prilagoditev mikroorganizma *T. thermophila* na nanodelce CuO v mediju. V študiji so preučevali vplive koncentracij nanodelce CuO, ki imajo predhodno ugotovljen toksičen vpliv na celice.

Namen naše študije je bil določiti in analizirati profile dolgoverižnih maščobnih kislin praživali *Tetrahymena thermophila* po izpostavitvi različnim koncentracijam delcev titanovega dioksida (nanodelcem TiO_2 in TiO_2 delcem, večjih od 100nm). *T. thermophila* je bila delcem izpostavljena v mediju, in zaradi požiranja delcev so le-ti vstopili tudi v organizem. Da bi ugotovili možne povezave med spremembo profilov dolgoverižnih maščobnih kislin in potencialnim citotoksičnim učinkom delcev TiO_2 na praživalo *T. thermophila*, smo vzporedno ugotavljali tudi nekatere biomarkerje stresa. Izbrali smo naslednje biomarkerje stresa: lipidna peroksidacija, koncentracija molekul ATP, spremembe v morfologiji celic in polnjenje prebavnih vakuol z delci. V študiji smo primerjali učinke nanodelcev TiO_2 in TiO_2 delcev, večjih od 100 nm.

Predpostavili smo, da je v primeru, da nanodelci TiO_2 nimajo citotoksičnega učinka na modelni organizem, sprememba profila dolgoverižnih maščobnih kislin neposredni dokaz za aklimacijo organizma *T. thermophila* in ne toksičen vpliv delcev TiO_2.

1.1.2 Interakcije nanodelcev z DNA

1.1.2.1 Mehanizmi genotoksičnosti nanodelcev

Kljub številnim opravljenim toksikološkim raziskavam, je trenutno malo znanega o genotoksičnih učinkih nanodelcev (Landsiedel in sod., 2009; Pfaller in sod., 2010). Genotoksičnost delcev je odvisna tako od njihove kemijske sestave, kot tudi od njihove velikosti in oblike (Yang in sod., 2009), pri tem pa ni povsem jasno, kakšen vpliv ima posamezna lastnost na biološke odzive. Na splošno velja, da se z zmanjševanjem velikosti delcev povečuje njihov biološki učinek (Lai, 2012).

Genotoksični vplivi lahko nastanejo že pri znatno nižjih koncentracijah nanodelcev od tistih, ki izzovejo direktno citotoksičnost (Pfaller in sod., 2010). Poznanih je več možnih mehanizmov, preko katerih lahko nanodelci povzročijo poškodbe DNA: preko primarnega (direktnega ali indirektnega) ali preko sekundarnega vpliva (Donaldson in sod., 2010).

Primarni direktni vpliv nastane, ko nanodelci vstopijo v celično jedro in pridejo v neposredni stik z genomsko DNA, ali s proteini, povezanimi z DNA. V celično jedro lahko prodrejo nanodelci, ki imajo premer med 8 in 10 nm (Barillet in sod., 2010) ali so namensko oblikovani za prodor v celice kot so npr. nanodelci za vnos zdravil ter nanodelci, ki se uporabljajo za diagnostično vizualizacijo (Tkachenko in sod., 2003; Nativo in sod., 2008; Chen in von Mikecz, 2005). Nanodelci, ki preidejo skozi celično membrano, lahko v jedro vstopijo pasivno z difuzijo (Geiser in sod., 2005) ali aktivno s transportom preko por v jedrni membrani (Magdolenova in sod., 2013). Z genomsko DNA nanodelci v celici lahko pridejo v stik tudi ob mitozi, po razpadu jedrne ovojnice (Magdolenova in sod., 2013). Mnogo avtorjev na podlagi svojih opazovanj predvideva, da prisotnost nanodelcev v jedru povzroča poškodbe na dvojni vijačnici (Karlsson, 2010; Karlsson in sod., 2004; Shukla in sod., 2011; Stone in sod., 2009). Ugotovljeno je bilo tudi, da direkten stik nanodelcev s proteini, povezanimi z DNA, povzroči poškodbe dednega materiala (Vandghanooni in Eskandani, 2011).

Številni avtorji so pri *in vitro* izpostavitvi celic ugotovili prisotnost nanodelcev v jedru. Poleg nanodelcev srebra in cinkovega oksida (AshaRani in sod., 2009; Hackenberg in sod., 2011a,b), tudi nanodelce TiO_2 (Shukla in sod., 2011). Hackenberg in sod. (Hackenberg in sod., 2010) so v jedru opazovali celo agregate TiO_2, velikosti 285±52 nm, pri tem pa niso ugotovili genotoksičnosti s kometnim testom. To je v nasprotju s številnimi študijami, ki so s kometnim testom in tudi nekaterimi drugimi testi dokazale genotoksičnost nanodelcev TiO_2 (za pregled glej (Magdolenova in sod., 2013)). Poleg tega je v nasprotju tudi z ugotovitvami naše študije, kjer smo opazovane prelome pripisali prav neposredni interakciji TiO_2 nanodelcev z DNA med potekom samega testa (Rajapakse in sod., 2013). Veliki agregati nanodelcev lahko povzročijo tudi deformacijo jedra (Di Virgilio in sod.,

2010), ki lahko vodi do nepravilnosti pri mitozi, saj prostorsko ovira pravilno razhajanje kromosomov kar privede do nastanka mikrojeder (AshaRani in sod., 2009; Gonzalez in sod., 2008) in nepravilnosti pri delitvi celice (Magdolenova in sod., 2013). Prav tako lahko mehansko poškodujejo kromosome, avtorji pa poročajo tudi o povečani frekvenci prenosa sestrskih kromatid po izpostavitvi nanodelcem TiO_2 (Di Virgilio in sod., 2010). Obsežen pregled možnih mehanizmov delovanja nanodelcev pri neposredni in posredni izpostavitvi nanodelcev so objavljeni v preglednem članku Magodolenova in sod. (2013).

Indirektna genotoksičnost nanodelcev je najpogosteje posledica oksidativnega stresa, ki je definiran kot porušeno ravnotežje med tvorbo prostih radikalov in intracelularno vsebnostjo antioksidantov (Betteridge, 2000). Pri oksidativnem stresu pride do povečane znotrajcelične produkcije reaktivnih kisikovih spojin (ROS – angl. Reactive Oxygen Species) ali reaktivnih dušikovih spojin (RNS – angl. Reactive Nitrogen Species), kar lahko vodi do oksidativnih poškodb proteinov, lipidov in DNA (Pfaller in sod., 2010; Yang in sod., 2009). Obstajajo pa tudi dokazi, da nanodelci lahko vplivajo na zmanjšano vsebnost antioksidantov v celici (npr. glutationa), s čimer se poveča verjetnost nastanka oksidativnih poškodb DNA, povzročenih s prostimi radikali (Park in sod., 2008; Li in sod., 2009; Wang in sod., 2009). Indirektni genotoksični vplivi z nanodelci lahko nastanejo tudi preko inhibicije proteinov, ki sodelujejo pri popravljalnih mehanizmih DNA (Beyersmann in Hartwig, 2008).

Sekundarna genotoksičnost nanodelcev je posledica sekundarnega odziva organizma, ki se sproži preko aktivacije molekularnih poti (Pfaller in sod., 2010). Sekundarno genotoksičnost pri sesalcih vodijo vnetnostne celice (Trouiller in sod., 2009), ki na mestu odlaganja nanodelcev sproščajo reaktivne spojine, ki lahko poškodujejo DNA. Sekundarna genotoksičnost je torej posledica oksidativnega stresa, vendar v tem primeru predstavljajo levkociti vir oksidantov (Donaldson in sod., 2010).

1.1.2.1 Študije interakcij nanodelcev z DNA

Z razvojem nanotehnologije in večanjem uporabe nanodelcev v industriji, je pomembno razviti zanesljive teste za ugotavljanje genotoksičnosti nanodelcev (Warheit in Donner, 2010; Gonzalez in sod., 2011). Navodila organizacije OECD (Organisation for Economic Cooperation and Development) narekujejo testiranje genotoksičnosti v *in vitro* sistemih, vendar pa so te zahteve vpeljane iz znanja o testiranju vodotopnih kemikalij, in zato niso primerne za testiranje genotoksičnosti nanodelcev. Znano je, da nanodelci lahko reagirajo s testnim medijem, s čimer se spremeni biološki potencial nanodelcev, še več - interagirajo lahko tudi s sestavinami testov za ugotavljanje genotoksičnosti (Greim in Norppa, 2010; Sathya in sod., 2010) in pomembno je, da se možnosti nastanka tovrstnih artefaktov zavedamo (Stone in sod., 2009). Popolna odstranitev nanodelcev iz medija ali celic, ki so

bile izpostavljene nanodelcem, je zaradi velikosti, mase in dispergiranosti nanodelcev v suspenziji praktično nemogoča. Prav tako je nemogoče odstraniti nanodelce, ki so v celico vstopili med tretiranjen s suspenzijo nanodelcev. V naši raziskavi smo želeli preveriti ali nanodelci TiO_2 vstopajo v *T. thermophila* z endocitozo, ter so posledično prisotni v prebavnih vakuolah.

Kometni test je med najpogosteje uporabljenimi testi genotoksičnosti nanodelcev (Karlsson, 2010; Landsiedel in sod., 2009; Magdolenova in sod., 2013). V preglednem članku je Karlsson (2010) pregledala 46 znanstvenih raziskav, kjer so za ugotavljanje genotoksičnosti nanodelcev uporabili kometni test in ugotovila, da je zaključek večine avtorjev, da nanodelci povzročajo poškodbe DNA. Poudarila je, da obstaja možnost interakcije nanodelcev s testnimi sestavinami in predlagala, da je poleg kometnega testa za povečanje zanesljivosti potrebno uporabiti tudi druge metode za ugotavljanje poškodb DNA. Magdolenova je in sod. (2013) pregledala 112 študij o genotoksičnosti nanodelcev, kjer so v 67 primerih uporabili kometni test in večina avtorjev je poročala o poškodbah DNA. Landsiedel in sod. (2009) je predlagal uporabo nabora standardiziranih testov za ugotavljanje mutageneze, s čimer bi ugotovili specifične mehanizme genotoksičnosti, poleg tega pa še simultano uporabo vsaj dveh testov genotoksičnosti. Tudi v naših študijah smo pri ugotavljanju genotoksičnosti različnih nanodelcev pri kvasovki simultano uporabili kometni test in GreenScreen test (Bayat in sod., 2013).

Genotoksičnost nanodelcev lahko ugotavljamo v *in vivo* ali *in vitro* sistemih. *In vitro* pristop je primeren za ugotavljanje primarne genotoksičnosti (neposreden vpliv delcev na DNA), medtem ko *in vivo* modeli nudijo možnost zaznave sekundarnih učinkov nanodelcev, kot je npr. vnetje (Kisin in sod., 2007; Dusinska in sod., 2011; Vega-Villa in sod., 2008; Arora in sod., 2012).

Prvi presejalni test genotoksičnosti je običajno Ames test, kjer ugotavljamo pojavnost reverznih mutacij (Warheit in sod., 2007). Sledijo testi na sesalskih celičnih linijah ali sesalskih primarnih kulturah, kjer ugotavljamo kromosomske aberacije (Dandekar in sod., 2010) ter prisotnost in število mikrojeder (Li in sod., 2003; Estevanato in sod., 2011). Poškodbe DNA, kot so enojni in dvojni prelomi vijačnice, oksidacija pirimidinov in purinov ali izpadi baz, ugotavljamo s kometnim testom (Shukla in sod., 2011; Shukla in sod., 2011). (Za podrobnosti o metodi glej (Rajapakse in sod., 2013)).

GreenScreen test je komecialno dostopen test, ki se je uveljavil po letu 2000. Temelji na aktivaciji promotorja RAD54, do katere pride ob poškodbah DNA. V kvasovki *Saccharomyces cerevisiae* so ob ta promotor vnesli zeleni fluorescentni protein (GFP) (Billinton in sod., 1998; Afanassiev in sod., 2000). Njegovo izražanje, ki ga lahko merimo z jakostjo fluorescence v zelenem spektru, nakazuje poškodbe DNA. To je zelo učinkovita

metoda saj meri poškodbe celotnega genoma, v primerjavi npr. s testom Ames, kjer spremljamo mutacije na točno določenih mestih genomske DNA rodu *Salmonella sp*.

Prednosti kometnega testa pred drugimi so: 1) je zelo občutljiv in zazna že zelo majhne poškodbe DNA; 2) za izvedbo potrebujemo majhno število celic ; 3) je relativno poceni; 4) potrebujemo relativno majhne količine testnih kemikalij (Tice in sod., 2000). Glavne omejitve kometnega testa so, da z njim ne moremo meriti/ugotavljati aneugenih sprememb, epigenetskih mehanizmov pri poškodbah DNA (Dhawan in sod., 2009) in fiksiranih mutacij (Stone in sod., 2009).

Obstajajo dokazi, ki kažejo na to da je kometni test neustrezna metoda za ugotavljanje genotoksičnosti nanodelcev. Izsledki nekaterih študij so pokazali, da so bili nanodelci (nanodelci CuO in TiO_2) prisotni v glavah kometov v gelu, čeprav pred izvedbo kometnega testa (torej po opravljenem tretiranju celic z nanodelci) s transmisijsko elektronsko mikroskopijo niso ugotovili prisotnosti nanodelcev v celičnem jedru (Karlsson, 2010). Lin in sod. (2009) so poročali o značilno veliki poškodovanosti DNA po tretiranju celic z nanodelci Ge in zaključili, da zaradi adhezivnosti Ge na celične membrane obstaja verjetnost, da je prišlo do interakcij nanodelcev z DNA *post festum*, med samo izvedbo kometnega testa.

Cilj naših študij je bil eksperimentalno ugotoviti ali so nanodelci v resnici genotoksični ali pa gre za interakcijo nanodelcev in DNA *post festum*, med samo izvedbo kometnega testa V študiji smo uporabili nekatere biomarkerje citotoksičnosti in kometni test za ugotavljanje genotoksičnosti nanodelcev TiO_2 na podlagi treh možnih scenarijev: a) *in vivo* izpostavitev (*T.thermophila* smo izpostavili suspenziji nanodelcev TiO_2 in delcev TiO_2 večjih od 100 nm; b) *in vitro* izpostavitev (*T. thermophila* smo vklopili v gel, ki smo ga izpostavili suspenziji nanodelcev TiO_2 in delcev TiO_2 večjih od 100 nm); c) acelična izpostavitev (jedra, vklopljena v gel smo smo izpostavili suspenziji nanodelcev TiO_2 in delcev TiO_2 večjih od 100 nm).

Na podlagi predhodnih študij, kjer poročajo, da nanodelci TiO_2 povzročajo poškodbe DNA posredno, s tvorbo reaktivnih kisikovih zvrsti (Trouiller in sod., 2009; Petkovic in sod., 2011a,b) smo predpostavili, da v primeru *in vitro* izpostavitve in *acelične* izpostavitve nano-TiO_2 delcem ne bomo zaznali poškodb DNA s kometnim testom, saj bo indirektni primarni vpliv nanodelcev odsoten. V primeru ugotovljenih poškodb DNA pri izpostavitvi celic *T. thermophila in vitro* in pri *acelični* izpostavitvi, pa bi lahko sklepali na interakcijo delcev in DNA molekul, kar pomeni lažni pozitivni rezultat. Kometni test je v tem primeru pri ugotavljanju genotoksičnosti potrebno previdno uporabljati.

V vzporedni študiji smo ugotavljali genotoksičnost različnih nanodelcev in delcev večjih od 100 nm pri evkariontskem modelnem organizmu kvasovki *Saccharomyces cerevisiae*.

Uporabili smo v industriji najpogosteje uporabljane delce: TiO_2, ZnO, CuO, Ag in ogljikove nanocevke (SWCNT), ter dva različna testa za ugotavljanje genotoksičnosti: GreenScreen in kometni test.

1.1.3 Interakcije nanodelcev s proteini

Proteini imajo v celici pomembno vlogo; so encimi, signalne in strukturne molekule. Pravilna konformacija proteinov je ključna za njihovo neovirano delovanje. Interakcija nanodelcev s proteini lahko spremeni njihovo konformacijo. Doslej dokazani učinki nanodelcev na encime so; inhibicija (Fischer in sod., 2002), inaktivacija (Wang in sod., 2010), sprememba konformacije (Chandra in sod., 2010; Xu in sod., 2010), razvitje (Zhang in sod., 2009) in stabilizacija strukture (Zhang in sod., 2009). Zaključimo lahko torej, da so učinki nanodelcev na žive sisteme, povezani z interakcijo s proteini.

V interakcijo proteinov in nanodelcev so vpletene: Van der Waalsove sile, Londonove disperzijske sile, vodikove vezi, polarnost ali prosti elektronski pari (Xia in sod., 2010). Fenomen vezave različnih proteinov na nanodelce so poimenovali korona (Lynch in Dawson, 2008). Na moč interakcije med proteinom in nanodelcem vplivajo predvsem površinske lastnosti nanodelcev, kot so kemijska zgradba nanodelca, oblika in razgibanost površine, poroznost in površinska kristaliničnost, heterogenost, hidrofilnost ali hidrofobnost. Kemijska zgradba nanodelca je odločilna lastnost za vezavo proteinov na njegovo površino. Študija vezave proteinov na TiO_2 je pokazala, da sta ključni tudi oblika in velikost nanodelcev, in sama aglomeracija delcev, ki igra pomembno vlogo za vezavo proteinov (Deng in sod., 2009). Čas obstoja kompleksa nanodelec-protein je pogojen z različnimi dejavniki, npr. stabilnost pH okolja in prisotnost in koncentracija drugih proteinov, ki imajo večjo vezavno afiniteto (Goppert in Muller, 2005). Zaključimo lahko, da čas zadrževanja in usodo nanodelcev v celici ali telesu določajo kompleksne interakcije s proteini.

Ugotovljeno je bilo, da nanodelci TiO_2 vstopajo v različne tipe celic in se v njih kopičijo. Opazili so prisotnost agregatov nanodelcev TiO_2 vezanih na membrano in v citoplazmi (Stearns in sod., 2001; Muhlfeld in sod., 2007; Rothen-Rutishauser in sod., 2007). Gheslaghi in sod. (2008) poročajo, da nanodelci TiO_2 vplivajo na polimerizacijo tubulina tako, da se spremeni njegova struktura, konformacija in zmanjša njen obseg. V primeru vezave lizocima na TiO_2 je encim postal nefunkcionalen (Xu in sod., 2010).

Razvoj orodij, ki temeljijo na omikah, odpira nove možnosti za raziskave na področju preučevanja molekularnih mehanizmov pri interakcijah med nanodelci in celicami (Matranga in Corsi, 2012). Nanoproteomika je med najnovejšimi analitskimi pristopi, ki

obljubljajo nadgradnjo »omskih« orodij za raziskovanje mehanizmov delovanja pri nanodelcih (Ray in sod., 2010).

Številne študije, ki poročajo o visoko zmogljivih (hightroughput) pristopih k raziskovanju učinkov nanodelcev na organizme, uporabljajo tako transkriptomske kot tudi proteomske analize. Transkriptomske analize so tehnično dodelane do te mere, da že dlje časa veljajo za robustne, visoko zmogljive in cenovno ugodne tehnologije, ki simultano ovrednotijo desettisoče mRNA molekul v minimaliziranih platformah (Hedge in sod., 2003). Proteomika je na področju študij mehanizmov delovanja nanodelcev ustreznejša zaradi naslednjih razlogov: spremembe proteoma kažejo dejanski končni biološki odziv, poteini imajo daljšo življensko dobo v primerjavi z molekulami mRNA in proteini se funkcionalno dokončno izoblikujejo v procesu posttranslacijskih modifikacij, česar s transkriptomskim pristopom ne moremo zasledovati (Pratt in sod., 2002). Med pomembnejša odkritja »omskih« pristopov sodi ugotovitev o specifičnih odzivih celic na strukturo (69) in kemijsko zgradbo nanomaterialov (Griffitt in sod., 2009; Gou in sod., 2010). Analiza mikromrež, je pokazala da ob prisotnosti nanodelcev TiO_2 pride do spremenjenega izražanja številnih genov povezanih z ribosomi (Griffitt in sod., 2009), kar je povezano z inhibicijo sinteze proteinov ob stresu (Patel in sod., 2002). V študiji celotnega transkriptoma in proteoma na treh človeških celičnih linijah z nanodelci TiO_2 in nanocevkami MWCNT, se je izkazalo, da je po 24 urah izpostavitve odziv lasten vsaki posamezni celični liniji (Tilton in sod., 2013). Biološki procesi, ki so bili najbolj izraženi pri vseh celičnih linijah, tretiranih z nanodelci TiO_2, so povezani z vnetnimi signalnimi potmi, apoptozo, ustavitvijo celičnega cikla, obrambnimi mehanizmi pri podvajajnju DNA in genomski nestabilnosti. Pri nanocevkah MWCNT so bile izražene poti, ki uravnavajo povečanje celičnih delitev, popravljalne mehanizme DNA in proti-apoptotske procese (Tilton in sod., 2013).

Nedavne študije o učinkih nanodelcev TiO_2 kažejo na oksidativni stres, citotoksičnost, vnetje in druge kazalce strupenostnih odzivov (Ferin in sod., 1992; Park in sod., 2008; Monteiller in sod., 2007; Johnston in sod., 2009). Proteomske študije na področju nanotoksikologije so pokazale spremembe v izražanju proteoma po izpostavitvi različnih celičnih tipov nanodelcem TiO_2 (Gao in sod., 2011; Ge in sod., 2011; Jeon in sod., 2011a,b; Jeon in sod., 2010; Tilton in sod., 2013):
povečanje izražanja proteinov, ki jih povezujemo z rakom (Jeon in sod., 2011a), zmanjšanje izražanja proteinov povezanih z protimikrobnim delovanjem, povečano izražanje proteinov povezanih z metabolizmom lipidov in maščobnih kislin, spremembe v izražanju proteinov, ki sodelujejo pri procesiranju mRNA (Gao in sod., 2011).

Tudi, ko ni direktnega kontakta nanodelcev TiO_2 s specifičnim tkivom, lahko pride do spremenjenega izražanja proteinov, ki označujejo oksidativni stres (Jeon in sod., 2011c). Proteomske študije so torej v skladu s predhodnimi študijami *in vivo* in *in vitro*, doprinesle

pa so bolj poglobljen uvid v biološke odzive in razkrile tudi nekatere nove mehanizme delovanja nanodelcev.

Malo je znanega o interakcijah med nanodelci in celicami pred nastopom oksidativnega stresa (Ma in sod., 2009). Tilton in sodelavci (2013) so na podlagi izražanja genov in proteinov ugotovili, da je zgodnji odziv na nanodelce neodvisen od tipa delcev ali celic. Zaključili so, da je zaznan zgodnji odziv zelo verjetno splošni odziv celic na nanodelce, pri tem pa niso poročali o oksidativnem stresu po eno-urni izpostavitvi nanodelcem (Tilton in sod., 2013). V nasprotju z omenjeno študijo (Tilton in sod., 2013), so v proteomski študiji zgodnjega odziva pri miši v limfnih vozlih po injekciji nanodelcev TiO_2, ugotovili spremembe v izražanju proteinov, povezanih z imunskim odzivom, metabolizmom lipidov in maščobnih kislin ter spremembe v izražanju proteinov, ki sodelujejo pri procesiranju mRNA (Gao in sod., 2011).

V znanstveni literaturi zaenkrat ni zaslediti splošne definicije zgodnjega celičnega odziva na neugodne pogoje. Zgodnji odziv omenjajo pri različnih eksperimentalnih zasnovah oziroma pri širokem naboru odzivov celic po izpostavitvi nanodelcem TiO_2. Nekateri avtorji zgodnji odziv obravnavajo v časovnih okvirih izpostavitve celic nanodelcem TiO_2, ki je v razponu od ene ure (Tilton in sod., 2013) do 24 ur (Gao in sod., 2011). Drugi avtorji pa ga opredeljujejo na podlagi relativno majhne izpostavitvene koncentracije (0,2 mg TiO_2/kg telesne teže), oziroma na podlagi sprememb pri izražanju proteinov, ki so vključeni v signalne poti (Ge in sod., 2011). Zgodnji odziv na nanodelce TiO_2 smo omenili tudi v ne-proteomski študiji, kjer je prišlo ob izpostavitvi nano delcem TiO_2 in delcem TiO_2 večjim od 100 nm do aklimacije membran pri mikroorganizmu *T. thermophila*. Pri tem ni bilo prisotnega oksidativnega stresa in sprememb v koncentraciji ATP (Rajapakse in sod., 2012). Na podlagi znanstvene literature smo v naših študijah zgodnji odziv definirali kot predlagamo naslednjo definicijo zgodnjega odziva: »**Zgodnji odziv je merljiv fiziološki odziv celice, ki ga NE spremljajo dejavniki citotoksičnosti, kot je npr. oksidativni stres.«** Če je prisoten oksidativni stres, potem proteomska študija nakazuje mehanizme, ki spremljajo ali so povezani s fiziološkim stanjem stresa v celici.

Znotrajcelična razporeditev proteinov določa funkcijo celic in tkiv (Guillemin in sod., 2005), zato je v proteomskih analizah ugotavljanje sprememb v različnih razdelkih celice ključnega pomena (Kultz, 2005). Na področju nanotoksikoloških raziskav učinkov TiO_2 so bile doslej opravljene le študije proteoma tkiv ali celičnih linij (Gao in sod., 2011; Ge in sod., 2011; Jeon in sod., 2011a,b,c; Jeon in sod., 2010; Tilton in sod., 2013), brez frakcioniranja na subproteome. V naši študiji smo izbrali citosolno celično frakcijo, saj leta vsebuje pomembne proteine, ki nakazujejo različne mehanizme odziva celice. Pri tem so nas zanimali predvsem proteini, pri katerih pride do sprememb v izražanju in so vključeni v energetski metabolizem ali metabolizem maščobnih kislin in njegovo regulacijo. Vsi trije našteti sklopi proteinov so prisotni v citosolu celice. Posebno pozornost smo namenili

proteinom, ki sodelujejo pri metabolizmu maščobnih kislin, saj smo na podlagi znanstvene literature (Mortimer in sod., 2011; Tilton in sod., 2013) in lastnih raziskav (Rajapakse in sod., 2012) predvidevali, da je le-ta vključen v zgodnji odziv celic pri izpostavitvi nanodelcem TiO_2. To je v skladu z vlogo maščobnih kislin v celicah, saj poleg zagotavljanja energije sodelujejo tudi pri celični signalizaciji s spremembo v strukturi membran, vplivajo stanje lipidnih modifikacij na proteinih in na aktivnosti jedrnih receptorjev (Wolfrum, 2007). Vsi opisani procesi so lahko del zgodnjega odziva na spremenjene okoljske pogoje.

Ena od prednosti pri frakcionaciji celičnega proteoma je zmanjšanje kompleksnosti vzorca, saj se zaradi odstranitve proteinov celičnega skeleta in migetalk, ki so bogato zastopani, s tem poveča verjetnost detekcije sprememb v izražanju proteinov, ki so v celici prisotni v majhnih koncentracijah.

Ta del raziskav je nadaljevanje ugotavljanja sprememb, ki jih nanodelci TiO_2 povzročajo pri praživali *T. thermophila*. Naš glavni namen je ugotoviti katere biokemijske poti so vključene v zgodnji odziv *T. thermophila* na delce TiO_2, še preden pride do oksidativnega stresa. Predpostavljamo, da bomo z izpostavitvijo *T. thermophila* nanodelcem TiO_2 pri subtoksičnih koncentracijah, zabeležili nano-specifični zgodnji odziv. Pri tem bomo opazili spremembe spremenjenega fiziološkega stanja celic, stresni odziv ali nanospecifični odziv celic. Na podlagi znanstvene literature (Mortimer in sod., 2011; Tilton in sod., 2013) in naših predhodnih raziskav (Rajapakse in sod., 2012) pričakujemo spremenjeno izražanje proteinov, ki so vključeni energetski metabolizem ali metabolizem in regulacijo maščobnih kislin. Izbrali smo koncentracije, ki na praživa l *T. thermophila* niso imele toksičnega vpliva, niso povzročile sprememb koncentracije ATP, niti oksidativnega stresa, ki smo ga ocenjevali z lipidno peroksidacijo (Rajapakse in sod., 2012).

1.2 MODELNA EVKARIONTSKA MIKROORGANIZMA

1.2.1 Pražival *Tetrahymena thermophila*

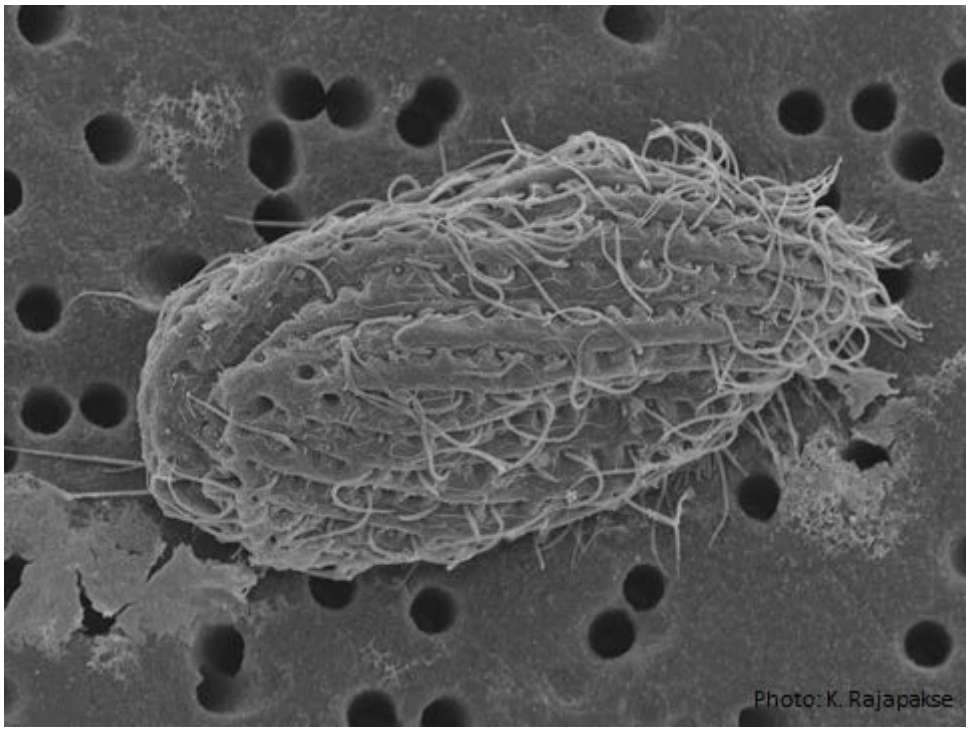
T. thermophila je ekološko evrieka sladkovodna vrsta migetalkarjev, ki jo sistematsko umeščamo v razred Oligohymenophorea, podrazred Hymenostomia, red Hymenostomatida, podred *Tetrahymenina* (Corliss, 1994).

T. thermophila laboratorijsko že več desetletij služi kot modelni evkariontski mikroorganizem v številnih molekularno-bioloških, biotehnoloških in toksikoloških študijah. Številne raziskave so pokazale, da sta vrsti *T. thermophila* in *T. pyriformis* ustrezna in zanesljiva modela za oceno toksičnosti snovi kot so insekticidi, fungicidi, mikotoksini, kancerogene snovi, organska onesnaževala, težke kovine in farmacevtske učinkovine (Sauvant in sod., 1999).

Po letu 2009 se je pražival *T. thermophila* uveljavila tudi kot modelni organizem v nanotoksikologiji (Mortimer in sod., 2010; Mortimer in sod., 2011; Rajapakse in sod., 2013; Rajapakse in sod., 2012; Kim in sod., 2010; Shi in sod., 2012; Bondarenko in sod., 2013).

V naši študiji smo jo kot modelni organizem za ugotavljanje potencialnih toksičnih učinkov nanodelcev izbrali zaradi naslednjih lastnosti:

- *T. thermophila* je enocelični evkariontski mikroorganizem in kot tak potencialna alternativa testiranju na živalih (Dayeh in sod., 2005),
- Kratek generacijski čas in aksenični pogoji gojenja praživali *T. thermophila* so še posebej ugodni pri proteomskih in membranskih študijah.
- V primerjavi s kvasovkami, mikroalgami in bakterijami *T. thermophila* nima celične stene, ki je ovira pri prehajanju onesnažil, zato so zelo občutljiv modelni organizem za preučevanje različnih onesnažil in strupenih snovi,
- Genom tega mikroorganizma je bil sekvenciran in objavljen v prostem dostopu (Database T. G.; Tetrahymena Genome Database; www.ciliate.org).
- Geni obravnavanega mikroorganizma so na nivoju aminokislin bolj podobni genom človeka kot genom drugih evkariontskih mikrobnih celic (Turkewitz in sod., 2002).
- Proteom tega mikroorganizma je dobro poznan; proteom mitohondrija (545 proteinov) (Smith in sod., 2007), proteom fagosoma (73 proteinov) (Jacobs in sod., 2006) in proteom cilioma (223 proteinov cilij - migetalk) (Smith in sod., 2005).
- Prehranjuje se z bakterijami, tako suspendiranimi kot tudi pritrjenimi v biofilmih (Hahn in Hofle, 2001; Jurgens in Matz, 2002; Sherr in Sherr, 2002), torej ima pomembno vlogo v vodnem prehranjevalnem spletu.



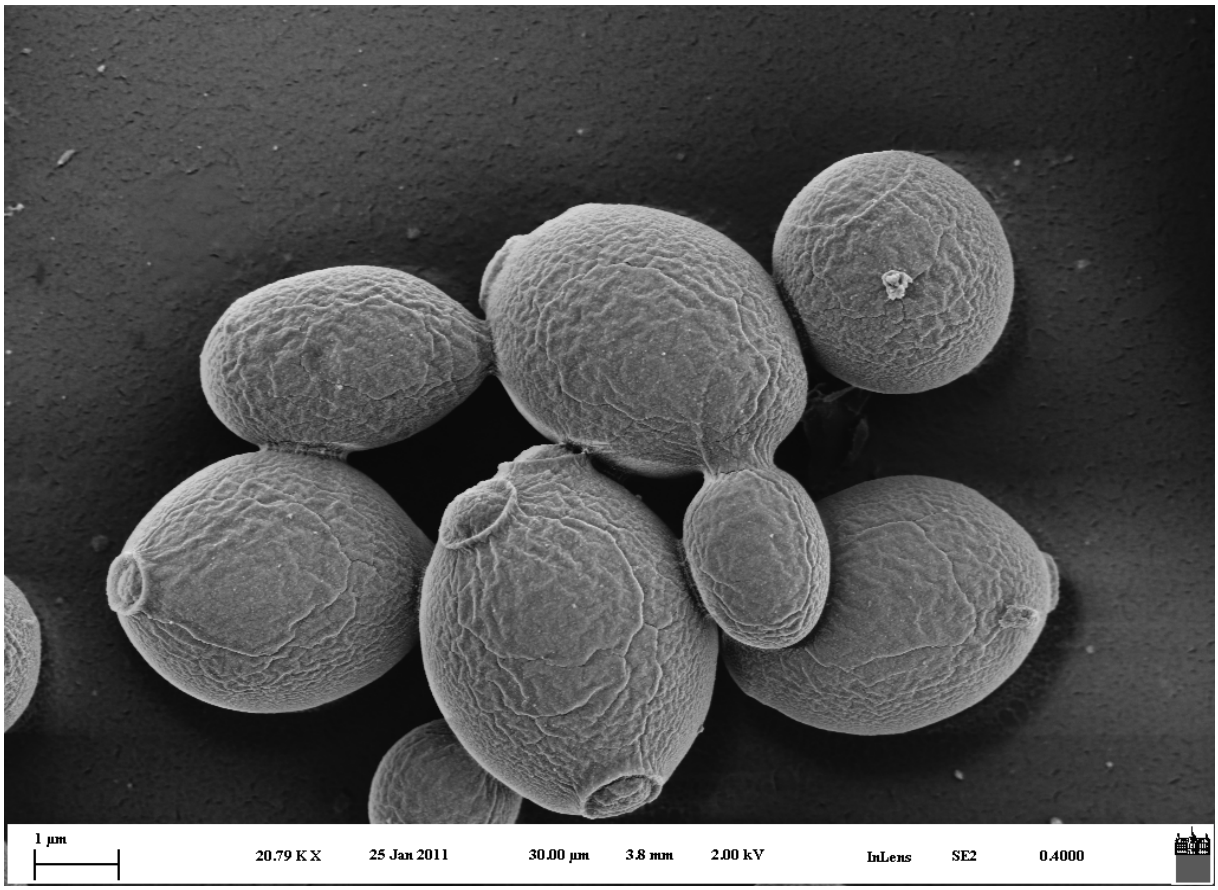
Slika 2: Slika praživali *Tetrahymena thermophila* posneta s SEM mikroskopom (Foto: Matej Hočevar), premer posamezne pore je 3 µm.
Fig. 2: *Tetrahymena thermophila* protozoan photographed with SEM microscopy (Photo by Matej Hočevar). Pore diameter is 3 µm.

1.2.2 Kvasovka *Saccharomyces cerevisiae*

Kvasovke so enocelične askomicetne ali bazidiomicetne glive, ki se nespolno razmnožujejo z brstenjem ali delitvijo, pri spolnem razmnoževanju pa ne tvorijo večceličnih tvorb (Wery in sod., 1996). Taksonomsko kvasovko *S. cerevisiae* umeščamo med askomicete (družina: *Saccharomycetaceae*, poddružina: *Saccharomycetoideae*, rod: *Saccharomyces*) (Walker, 1999). Kvasovke *S. cerevisiae* so med najbolj intenzivno preučevanimi enoceličnimi evkariontskimi organizmi na področju molekularne in celične biologije. Glavni razlog za to je v tem, da je njihova celična struktura in funkcijska organizacija zelo podobna celicam organizmov višjih organizacijskih nivojev (Gromozova in Voychuk, 2007).

V zadnjem desetletju se je kvasovka uveljavila tudi kot pomemben organizem v toksikologiji. Kot modelni evkariontski organizem se pojavlja v najrazličnejših študijah toksičnosti, npr.: težkih kovin (Kungolos in sod., 1999; De Freitas in sod., 2004; Schmitt in sod., 2004; Lin in sod., 2011; Gardarin in sod., 2010), zdravil proti raku (Buschini in sod., 2003), herbicidov (Cabral in sod., 2003), fungicidov (Dias in sod., 2010) in sredstev za konzerviranje hrane (Kasemets in sod., 2006). Nesporno se kvasovka od leta 2009 pojavi

tudi kot modelni organizem v nanotoksikoloških študijah (Kasemets in sod., 2009; Garcia-Saucedo in sod., 2011; Bayat in sod., 2013; Nomura in sod., 2013).
V naši študiji smo kvasovko *S. cerevisiae* uporabili kot modelni evkariontski organizem za primerjavo rezultatov genotoksičnosti in citotoksičnosti s tistimi pri praživali *T. termophila*.



Slika 3: Slika kvasovke *Saccharomyces cerevisiae* posneta s SEM mikroskopom (Foto: Gorazd Stojkovič in Marjan Marinšek).
Fig. 3: Photograph of *Saccharomyces cerevisiae* yeast taken with SEM microscope (Photo by Gorazd Stojkovič and Marjan Marinšek).

1.3 NANODELCI, KI SMO JIH TESTIRALI V DOKTORSKI RAZISKAVI

V doktorski nalogi smo za študije interakcij nanodelcev s celico v treh raziskavah uporabili nanodelce TiO_2 in delce TiO_2 večje od 100 nm. V eni od študij smo poleg nanodelcev TiO_2 uporabili tudi druge nanodelce (ZnO, CuO, Ag, SWCNT) in delce večje od 100 nm (ZnO, CuO, Ag). Razlog za dodatno izbrane nanodelce je njihova široka uporaba v industriji, njihova vse večja prisotnost v komercialno dostopnih izdelkih in uporaba v medicinske namene. Zaradi njihove pogoste uporabe je ugotavljanje njihovih bioloških učinkov ali morebitne strupenosti velikega pomena za človeka in okolje.

1.3.1 Nanodelci titanovega oksida (nanodelci TiO_2)

Nanodelci TiO_2 so kemijsko in toplotno stabilni (Xu in sod., 2009). Uporabljajo jih za različne aplikacije: kot UV zaščitno sredstvo v sončnih kremah, za fotokatalitično čiščenje vode, v kozmetični, farmacevtski industriji ter industriji barvil (Trouiller in sod., 2009; Wang in sod., 2009). V prihodnosti se njihova uporaba predvideva v nanomedicini kjer bodo služili kot nosilci zdravil (Vandghanooni in Eskandani, 2011) pa tudi pri izdelavi nove generacije solarnih celic (Aruoja in sod., 2009).

1.3.1.1 Biološki učinki nanodelcev TiO_2

Nanodelci TiO_2 so kemijsko in temperaturno stabilni, zato velja splošno mnenje da predstavljajo majhno tveganje za organizme (Xu in sod., 2009). Vendar pa se na podlagi podatkov o strupenostnih učinkih nanodelcev TiO_2, širokega spektra uporabe in sproščanja v okolje, veča verjetnost tveganja za okolje in človeka (Iavicoli in sod., 2011; Lee in sod., 2007).

Nanodelci TiO_2 predstavljajo modelni anorganski kovinski oksid pri *in vivo* in *in vitro* študijah toksičnega učinka nanomaterialov na okolje in zdravje človeka (Zhang in sod., 2007; Iavicoli in sod., 2011; Lee in sod., 2007). Številne opravljene študije poročajo o potencialnih toksičnih učinkih nanodelcev TiO_2, vendar si njihovi zaključki nasprotujejo. Večina raziskav poroča o nastanku reaktivnih kisikovih zvrsti (ROS), tem pa sledijo različni tipi učinkov na celice, kot so oksidativni stres, citotoksičnost, vnetje in drugi (glej (Iavicoli in sod., 2011)).

Nekateri avtorji menijo, da oksidativni stres ni edini mehanizem delovanja nanodelcev na celice. Banaszak (2009) in Pal in sod. (2007) so poročali o neposrednih interakcijah med membranskimi lipidi in nanodelci, drugi (Gurr in sod., 2005; Wang in sod., 2009) pa destabilizacijo membrane pripisujejo lipidni peroksidaciji, ki je posledica oksidativnega stresa. Amezaga - Madrid in sod. (2003) poročajo, da so nanodelci TiO_2 povzročili

destabilizacijo celične membrane zaradi fotokatalitične aktivnosti. Sayes in Warheit (2008) sta destabilizacijo celičnih membran ob izpostavitvi nanodelcem TiO_2 interpretirala kot posledico zmanjšanega membranskega potenciala v mitohondrijih, zaradi katere pride do sprostitve encima laktat dehidrogenaza, ki je povezan s pojavom »puščajočih membran« (leaky membranes). Kot možen mehanizem delovanja je zmanjšano mitohondrijsko aktivnost zaradi vpliva nanodelcev TiO_2 potrdil tudi Barillet in sod. (2010). Na podlagi rezultatov Barillet in sod. (2010) zaključuje, da ni neposredne povezave reaktivnih kisikovih zvrsti s citotoksičnostjo TiO_2, zato oksidativni stres najverjetneje ni edini mehanizem delovanja nanodelcev kovinskih oksidov.

Obsežna znanstvena literatura, ki obravnava ekotoksikološke učinke nanodelcev TiO_2 v *in vivo* sistemih, možnih mehanizmov delovanja nanodelcev ne navaja (Menard in sod., 2011). Nekateri avtorji pa zaključujejo, da biološki učinki in mehanizmi škodljivih učinkov nanodelcev TiO_2 na organizme niso razjasnjeni (glej Valant in Drobne, 2012; Gao in sod., 2011), niti ni jasno ali so posledica velikosti, koncentracije ali sekundarnih lastnosti nanodelcev TiO_2 (Magdolenova in sod., 2013).

Še redkejše pa so objave o učinkih in o mehanizmih, ki potekajo ob interakcijah nanodelcev TiO_2 s celicami preden pride do stresa (Ma in sod., 2009; Gao in sod., 2011). Z napredovanjem "omskih" orodij, se odpirajo nove možnosti in razsežnosti pri raziskavah molekularnih mehanizmov interakcij nanodelcev s celicami. Analiza diferencialnega izražanja proteoma je ključni visoko zmogljivi pristop, ki omogoča vpogled v celične procese ob izpostavitvi celic nanodelcem (Haniu in sod., 2009), ne glede na obseg učinkov. Genomika, transkriptomika, proteomika in metabolomika so široko uporabljani pristopi, tako pri toksikoloških študijah, kot tudi pri ugotavljanju biomarkerjev bolezni in pri razjasnjevanju mehanizmov delovanja kemikalij v živih organizmih (Witzmann in Monteiro-Riviere, 2006; Haniu in sod., 2010), zato so pomembno orodje tudi pri študiji učinkov nanodelcev.

1.3.2 Nanodelci cinkovega oksida (nanodelci ZnO)

Nanodelci cinkovega oksida se zaradi svojih specifičnih lastnosti uporabljajo pri proizvodnji barv in UV zaščitnih sredstev ter kot nosilci zdravilnih učinkovin (Zhang in sod., 2012; Zhang in sod., 2013; Sultana in sod., 2012).

1.3.3 Nanodelci bakrovega oksida (nanodelci CuO)

Nanodelci bakrovega oksida se zaradi svojih specifičnih lastnosti uporabljajo v znanosti in različnih tehnoloških procesih. Zaradi učinkovite termične in električne prevodnosti se uporabljajo za izdelavo elektronskih vezij, baterij, plinskih senzorjev in tekočin za prenos

toplote (Chen in sod., 2012). Ker delujejo tudi antibiotično, se uporabljajo tudi v različnih protimikrobnih pripravkih (Aruoja in sod., 2009; Gomes in sod., 2012).

1.3.4 Nanodelci srebra (nanodelci Ag)

Nanodelce srebra veliko uporabljajo v tekstilni in prehrambeni industriji, uporabljajo jih pri proizvodnji barv, v kozmetiki, elektroniki in pri izdelovanju medicinskih pripomočkov (Cohen in sod., 2007; Lee in sod., 2007; Vigneshwaran in sod., 2007). Pomembni so zlasti zaradi protibakterijskega učinka, ki ga imajo srebrovi ioni in s srebrom obložene površine (Russell in Hugo, 1994; Silver, 2003; Klasen, 2000).

1.3.5 Ogljikove nanocevke (SWCNT)

Od njihovega odkritja naprej jih zaradi njihovih edinstvenih mehanskih, termičnih, fotokemijskih lastnosti in prevodnosti intenzivno uporabljajo pri izdelavi katalizatorjev in pri shranjevanju energije (Ilijima, 2006; Kumar in sod., 2011).

1.4 HIPOTEZE

Nanodelci lahko reagirajo z biološkimi sistemi in so zato potencialno nevarni, kar nakazujejo tudi številne študije. Bioreaktivnost po definiciji povezujemo s kinetiko reakcij, v doktorski nalogi pa smo se osredotočili na bioaktivnost delcev, kar pomeni vpliv, ki ga imajo le-ti na živ organizem ali tkivo. V naših študijah smo poglobljeno raziskovali učinke interakcij nanodelcev s celico, in sicer na nivoju membrane, DNA in proteinov.

V treh raziskavah smo uporabljali karakterizirane nanodelce TiO_2, pri eni študiji pa tudi nanodelce cinkovega oksida (ZnO), nanodelce bakrovega oksida (CuO), nanodelce srebra (Ag) in ogljikove nanocevke (SWCNT).

Študije so potekale na modelnem mikroorganizmu, enoceličnem migetalkarju *Tetrahymena thermophila*. Izbran modelni mikroorganizem se že več desetletij uporablja v različnih (eko)toksikoloških, molekularnih in celičnih raziskavah. Posledično je na voljo ogromno ekotoksikoloških in toksikoloških podatkov, ki so bili pridobljeni v teh študijah in jih bomo s pridom uporabili pri oblikovanju poskusov in razlagi rezultatov. Pri eni od študij smo želeli določena spoznanja preveriti na evkariontskem mikroorganizmu, kvasovki *Saccharomyces cerevisiae*.

Namen raziskovalnega dela je bil ugotoviti vplive, ki so posledica interakcije med nanodelci in celicami praživali *Tetrahymena thermophila*. Preveriti smo želeli naslednje delovne hipoteze:

a) Nanodelci TiO_2 vstopajo v interakcijo z različnimi komponentami celice praživali *Tetrahymena thermophila*. Učinki/Vplivi bodo merljivi na celični membrani, v izražanju citosolnih proteinov in na celičnem genetskem materialu (DNA).

b) Modelni organizem *T. thermophila* se bo na nanodelce TiO_2 odzval drugače kot na delce TiO_2 večje od 100 nm in odziv ne bo odvisen od koncentracije.

c) Izpostavitev mikroorganizma *T. thermophila* nanodelcem TiO_2 bo povzročila spremembe fiziološkega stanja, stres ali nanospecifični odziv.

Z delom bomo prispevali k razumevanju interakcij nanodelcev s celicami na molekularnem in celičnem nivoju in k razumevanju delovanja nanodelcev na organizem, bodisi da je to delovanje kvarno ali pa ne.

Rezultati bodo služili nadaljnjim raziskavam in novim spoznanjem na področju interakcij med nanodelci in biološkimi sistemi ter varnejši in bolj učinkoviti proizvodnji nanomaterialov za medicinsko, farmacevtsko in prehrambno uporabo.

2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Aklimacija protozoja *Tetrahymena thermophila* ob izpostavitvi nanodelcem in delcem večjim od 100 nm TiO_2 s spremembo profilov dolgoverižnih maščobnih kislin v membrani

Acclimation of *Tetrahymena thermophila* to bulk and nano-TiO_2 particles by changes in membrane fatty acids saturation

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V naši študiji smo eksperimentalno dokazali, da pri izpostavitvi mikroorganizma *Tetrahymena thermophila* nanodelcem TiO_2 in delcem TiO_2 večjim od 100 nm ne pride do nastanka reaktivnih kisikovih zvrsti ali do lipidne peroksidacije, pride pa do sprememb v profilu dolgoverižnih maščobnih kislin. Ugotovljene spremembe smo razložili kot aklimacijo na neugodne okoljske razmere in ne kot toksični učinek delcev TiO_2. Celice mikroorganizma *T. thermophila* smo izpostavili delcem TiO_2 za 24 ur pri 32°C. Spremembe v profilu dolgoverižnih maščobnih kislin so pokazale povečano rigidnost membrane. Velikost delcev TiO_2 (nanodelci in delci večji od 100 nm) ni vplivala na velikost sprememb profilov dolgoverižnih maščobnih kislin. Opazili smo reverzibilno polnjenje prebavnih vakuol z delci TiO_2, ki je bilo odvisno od velikosti delcev TiO_2. Naši rezultati kažejo, da so interakcije med delci in celično membrano neodvisne od oksidativnega stresa.

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Acclimation of *Tetrahymena thermophila* to bulk and nano-TiO_2 particles by changes in membrane fatty acids saturation

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ABSTRACT

We provide experimental evidence that changes in the membrane fatty acid profile of *Tetrahymena thermophila* incubated with nano- or bulk TiO_2 particle are not accompanied by ROS generation or lipid peroxidation. Consequently these changes are interpreted as acclimation to unfavorable conditions and not as toxic effects. *T. thermophila* cells were exposed to TiO_2 particles at different concentrations for 24 h at 32 °C. Treatment of cultures with nano- and bulk TiO_2 particles resulted in changes of membrane fatty acid profile, indicating increased membrane rigidity, but no lipid peroxidation or ROS generation was detected. There were no differences in membrane composition when *T. thermophila* was exposed to nanosized or bulk-TiO_2 particles. We also observed reversible filling of food vacuoles, but this was different in case of nano- or bulk TiO_2 exposure. Our results suggest that interactions of particles and cell membranes are independent of oxidative stress.

1. Introduction

There have been many studies on the effects of nanosized TiO_2 (nano-TiO_2) on aquatic and terrestrial organisms [1,2]. The most frequently studied consequences of exposure to nano-TiO_2 are cytotoxicity and genotoxicity *via* oxidative stress [3,4].

Irrespective of the exposure route, the first contact between the cells and nanoparticles must involve the cell membranes [5]. Accordingly, we investigated whether a suspension of TiO_2 particles could affect the cell membrane composition of a eukaryotic microorganism *Tetrahymena thermophila* in the absence of light (when ROS generation due to catalytic activity of TiO_2 was expected to be minimized) and whether this is evidence of a toxic response or of acclimation to unfavorable environmental conditions. A significant decrease in membrane fluidity after exposure of *Tetrahymena* sp. to TiO_2 has already been documented [6,7].

There are numerous reports on functional alterations of cell membranes occurring under unfavorable environmental conditions involving for example, temperature or chemicals. Bearden et al. [8] and Schultz et al. [9], described membrane fatty acid profile alterations in *Tetrahymena* sp. resulting from exposure to chemicals, such as pentachlorophenol and 1-octanol, acting noncovalently. Shug et al. [10] detected a marked effect of iron ions on the desaturation of fatty acids in the membrane of *T. thermophila*. When exposed to methyl mercury, small but distinct changes in the profile of membrane fatty acids were detected (personnel communication) and it was suggested that profiling of fatty acid methyl esters (FAME-s) could be used for identification of different groups of chemicals in *T. thermophila*. The study of effects of an aqueous suspension of fullerene (C60) on bacteria also showed changes in lipid composition which were dependent on the C60 concentration [11]. The same authors also successfully employed profiling of FAME-s. Alterations in membrane lipid profiles have been interpreted as a physiological adaptation or an acclimation to extreme conditions [10,12–14]. Mortimer et al. [7] demonstrated changes in the fatty acid profile of protozoon *T. thermophila* exposed to nano-CuO.

Functional alterations of cell membranes could also be interpreted as acclimation to unfavorable conditions. However, acclimation is not linked only to membranes. This phenomenon acclimation has been defined as a short-term phenotypic change, which allows survival in suboptimal environmental conditions, including pollution [8,15,16]. When suboptimal conditions result from exposure to chemicals, the primary stress response of an organism compensates for the potential adverse effects on cells, but with elevated concentrations of chemicals and prolonged exposure

Abbreviations: RM, nutrient rich medium; PM, nutrient poor medium.

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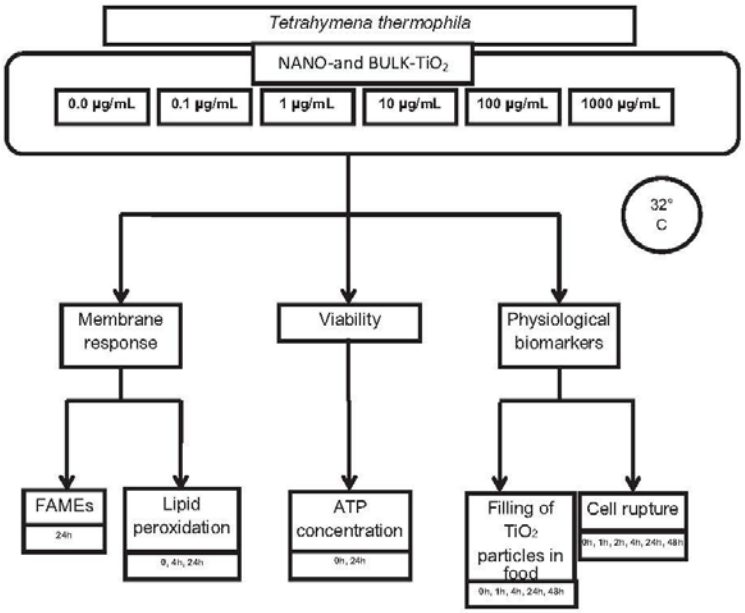

Fig. 1. Experimental arrangement. Three major toxicity endpoints were studied: changes in cell membrane, viability and physiological responses of *T. thermophila* to different exposure concentrations of TiO_2 particles. The culture of *T. thermophila* was preincubated in the PM for 24 h before beginning the exposure to TiO_2. Changes in membrane composition were assessed by FAME-s method (at 0.1, 10 and 1000 µg TiO_2/ml) and by the extent of lipid peroxidation (measuring MDA content). Viability was assessed by ATP concentration. Two physiological biomarkers distinctive of *T. thermophila*, namely filling of TiO_2 particles in food vacuoles and cell rupture were also assessed as described by Dai et al. [24].

to them, toxic effects are unavoidable. An association of alterations in membrane structure with cytotoxicity has been reported by Clarke et al. [17] for human breast cells and also by Mortimer et al. [7] for *T. thermophila*. So far, *Tetrahymena pyriformis* has been shown when exposed to temperature changes, to respond through changes in membrane fatty acid profiles [15] and to organic chemicals considered to act *via* a nonpolar toxic action [16] [8]. Very recently, changes in membrane lipid composition in terms of lowering membrane fluidity were demonstrated by Mortimer et al. [7] and explained as an adaptation mechanism to exposure to CuO nanoparticles [7]. These authors studied the effects of toxic concentrations of nano-CuO on the membrane of *T. thermophila* [7].

The aim of this research was to assess the total membrane fatty acid profile of *T. thermophila* after exposure to TiO_2 particles in a range of concentrations. Organisms were exposed to particles *via* food as well as substratum. In parallel experiments, some additional biomarkers such as lipid peroxidation, ATP concentration, cell morphology and filling of particles in vacuoles were analyzed in an attempt to correlate changes in membrane fatty acid saturation with potential cytotoxic effects. The effects of nano- and bulk TiO_2 particles were compared. We hypothesize that if changed membrane fatty acid profile of *T. thermophila* exposed to TiO_2 particles is not accompanied by a cytotoxic response this is direct evidence of acclimation to the particles present in media and not an indication of particle toxicity.

2. Materials and methods

2.1. Chemicals

All reagents were purchased from Sigma Aldrich Co. (St. Louis, MO, USA), Merck (Darmstadt, Germany) or Biolife (Milan, Italy), unless specifically stated otherwise.

2.2. T. thermophila growth and exposure conditions

Axenic cultures of *T. thermophila* from the Protoxkit F™ (MicroBioTests Inc.) were grown for 24 h in the dark at 32 °C in a semidefined proteose-peptone based medium [18] – a nutrient rich medium; RM. The nutrient rich medium contains 5 g D-glucose, 5 g proteose-peptone, 1 g yeast extract, 1.2 g Trisma-base, chlorides (2.28 µM $CaCl_2 \cdot 2H_2O$, 0.29 µM $CuCl_2 \cdot 2H_2O$, 0.05 µM $FeCl_3 \cdot 6H_2O$, 0.03 µM $MnCl_2 \cdot 6H_2O$, 0.004 µM $ZnCl_2$) and sulphates (4.1 µM $MgSO_4 \cdot 7H_2O$, 0.64 µM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$), up to 1000 ml doubly distilled H_2O, pH corrected to 7.35 with HCl. The cell density obtained in these culture conditions was approximately 10^5 cells/ml.

The cells were harvested by centrifugation (3 min, 60 rcf), washed and resuspended in a medium specifically modified for this experiment: semidefined proteose-peptone based medium by Schultz [18] lacking yeast extract and bacteriological peptone – a nutrient poor medium; PM. This nutrient poor medium contains 5 g D-glucose, 1.2 g Trisma-base, 1000 ml doubly distilled H_2O. The pH of the medium was adjusted to 7.4 with HCl and temperature was held constant at 32 °C for the entire experiment. All experiments were performed in batch cultures of 100 ml in Erlenmeyer flasks, and aerated by shaking (90 rpm) in darkness.

After 24 h in the PM, cells were treated with bulk or nano-TiO_2. The final concentrations of particles in the medium were: 0.1, 1, 10, 100, 1000 µg/ml. Following the addition of TiO_2, *T. thermophila* cultures were incubated at 32.0 °C for 48 h. Assays of ATP concentration, total protein concentration, filling of vacuoles with TiO_2 and morphological characterizations were performed at several time intervals (Fig. 1). For each concentration of nano- or bulk TiO_2, three independent assays were carried out. A supplementary set of three replicates, without TiO_2 particles, was set up for each assay as a control.

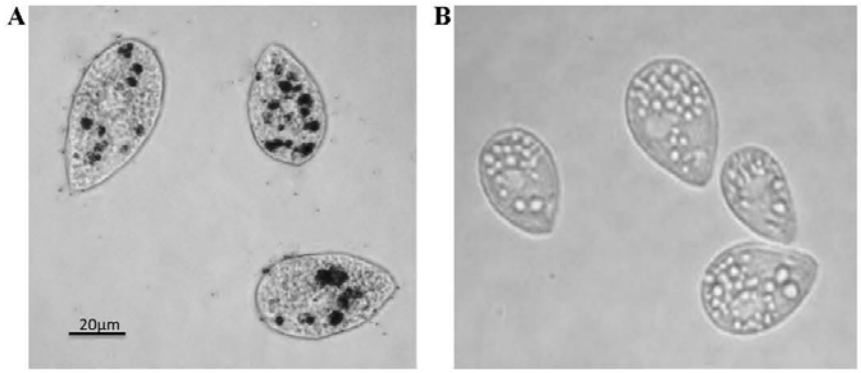

Fig. 2. Food vacuoles filled with particles were first visible under a light microscope after 4 h at exposure concentrations higher than 10 µg/ml of TiO_2 (A). Control cells with visible vacuoles, however were not filled with dark matter (TiO_2) (B).

2.3. Bulk and nano-TiO_2 tested suspension

The TiO_2 nanoparticles were supplied in the form of a powder with guaranteed 99.7% purity having the following characteristics provided by the manufacturer: Anatase crystalline structure; average particle size 15 nm; and surface area, 190–290 m^2/g. Bulk and nano-TiO_2 particles were dispersed in PM using bath sonication for 30 min.

The dispersions of nanoparticles (1000 µg/ml) were inspected by dynamic light scattering (DLS) using a 3D DLS–SLS (dynamic light scattering–static light scattering spectrometer; LS Instruments, Fribourg, Switzerland). This allows the determination of hydrodynamic radii of particles in extremely turbid suspensions by a so-called 3D cross-correlation technique that successfully eliminates multiple scattering of light. As the light source a HeNe laser operating at a wavelength of 632.8 nm was used. Scattering was measured at an angle of 90°.

Zeta potentials of nano-TiO_2 suspensions (1000 µg/ml) were measured with ZetaPals (Brookhaven Instrument Corporation) in the PM medium, and used to assess the exposure to living cells. Zeta potentials were measured at different pH values, adjusted by adding NaOH or HCl to the suspension.

2.4. Assessment of cellular fatty acid composition by gas chromatography

T. thermophila cells were harvested by centrifugation, pellets were resuspended in sterile doubly distilled water (1 ml), frozen at −20 °C and then lyophilized. Dried samples were pulverized and transferred to HACH screw cap test tubes. First, the sample was mixed with hexane (0.5 ml) and then 1.5 M HCl in MeOH (1 ml) and pure MeOH (1 ml) were added. The test tubes were filled with N_2 and incubated at 80 °C for 10 min. The reaction was stopped by cooling the tubes in ice. Following the addition of doubly distilled water (2 ml), each reaction mixture was vigorously mixed for 1 min and centrifuged (30 s, 670 rcf). The organic phase containing FAMES extracted in hexane was transferred to a clean vial and filled with N_2. The samples were stored at −20 °C until analysis.

Fatty acid methyl esters (FAME-s) were separated by capillary gas chromatography using Omegawax TM 320 (30 m × 0.32 mm ID × 0.25 mm) capillary column with polyethylene glycol as the stationary phase. Helium was used as a carrier gas with a flow rate of 2.0 ml/min, and a split ratio of 10:1. The initial temperature for analysis was 185 °C and the final temperature was 215 °C with a temperature increase rate of 1 °C/min. The run time was 54 min and volume injected was 2 µl. Identification of fatty acid methyl esters was achieved by comparison of retention times and results were calculated using response factors derived from chromatographic standards of known composition (Nu Chek Prep, GLC-85, Nu-Chek Prep Inc., Elysian, USA). The gas chromatography system used was an Agilent 6890 series GC equipped with Agilent 7683 Automatic Liquid Sampler, 7683 Injector and FID detector.

Results were analyzed using ChemStation Plus® software. Membrane fatty acids which were present as less than 0.5% of total fatty acids were designated as "trace fatty acids" and were not considered further. The data were analyzed statistically applying Student's *t*-test with significance level of 0.05.

2.5. Assessment by MDA measurement of the extent of lipid peroxidation

Lipid peroxidation was measured as the formation of malondialdehyde (MDA) in *T. thermophila* samples at 32 °C after 0, 4 and 24 h of incubation [19]. Cells were homogenized and total protein concentration was measured spectrophotometrically at 280 nm, and used as a measure of biomaterial in the experiments. For measurement of MDA concentration, 500 µl of homogenized sample was mixed with 500 ml of buffer A (30% trichloroacetic acid, 0.75% 2-thiobarbituric acid, 0.5 M HCl and 0.02% butylated hydroxytoluene), incubated at 90 °C for 30 min, then chilled on ice. n-Butanol (1.5 ml) was mixed with the sample and centrifuged (10 min, 6700 rcf). The absorbance of the resulting chromophore was measured at 535 nm and 600 nm. The latter was subtracted from the former to correct for nonspecific turbidity. The concentration of MDA was calculated using an extinction coefficient of 156 mM^{-1} cm^{-1} [19]. For statistical analysis, each concentration of MDA was divided by the total protein concentration of the corresponding sample.

2.6. Viability assessment by ATP assay

Adenosine 5′-triphosphate (ATP) Bioluminescent Assay Kit (Sigma Aldrich, Germany) was employed for the quantitative bioluminescent determination of ATP. For ATP extraction, protozoa culture samples (100 µl) were added to boiling extraction buffer (900 µl), as described previously [20], 0.1 M Tris and 2 mM EDTA were added and the pH was adjusted to 7.8 with acetic acid [21]. The relative light units (RLU) were recorded in JUNIOR LB 9509 (Berthold technologies). The ATP concentration (measured in µmol ATP/l) was calculated using the equation:

$$[ATP] = 10^{(\log RLU - b)/a} \times 10$$

where a and b are factors calculated from the calibration curve with a correlation coefficient $R = 0.99$.

2.7. Assessment by light microscope of percentage of T. thermophila cells containing at least one vacuole filled with TiO_2

Total and dead cell numbers were estimated by conventional direct microscopic counting under observation at 200 times magnification in a Neubauer chamber. The uptake of TiO_2 by food vacuoles (Fig. 2) was studied from two perspectives: treatment with several TiO_2 exposure concentrations (at 0.1, 1, 10, 100 and 1000 μg TiO_2/ml) and with different time exposures (0, 1, 2, 4, 24 and 48 h). A 10 μl sample of culture was put into a Neubauer chamber, and dead cells, including non-motile cells and cells with changed morphology were counted, then 5 μl of 4% formalin was added to kill the cells and a total cell count was performed.

The percentage of cells containing at least one food vacuole filled with TiO_2 particles was calculated as follows:

$$\text{Vacuole formation}(\%) = \frac{\text{number of cells containing at least one } TiO_2 \text{ vacuole}}{100 \text{ cells}}.$$

3. Results

3.1. Characteristics of TiO_2 nanoparticle suspensions

The primary and secondary characteristics of the nanoparticles used in the experiments were assessed. Primary characteristics include particle size, shape and crystallinity and have been described previously and the same particles were used in the current experiments [22]. Secondary characteristics are those of nanoparticles in a suspension. In our study, particles were suspended in nutrient poor medium (PM) and analyzed. DLS analysis showed that the average value of the hydrodynamic radius of TiO_2 nanoparticles suspended in test medium was 820 nm. The average particle size of bulk TiO_2 could not be accurately measured by this approach because of the presence of many larger agglomerates.

Zeta potentials of TiO_2 nanoparticle suspension (1000 μg/ml) were measured in the same medium used to expose cells, at different pH values. The Zeta potential recorded at pH 7.4 was −15 mV, which is equivalent to a suspension of incipient stability (Fig. 3).

3.2. Cellular fatty acid composition (FAME-s)

A 24 h exposure of *T. thermophila* to TiO_2 at 32 °C resulted in differences of relative percentages of straight chain saturated, monounsaturated, saturated *iso* and *ante-iso* fatty acids in cell membranes of *T. thermophila* between TiO_2 treated populations and controls (Table 1). A significant increase of straight chain saturated fatty acids in membrane cell lipids was detected in exposed cells (Fig. 4). The increase of saturated fatty acids was mostly due to the increase of 15:0, 16:0, 17:0 and 18:0 fatty acids (data not shown). All these changes were independent of TiO_2 size (nano- or bulk) and exposure concentration (0.1, 10 and 1000 μg TiO_2/ml) but compared to control cells, the proportions of unsaturated fatty acids remained unchanged (Fig. 4).

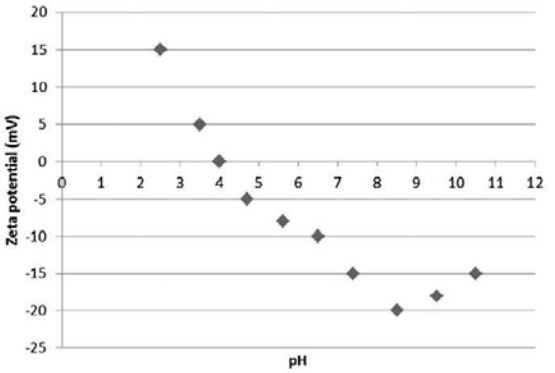

Fig. 3. Zeta potentials of TiO_2 nanoparticle suspensions (1000 μg/ml) measured in the nutrient poor medium and used in experimental exposures to living cells.

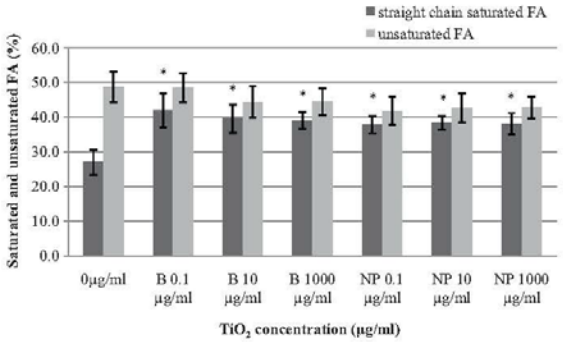

Fig. 4. Changes of straight chain saturated and unsaturated fatty acids in *T. thermophila* after exposure to different concentrations of TiO_2 at 32 °C after 24 h. Bars represent the standard deviation. *Indicates significant differences of straight chain fatty acids from control values ($p < 0.05$). B (bulk concentration), NP (nanoparticle concentration).

Table 1
Percentage composition of membrane fatty acid samples from *T. thermophila* exposed to TiO_2 particles at 32 °C after 24 h. The data are presented as total sums of different types of fatty acids of lipid extracted from the three independent cultures. Percentages are expressed as means ± standard deviation (SD). B (bulk concentration), NP (nanoparticle concentration), FA (fatty acid).

Particle type		Bulk (B)			Nanoparticles (NP)		
Particle concentration (μg TiO_2/ml)	0	0.1	10	1000	0.1	10	1000
Unsaturated FA (%)	48.9 ± 2.5	48.6 ± 3.2	44.3 ± 2.5	44.5 ± 3.9	41.8 ± 3.1	42.7 ± 2.2	42.8 ± 2.2
Monounsaturated FA (1×) (%)	16.3 ± 2.5	18.6 ± 3.3	18.5 ± 2.8	18.2 ± 1.6	23.5 ± 2.9	24.7 ± 2.3	23.0 ± 2.0
Polyunsaturated FA (2×) (%)	8.6 ± 0.8	8.1 ± 0.6	10.0 ± 0.5	8.5 ± 0.3	9.7 ± 0.2	9.3 ± 0.3	11.1 ± 0.2
Polyunsaturated FA (3×) (%)	19.0 ± 4.1	17.6 ± 0.4	19.1 ± 1.3	19.6 ± 2.0	18.4 ± 1.0	17.5 ± 1.6	16.2 ± 0.9
Saturated FA (%)	47.8 ± 3.1	47.5 ± 4.2	44.3 ± 3.5	44.5 ± 1.9	41.8 ± 2.7	42.7 ± 2	42.8 ± 3.1
Straight chain saturated FA (%)	23.1 ± 3.6	42.0 ± 4.9	39.6 ± 4.1	39.0 ± 2.4	37.9 ± 2.6	38.4 ± 2.0	38.1 ± 3.1
Branched chain *iso* and *ante-iso* saturated FA (%)	24.7 ± 3.5	6.5 ± 0.7	4.7 ± 1.1	5.5 ± 0.6	3.9 ± 0.6	4.3 ± 0.6	4.7 ± 0.5
Saturated *iso* FA (%)	8.6 ± 1.1	5.8 ± 0.7	4.6 ± 0.7	5.1 ± 0.5	3.7 ± 0.7	4.1 ± 0.8	4.5 ± 0.6
Saturated *ante-iso* FA (%)	16.1 ± 0.2	0.7 ± 0.1	0.1 ± 0.2	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
Average number of C-atoms in membrane FA	15.3	16.0	15.9	15.5	16.1	16.2	16.0

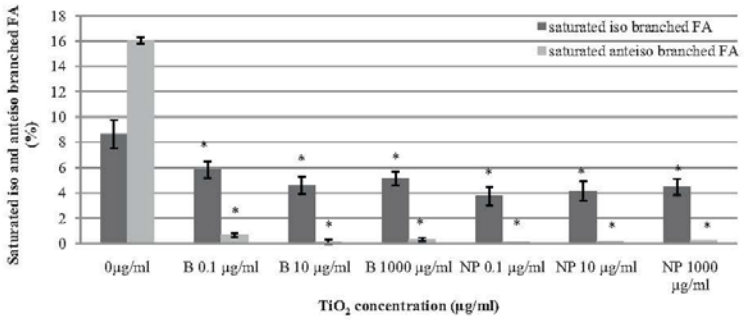

Fig. 5. Changes of portions of *iso* and *ante-iso* branched fatty acids in *T. thermophila* at 32 °C after 24 h. Bars represent standard deviation. *Indicates significant differences from control values ($p < 0.05$). B (bulk concentration), NP (nanoparticle concentration).

A certain proportion of saturated membrane fatty acids has a branched-chain structure, which exist in either *iso* or *ante-iso* forms. These two isoforms are known to differ in their influence on membrane fluidity, and consequently we further analyzed the potential association between the relative amount of each branched-chain isomer and the exposure conditions. The results showed that both isoforms were decreased in cells exposed to bulk or nanoTiO_2 (*iso* mostly on account of 15:0 *iso* and 16:0 *iso* fatty acids) in comparison to controls, regardless of the concentration (data not shown). However, the decrease in *ante-iso* saturated fatty acids was much more pronounced (Fig. 5), mostly on the account of 15:0 *ante-iso* fatty acid (data not shown).

Another difference between controls and TiO_2-treated populations of *T. thermophila* was in the average length of membrane fatty acids (Table 1). An increase in the average length of fatty acids from 15.3 C atoms in control samples to 16.0, 15.9 and 15.5 in cultures treated with bulk TiO_2 in exposure concentrations of TiO_2 of 0.1, 10 and 1000 µg TiO_2/ml, respectively, was noted. An increase to 16.1, 16.2 and 16.0 C atoms in nano-TiO_2 treated cultures (in exposure concentrations of TiO_2 of 0.1, 10 and 1000 µg TiO_2/ml, respectively, was detected (Table 1). Thus, the lipid bilayer in cultures exposed to TiO_2 particles appears, in comparison to controls, to be wider [23], namely from 0.2 to 0.7 C atoms in cultures treated with bulk TiO_2 and from 0.7 to 0.9 C atoms in nano-TiO_2 treated cultures.

3.3. Lipid peroxidation

When *T. thermophila* is exposed to TiO_2, elevation of the amount of MDA, compared to controls, is not detected. The average content of MDA in the control sample is 140 ± 23 nM of MDA per mg of protein.

3.4. ATP concentration in T. thermophila cultures

When *T. thermophila* is exposed to bulk or nano-TiO_2 for 24 h, no significant alterations in ATP could be observed. ATP concentrations were similar to that measured in controls (Fig. 6).

3.5. Filling of vacuoles with TiO_2

Filling of vacuoles with dense material was observed in cells exposed to nano- or bulk TiO_2 present in the media at concentrations higher than 10 µg/ml. At concentrations of TiO_2 particles below 10 µg/ml and in control samples, no vacuoles filled with dark material were observed.

Significant differences were observed in the proportion of cells containing vacuoles filled with TiO_2 that were exposed to bulk TiO_2 as opposed to nano-TiO_2. *T. thermophila* cells exposed to bulk TiO_2 contained a higher percentage of vacuoles filled with TiO_2 than those exposed to nano-TiO_2. This was recorded at all observation times throughout the experiment (Fig. 7). A relatively small amount (<5%) of cells containing vacuoles filled with TiO_2 was observed after 24 h compared to those exposed for 1, 2 or 4 h at the highest exposure concentrations of bulk and nano-TiO_2 indicates clearing of *T. thermophila* after 24 h (Fig. 7).

3.6. Morphological alterations characteristics of T. thermophila

In investigated samples of *T. thermophila* treated with, bulk or nano-TiO_2, the type of cell rupture as described previously by Dai et al. [24] was observed. The proportion of ruptured cells in all experiments was between 0% and 5%. The pattern of cell rupture was not related either to particle exposure concentration, or to the duration of exposure. These cells were enlarged, round shaped and vacuoles appeared more pronounced. The oral apparatus was disrupted and formed a large vacuole, from which the content of cells leaked. This phenomenon was not observed in control samples.

Further, we did not observe either normal or abnormal mature resting cysts in cultures exposed to nano- or bulk TiO_2, as some authors have reported in stress conditions for ciliates [24].

4. Discussion

Our results showed that exposure of *T. thermophila* to nano-TiO_2 or bulk TiO_2 in a range of exposure concentrations of 0.1, 10 and

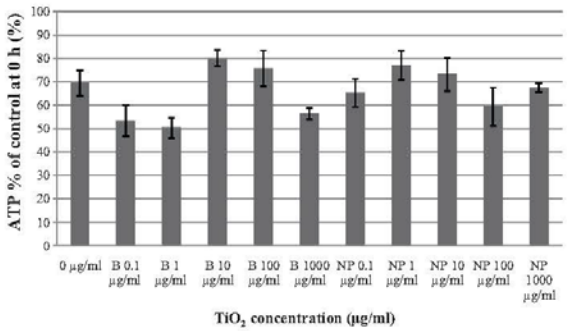

Fig. 6. Variation of ATP concentration (measured in µmol/l) in cultures *of T. thermophila* exposed to TiO_2 for 24 h at 32 °C in one of the two independent assays. The results are expressed as a percentage compared to time (h) of exposure to TiO_2 particles (100% viability). Values were calculated from the averages of three subsamples, standard deviations are given. B (bulk concentration), NP (nanoparticle concentration).

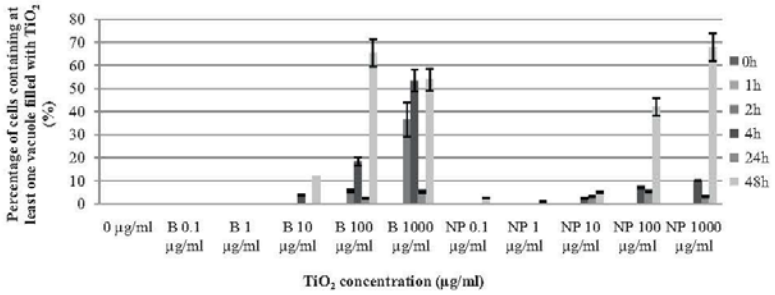

Fig. 7. The percentage of cells containing food vacuoles filled with TiO_2 after exposure of *T. thermophila* to different nano- and bulk TiO_2 exposure concentrations. Data are means of three independent assays. Standard deviations are given. B (bulk concentration), NP (nanoparticle concentration).

1000 µg/ml caused changes in membrane lipid composition but no differences in ROS concentration or lipid peroxidation. Since changes in membrane lipid composition were not correlated with toxicity markers they are interpreted as acclimation to unfavorable conditions, in this case the presence of TiO_2 particles in the medium. However, in groups exposed to either nanoparticles or bulk TiO_2, up to 5% of ruptured cells were observed. Ruptured cells were never observed in control populations. Obviously, for a certain small portion of cells, TiO_2 particles caused destruction.

We were able to detect the effects of TiO_2 particles on cells before toxic responses occurred. Both nano- and bulk TiO_2 caused significant decrease in the ratio of unsaturated to saturated straight chain fatty acids. Further, a significant decrease in percentage of *iso* and *ante-iso* branched fatty acids in cultures treated with nano- and bulk TiO_2 (Fig. 5) was detected. Both structural and geometrical isomers of fatty acids are known to affect membrane fluidity [25] and modes of modulation of membrane fluidity by inducing changes in fatty acid isomerization are well known for bacteria exposed to extreme environmental conditions [26] [27]. Our results are in line with these findings where for example, a decrease of ante-isoforms is reported to lead to a decrease in the fluidity of membranes.

Changes in membrane composition leading to its increased rigidity have been reported often and associated with acclimation to suboptimal environmental conditions, such as rise in temperature [25,28], hyperosmotic stress [29], particles and UV light [6] as well as nanoparticles [7]. Most authors report, that cell membrane rigidity is accompanied by lipid peroxidation [30]. Consequently, it is difficult to differentiate whether the response is acclimation to suboptimal conditions or a result of their adverse effect. In the current study, we succeeded in documenting membrane lipid changes in the absence of any lipid peroxidation. In this way, we were able to confirm that changes in membrane lipid composition represent an acclimation to incubation of cells with both nano- and bulk particles. In our study no lipid peroxidation was detected in cultures with increased membrane rigidity. Observed membrane changes associated with TiO_2 particles and resulting in increase of membrane rigidity. In our experiments the choice of temperature was based on literature data, where optimal growth temperature for *T. thermophila* in the semidefined proteose-peptone based medium is 32 °C [18,31]. Growth of *T. thermophila* at the temperature selected is accompanied by *de novo* fatty acid synthesis, which is necessary in the type of membrane acclimation observed. The degree of change in membrane composition associated with the increase in rigidity indicated was not dose-dependent in relatively wide concentration range, from 0.1 µg/ml to 1000 µg/ml.

In published studies, different mechanisms of interaction between nanoparticles and cell membranes have been proposed. Hussain et al. [32] report that cell membranes associate with nanoparticles, some of which are internalized by the cells. Sayes et al. [33] interprets the affected cell membrane stability (lactate dehydrogenase release) and the decrease in mitochondrial membrane potential, assessed by the MTT method, after nanosized TiO_2 exposure as symptoms of 'leaky membranes', a mechanism in which cytoplasmic membrane rupture is a possible defence mechanism/strategy in case of overload concentrations of particles. Other authors explain disruption of membrane structures and stability either as a result of direct physical interactions between particles and membranes [5] or as a result of lipid peroxidation, itself a consequence of oxidative stress provoked by particles [34,35]. Our results provide evidence that the interactions between TiO_2 particles and *T. thermophila* are independent of oxidative stress.

Ingested particles were observed in food vacuoles where the percentage of cells containing at least one vacuole filled with TiO_2 recorded at exposure concentrations 10, 100 and 1000 µg TiO_2/ml was dose- and time-dependent (Fig. 7). However, the amount of dense material in vacuoles failed to correlate with the detected membrane effect or alter ATP or protein concentration. This suggests that ingestion of nanoparticles and their sequestration into vacuoles does not pose a serious threat to the organism at exposure concentrations up to 1000 µg/ml and exposure times of up to 48 h. Mortimer et al. [36] observed a more rapid uptake by *T. thermophila* of CuO nanoparticles than bulk CuO particles. This is not consistent with our results, where the uptake was higher for the bulk form when compared with equal exposure concentrations of nano-TiO_2. The CuO particle uptake reported by Mortimer et al. [36] was also much faster (requiring only 4 h) when compared to uptake of TiO_2, and further, in our study, cells were never completely filled with particles as they were in the CuO study.

We observed clearing of vacuoles filled with TiO_2 after 24 h of exposure. There are no similar previous reports on nanoparticle ingestion and clearing by protozoa, and we consider clearing to be a phase of feeding activity. Initially, a high percentage of cells containing at least one TiO_2-filled vacuole is observed and subsequently, almost no cells are observed containing food vacuoles filled with TiO_2. This clearance is then followed by another intense period of ingestion (Fig. 7). The increase of percentage of cells containing at least one food vacuole filled with TiO_2 (after 0, 1, 2, 4, 24 and 48 h) correlated with increased particle concentration in the media (aggregate size and amount 10, 100 and 1000 µg TiO_2/ml) (Fig. 7). The presence of particles in the food vacuoles indicates an exposure route of cells, in addition to the body surface, to nanoparticles.

We used a modified exposure medium for *T. thermophila* in order to reduce the effects of media on nanoparticle behavior. The exposure media for *T. thermophila* was modified in order to reduce interactions between nanoparticles and biological media, TiO_2 toxic buffering with organic compounds and ions in media, but it still provided satisfactory energetic conditions for

avoidance of starvation and competition for food. Murdock et al. [37] showed that particle size changed dramatically in media containing organic molecules as compared to distilled water. The rich proteose-peptone media described by Schultz [18] was modified by complete reduction of protein content.

The exposure concentrations used in our experiments (0.1, 1, 10, 100 and 1000 μg/ml) were higher than those predicted to be found in the environment [38,39] and consequently, we were able to conclude that TiO_2 particles at levels to be expected in the environment pose little threat to a protozoan *T. thermophila*.

5. Conclusions

Our results show that effects of nano- and bulk TiO_2 particles in exposure concentrations ranging from 0.1, 10, to 1000 μg/ml result in acclimation of a protozoan *T. thermophila* by its changing its membrane composition in a manner associated with increased membrane rigidity. This study is the first nanoparticle-related study successfully employed fatty acid profiling in eukaryotic cells to elucidate the acclimation response to suspended particles, independent of oxidative stress. In *T. thermophila* cultures treated with TiO_2 concentrations above 10 μg/ml, we have observed a deliberate filling of TiO_2 particles into food vacuoles, and this is followed by clearing. The expected environmental concentrations of TiO_2 particles do not pose a threat to a protozoan *T. thermophila* but chronic exposure may result in undesirable effects on protozoans. The novelty of this study is the finding that changed fatty acid profile lead to increased membrane rigidity while exposure of *T. thermophila* is not accompanied by cytotoxicity. This is a proof that cell–TiO_2 particle interactions are also independent of ROS and oxidative stress.

Acknowledgements

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2.1.2 Eksperimentalni dokaz o lažno-pozitivnih rezultatih kometnega testa zaradi interakcije nanodelcev TiO_2 s sestavinami testa

Experimental evidence of false-positive Comet test results due to TiO_2 particle – assay interactions

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V naši študiji smo ugotavljali genotoksičnost delcev TiO_2 pri mikroorganizmu *Tetrahymena thermophila* s kometnim testom. Celice smo dvema različnima koncentracijama nanodelcev TiO_2 in delcev TiO_2 večjih od 100 nm izpostavili na tri različne načine: a) celice v suspenziji, b) celice vklopljene v gel c) jedra vklopljena v gel. Pri vseh treh izpostavitvah smo izmerili pozitivni rezultat kometnega testa, nismo pa dokazali drugih kazalcev citotoksičnosti, kot so: lipidna peroksidacija, koncentracija reaktivnih kisikovih zvrsti, spremembe v profilih dolgoverižnih maščobnih kislin. Znanstavena poročila navajajo, da je v primeru odsotnosti citotoksičnosti, genotoksičnost posredna. Možna razlaga za pozitivne rezultate genotoksičnosti v naši študiji je lažno pozitivni rezultat kometnega testa zaradi interakcij med delci TiO_2 in DNA po opravljeni izpostavitvi mikroorganizma *T. thermophila*. Predlagamo uporabo acelularnega kometnega testa za preverjanje možnosti nastanka lažno pozitivnih rezultatov pri kometnem testu, saj je glede na dokaze v pričujočem delu, pomembno predvideti interakcije nanodelcev z DNA in posledično poškodbe.

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Experimental evidence of false-positive Comet test results due to TiO_2 particle – assay interactions

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Abstract

We have studied the genotoxicity of TiO_2 particles with a Comet assay on a unicellular organism, *Tetrahymena thermophila*. Exposure to bulk- or nano-TiO_2 of free cells, cells embedded in gel or nuclei embedded in gel, all resulted in a positive Comet assay result but this outcome could not be confirmed by cytotoxicity measures such as lipid peroxidation, elevated reactive oxygen species or cell membrane composition. Published reports state that in the absence of cytotoxicity, nano- and bulk-TiO_2 genotoxicity do not occur directly, and a possible explanation of our Comet assay results is that they are false positives resulting from *post festum* exposure interactions between particles and DNA. We suggest that before Comet assay is used for nanoparticle genotoxicity testing, evidence for the possibility of *post festum* exposure interactions should be considered. The *acellular* Comet test described in this report can be used for this purpose.

Keywords: *Tetrahymena thermophila*, DNA damage, nanoparticles, nanotoxicity

Introduction

Genotoxicity has been defined by the International Conference of Harmonization in an ICH-Guideline as deleterious change in the genetic material induced by any mechanism. Damage to DNA results in cellular dysfunction and may therefore initiate and promote mutagenesis and carcinogenesis, or impact fertility (Sathya et al. 2010). Because of this, data on genotoxicity are of great importance in regulatory health risk assessment.

Genotoxicity of nanoparticles (NP) has frequently been documented (Sathya et al. 2010) (Landsiedel et al. 2010; Karlsson 2010) and the mechanisms of this genotoxicity include direct primary genotoxicity driven by direct interaction of NPs with DNA (Donaldson et al. 2010) and indirect primary genotoxicity resulting from oxidative stress (Nel et al. 2006). Oxidative stress occurs when NPs are transported into the nucleus (Chen & von Mikecz 2005) (AshaRani et al. 2009) or when the nuclear membrane breaks down during mitosis (Karlsson 2010). An example of an indirect mechanism is enhancement of the permeability of the lysosomal membrane, leading to release of DNase, which, transported to the nucleus, can degrade DNA (Banasik et al. 2005). Secondary indirect mechanisms of nanoparticle genotoxicity are associated with inflammation (Trouiller et al. 2009).

With the advent of nanotechnology, it is essential to define a reliable test system with which the genotoxic potential of engineered NPs can be assessed (Warheit & Donner 2010; Gonzalez et al. 2011). Guidelines provided by the Organisation for Economic Cooperation and Development (OECD) include *in vitro* genotoxicity testing, but these tests are designed basically for water-soluble chemicals and so may not be suited to the testing of the genotoxicity of NPs. Nanoparticles interfere with test media, modifying the biological potential of the NPs, and they may also interact with the test system, affecting the test results (Sathya et al. 2010); (Greim & Norppa 2010). In an attempt to clarify this issue, the OECD has established projects designed to evaluate the relevance and reproducibility of genotoxicity assays (see (Warheit & Donner 2010); Stone et al. (2009)) have shown the importance when assessing direct primary genotoxicity of accurate distinction of artefacts and the possible interaction of test components with nanoparticles remaining in the test system after exposure. Residual NPs may come into contact with nuclear DNA during tests affecting the test, and this may also happen when NPs present inside cells in the cellular lysosomes or food vacuoles are released during the tests.

In recent research of genotoxicity and nanoparticles, the Comet assay has been one of the most frequently used tests (Landsiedel et al. 2009) (Karlsson 2010). Recently, Karlsson (2010) reviewed 46 papers dealing with the genotoxicity of NPs by the Comet assay and concluded that majority of

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the NPs tested caused DNA strand breaks. However, the possibility of interaction of NPs with the chemicals used in the assay was cited and the use of additional methods, distinct from the Comet assay, was suggested for the measurement of DNA damage. Further mutagenicity studies have also been recommended. Landsiedel et al. (2009) suggested the use of a battery of standardised genotoxicity tests covering a wide variety of potential mechanisms and suggested that at least two genotoxicity tests should always be implemented.

At present, there are four techniques in common use for *in vitro* testing the genotoxicity of nanoparticles. These are the Ames test, the Chromosomal Aberration Test, the Comet assay and the Micronucleus test. Of these, the Comet assay is the most popular because (1) it is sensitive and capable of detecting low levels of DNA damage, (2) it requires only small numbers of cells per sample, (3) it is relatively inexpensive and (4) it requires relatively small amounts of test substance (Tice et al. 2000). Among the limitations and disadvantages of the Comet assay is its failure to detect: (1) aneugenic effects, (2) epigenetic mechanisms of DNA damage (Dhawan et al. 2009) and (3) fixed mutations (Stone et al. 2009). There are also some serious obstacles to the use of the Comet assay for NP genotoxicity studies. Karlsson (2010), for example, has shown the presence of nanoparticles (nano-TiO_2 and nano-CuO), in heads of the comets in the gels, while intracellular localisation of particles investigated by TEM did not reveal particles in cell nuclei. The possibility of post-exposure particle DNA interactions was also discussed by Lin et al. (Lin et al. 2009), studying the genotoxicity of Ge nanoparticles by the Comet assay. They noted a statistically higher level of DNA damage in exposed cells when compared with control cells and speculated that since nanoparticles of Ge readily adhere to cell surfaces, nanoparticles in or attached to the cells caused the damage during the assay process.

The features that motivated the selection of *Tetrahymena thermophila* as a model organism for this study are as follows: (1) it is a one-cell eukaryotic organism. Thus, the data obtained by Comet assay correspond to the impact of TiO_2 on whole-organism DNA, and in summary the effects are measured on the genome of the entire cell population. (2) Its short generation time and its axenic culture are especially advantageous for studying genotoxicity. (3) As protists have highly developed systems for internalisation of nanoscale (100 nm or less) and microscale (100 – 100,000 nm) particles (Frankel 2000), they are very good model organisms for nanotoxicology (Holbrook et al. 2008) (Kahru et al. 2008). (4) It has been used in toxicology for decades as a useful model organism for cellular and molecular biologists as well as for environmental research (Sauvant et al. 1999; Gutiérrez et al. 2003).

The aim of the present study was to provide experimental evidence on the possibility that NPs interact with the DNA *post festum*, during a Comet assay. We used a unicellular model organism *T thermophila* to assess genotoxicity by a Comet assay and cytotoxicity by conventional markers. In our study, three exposure scenarios were used: (1) *in vivo* exposure: *T. thermophila* was incubated in a suspension of particles – both nanoparticles and bulk-TiO_2; (2) *in vitro* exposure: *T. thermophila* was embedded in gels that were incubated in a suspension of particles; (3) *acellular* exposure: only nuclei were embedded in gels and the gels with embedded nuclei were incubated in a suspension of particles. We chose to examine nano-TiO_2 particles for which a substantial amount of genotoxicity data already exists. (Trouiller et al. 2009) suggested that DNA damage results not from direct primary effects of nano-TiO_2 but rather from reactive oxygen species (ROS) generation and is therefore a primary indirect effect. Very same was confirmed also by Petkovic et al. (2011a, b) (Petkovic et al. 2011a) (Petkovic et al. 2011b). Consequently, we hypothesise that *in vitro* exposure (cells embedded in gel) and *acellular* exposure of only nuclei to nano-TiO_2 would fail to produce a positive result in a Comet assay since ROS generation, a primary indirect effect, would be absent. If *in vitro* and *acellular* exposure were to lead to a positive Comet assay, this would suggest that particles could damage DNA during the tests, producing the positive Comet test result. In such cases, the use of Comet assay would have to be critically reconsidered.

Materials and methods

Chemicals

Unless otherwise specified, reagents were purchased from Sigma Aldrich Co (St. Louis, MO, USA), Merck (Darmstadt, Germany) or Biolife (Milan, Italy). TiO_2 nanoparticles with 99.7% purity were supplied in the form of a powder.

T. thermophila growth conditions

Axenic cultures of *T. thermophila* from the Protoxkit FTM (MicroBioTests Inc.) were grown for 24 h in the dark at 25 C in a semidefined proteose-peptone-based "rich" medium (RM) (Schultz 1997). The cell density obtained after incubation in these culture conditions was approximately 10^5 cells/ml.

Exposure conditions

The cells were harvested by 3 min centrifugation at 60 rcf. Cells were washed and resuspended in a "poor" medium (PM), which consisted of the semidefined proteose-peptone-based medium used by Schultz (Schultz 1997), but lacking yeast extract and bacteriological peptone. The pH of the medium was adjusted to 7.4 and temperature was maintained at 25 C for the entire experiment. All experiments were performed in 100 ml batch cultures that were maintained in Erlenmeyer flasks and aerated by shaking at 90 rpm in an incubator in the dark.

After 1 h in the PM, cells were exposed to bulk- or nano-TiO_2. The final concentration of particles in the medium, either bulk- or nano-, was 0.1 and 100 μg/ml. Following the addition of TiO_2, *T. thermophila* cultures were incubated at 25 C for 4 h. For each concentration of bulk- or nano-TiO_2, three independent assays were carried out. A supplementary set of three replicates without TiO_2 was set up as a control for each assay. After 4-h treatment with TiO_2 bulk- or nanoparticles, 15 ml of cell suspension was harvested for the purpose of cellular fatty acid composition analysis by gas chromatography.

Bulk- and nano-TiO_2 tested suspension

Aqueous dispersions of nanoparticles were put on carbon-coated grids, dried at room temperature, examined with a 200-keV field emission transmission-electron microscope (Philips CM 100; Koninklijke Philips Electronics, Eindhoven,

The Netherlands) and analysed by transmission-electron diffraction to identify the TiO_2 crystal phase.

Bulk-TiO_2 and 15-nm TiO_2 nanoparticles were dispersed in PM before treating the cell cultures. Bath sonication for 30 min was used to disperse particle agglomerates in stock solutions.

The suspensions of nanoparticles (1000 µg/ml) were inspected by dynamic light scattering (DLS) using a 3D DLS-SLS (dynamic light scattering – static light scattering spectrometer: LS Instruments, Fribourg, Switzerland). This allows the assessment of hydrodynamic radii of particles in extremely turbid suspensions by a so-called 3D cross-correlation technique that eliminates multiple scattering of light. As the light source, a HeNe laser operating at a wavelength of 632.8 nm was used and scattering was measured at an angle of 90°.

Zeta potentials of TiO_2 nanoparticle suspensions (1000 µg/ml) were measured with ZetaPals, (Brookhaven Instrument Corporation) in the PM medium, and were used to assess the exposure to living cells.

Assessment of cellular fatty acid composition by gas chromatography

T. thermophila cells were harvested by centrifugation at 60 rcf for 10 min of 15 ml culture samples. The pellets were resuspended in sterile double-distilled water (1 ml) then frozen at -20 C and lyophilised. Lipids were transesterified using an HCl/MeOH procedure (Dionisi et al. 1999). Dried samples were pulverised and transferred to screw cap test tubes. First, the sample was mixed with hexane (0.5 ml). Then 1.5M HCl in MeOH (1 ml) and pure MeOH (1 ml) were added and the test tubes were filled with N_2 and incubated at 80 C for 10 min. The reaction was stopped by cooling the tubes in ice. Following the addition of double-distilled water (2 ml), each reaction mixture was vigorously mixed for 1 min and centrifuged for 30 s at 670 rcf. The organic phase was transferred to a vial under N_2 and the samples were stored at -20 C prior to analysis.

Fatty acid methyl esters were separated by capillary gas chromatography using Omegawax TM 320 (30 m × 0.32 mm ID × 0.25 mm) capillary column with polyethylene glycol as the stationary phase. The gas chromatography system used was an Agilent 6890 series GC equipped with Agilent 7683 Automatic Liquid Sampler, 7683 Injector and FID detector and helium as the carrier gas with a flow rate of 2.0 ml/min, split ratio 10:1. The initial temperature for analysis was 185 C and the final temperature was 215°C. The injected volume was 2 µl and the run time was 54 min. Fatty acid methyl esters were identified from their retention times and results were calculated using response factors derived from chromatographic standards of known composition (Nu Chek Prep, GLC-85, Nu-Chek Prep Inc., Elysian, MN, USA).

Results were analysed using ChemStation Plus® software. Membrane fatty acids that were less than 0.5% of total fatty acids were designated as trace fatty acids and were not considered further. Statistical analysis of the compositional data was used to evaluate differences in average fatty acid composition between different treatments (size and concentration of particles). Multivariate analysis of variance on isometric log-ratio transformations of the composition data was carried out.

Assessment of the extent of lipid peroxidation by quantitation of malondialdehyde

Lipid peroxidation was tracked by the formation of malondialdehyde (MDA), a lipid peroxidation by-product that reacts with thiobarbituric acid (Ortega-Villasante et al. 2005). An aliquot of the culture (15 ml) was harvested by centrifugation at 6700 rcf for 10 min. Cells were homogenised by sonication for 3 min in an ice-cold water bath. To measure total protein concentration, 5 µl of sample was taken, and distilled water (995 µl) was added. The sample was then diluted by a factor of 10 and total protein concentration was measured spectrophotometrically at 280 nm. The total protein concentration was used as a measure of the biomaterial in the experiments. For the measurement of MDA concentration, homogenised sample (500 µl) was mixed with buffer A, 30% trichloroacetic acid, 0.75% 2-thiobarbituric acid, 0.5 M HCl and 0.02% butylated hydroxytoluene (500 ml), incubated at 90°C for 30 min, then chilled on ice. n-Butanol (1.5 ml) was mixed with the sample, and the mixture was centrifuged at 6700 rcf for 10 min at 4°C. The absorbance of the resulting chromophore was measured at 535 and 600 nm and the latter was subtracted from the former to correct for nonspecific turbidity. The concentration of MDA was calculated using an extinction coefficient of 156 mM^{-1} cm^{-1} (Ortega-Villasante et al. 2005). For statistical analysis, each concentration of MDA was divided by the total protein concentration of the corresponding sample.

Reactive oxygen species assessment

Assessment of ROS was performed by using the OxiSelect Intracellular ROS Assay Kit™ (Cell Biolabs) measuring green fluorescence as described by Petkovic et al. (2011b). DCFH-DA (2′,7′-dichlorodihydrofluorescin diacetate), standards, H_2O_2 and TiO_2 suspension were prepared in cell media (PM). *T. thermophila* cells were first pretreated with 100 µM solution of DCHF-DA in the PM cell culture media for 60 min at 30 C. Cells were then treated with 250 µM H_2O_2 and 0.1 and 100 µg/ml nano-TiO_2 particles or 0.1 and 100 µg/ml bulk-TiO_2 particles for 4 h. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly. H_2O_2 is the principal ROS, responsible for the oxidation of DCFH-DA to DCF (LeBel et al. 1992). Negative (nontreated cells) and positive (H_2O_2-treated cells) controls were included in each experiment. For kinetic analysis of ROS formation, the plates were maintained at 25°C and the fluorescence intensity (480 nm excitation/530 nm emission wavelengths) of the DCF formed was recorded every 5 min (for the first 30 min) and then every 30 min during the remainder of the 4-h incubation, using a Synergy H4 hybrid fluorescence plate reader (BioTrek). The statistical significance between treated groups and controls was determined by two-tailed Student's t-test and $p < 0.05$ was considered as statistically significant. For each concentration of nano- or bulk-TiO_2, three independent assays and two technical replicates were carried out.

Comet assay

Different protocols and versions of Comet assay were used to assess the extent and the type of DNA damage as shown in Figure 1.

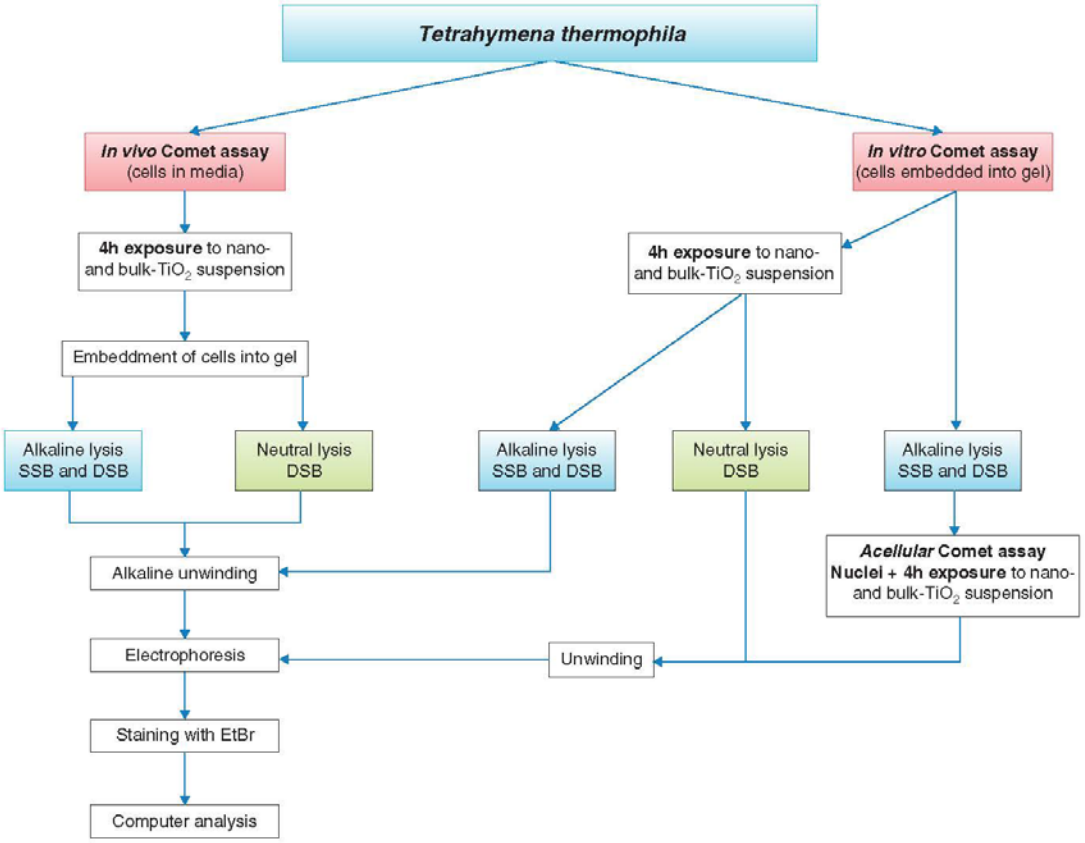

Figure 1. The protocols and types of exposure used in our genotoxicity study. These indicate where and when the bulk- and nano-TiO_2 particles may remain in close proximity to nuclei in the final steps of the Comet assay, leading to an overestimate of genotoxicity and type of DNA damage (DSB, double DNA strand breaks, SSB, single DNA strand breaks).

Comet assay with alkaline lysis *in vivo*

The alkaline version of the Comet assay was performed by modifications of the original protocol (Lah et al. 2004). After exposure to TiO_2 particles in PM (as described in "Exposure Conditions, above), cells were harvested by 5-min centrifugation at 60 rcf washed with PM and resuspended in PM. To achieve a uniform background, rough microscope slides were coated with 400 µl of 0.5% normal melting point (NMP) agarose and were left to air-dry overnight. Cells were mixed with 3.0% low melting point (LMP) agarose and spread over the slides as the second layer, giving a final concentration of 140 cells/µl. After removing the cover glasses, the slides were covered with a third layer of 300 µl of 3.5% LMP agarose, to prevent escape of *T. thermophila* DNA during cell lysis and electrophoresis.

T. thermophila cells embedded in agarose were dipped in phosphate-saline buffer (PBS; 80 g NaCl, 8 g NaCl, 2 g KCl, 2 g KH_2PO_4 in 1 L doubly distilled H_2O at pH 7.2–7.4) for 20 min on ice and then washed twice with PBS. Slides were incubated overnight in lysis solution (30 mM NaOH, 1.2M NaCl, 1% (w/v) lauroylsarcosine, 0.05% Triton X 100, 1% DMSO pH 12.4). The slides were rinsed three times for 20 min each in electrophoresis buffer (30 mM NaOH, 10 mM EDTA, pH 12.4) to remove lysis solution and to unwind the nuclear DNA. The samples were then subjected to electrophoresis for 20 min at 25 V and 300 mA in the same buffer. Following the electrophoresis, the gels were neutralised in 400 mM Tris-HCl, pH 7.5 for 15 min. For visualisation in a fluorescence microscope, the slides were stained with ethidium bromide (10 µg/ml) and 60 randomly selected nuclear images of each slide were acquired with an epifluorescent microscope (Olympus BX50), using a BP 515–560 nm excitation filter and a barrier filter of LP 590 nm at 400× magnification (Figure 2). Microscopic images of comets were captured by a digital camera (Hamamatsu Orca 2), connected to a computer. Detected comets were scored by Komet 5.0 Computer Software (Kinetic Imaging Ltd., 2001). The tail lengths and percentage of DNA in the comet's tails and heads were determined and further used to analyse the nuclear DNA damage.

Comet assay with alkaline lysis *in vitro*

After culture growth in RM for 24 h in the dark at 25°C, cells were harvested by 5 min centrifugation at 60 rcf, washed with

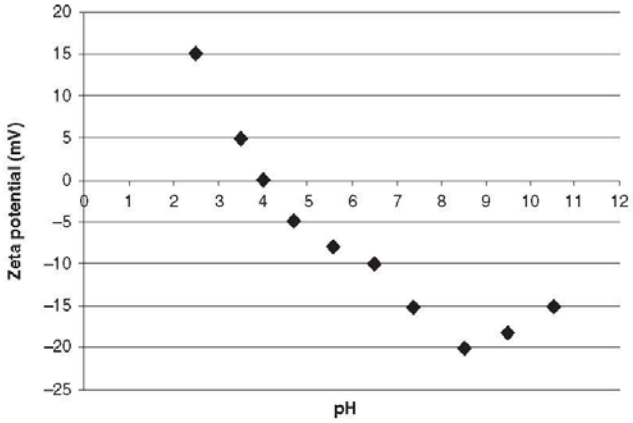

Figure 2. Zeta potentials of TiO_2 nanoparticle suspensions (1000 µg/ml) measured in the poor medium and used in experimental exposures.

PM and resuspended in PM. The cells were embedded into 3.0% low-melting point agarose, the first and the third layers prepared as described above. Glass slides with embedded cells were then exposed to TiO_2 particles in PM for 1 h and then treated with TiO_2 nano and bulk particles (0.1 and 100 µg/ml) for 4 h. *T. thermophila* cells embedded in agarose were dipped in PBS for 20 min on ice and then washed twice with PBS. Glass slides were treated in alkaline lysis and all further steps were the same as described in the section "Comet assay with alkaline lysis *in vivo*" above.

Acellular Comet assay with alkaline lysis

After culture growth in RM for 24 h in the dark at 25°C, cells were harvested by 5 min centrifugation at 60 rcf, then washed with PM and resuspended in PM. The cells were embedded into 3.0% LMP agarose and the first and the third layers were prepared as described above. *T. thermophila* cells embedded on glass slides were dipped in PBS for 20 min on ice and then washed twice with PBS. Glass slides were treated after alkaline lysis and washed three times with PBS buffer for 10 min. One-hour exposure of embedded nuclei to PM in the dark at 25°C was followed by the exposure to TiO_2 particles at two selected concentrations (TiO_2 nano- and bulk particles; 0.1 and 100 µg/ml) for 4 h, in the dark at 25°C. After exposure, the glass slides were washed with electrophoresis buffer (EF buffer; 6 mL NaOH, 4 mL EDTA, 1990 ml MQ), and all further steps were the same as in section "Comet assay with alkaline lysis *in vivo*" above.

Comet assay with neutral lysis *in vivo* and *in vitro*

Both *in vivo* and *in vitro* exposures to TiO_2 particles were tested as described in the sections "Comet assay with alkaline lysis *in vivo*" and "Comet assay with alkaline lysis *in vitro*" above. As a positive genotoxic control toxicant, 100 µM methyl methanesulfonate (MMS) was used. For the Neutral Comet assay, a modification of the protocol by Wojewodzka et al. (2002) was used. The cell suspension was mixed with low-melting point agarose (LMP agarose) at a final concentration of 0.75%. After the preparation of the third layer, the slides were left at 4°C in the dark for 1–2 h in the lysing buffer, which consisted of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 1% *N*-lauroylsarcosine, pH 9.0. Immediately before use, 0.5% Triton X-100 and 10% dimethylsulfoxide (DMSO) were added to the buffer and mixed for 20 min. After 1 h of lysis, the slides were washed three times with the electrophoresis buffer (300 mM sodium acetate, 100 mM Tris–HCl, pH 8.3) and left in fresh buffer solution for 1 h, then placed in a horizontal gel electrophoresis unit filled with a fresh electrophoretic buffer. The slides were electrophoresed for 1 h at 14 V (0.5 V/cm, 11–12 mA) at 8°C.

Statistical analysis of Comet assay results

The average percentage of tail DNA was compared in an incomplete four-factor experimental design using analysis of variance (ANOVA). The first factor was "lysis" with two levels: alkaline and neutral; the second factor was "method" with four levels: *acellular* Comet assay (only by alkaline lysis), *in vivo* Comet assay, *in vitro* Comet assay and control. The third factor was the "size" of TiO_2 particles, either nano or bulk, and the fourth factor was concentration of TiO_2 particles, either 0.1 or 100 µg/ml. The experiment was carried out in three biological replicates. At least 60 nuclei were examined in each replicate and the medians of percentage of tail DNA were calculated for each biological replication. The ANOVA calculations were made on the basis of the medians of percentage of tail DNA. The Duncan's multiple comparison test was used to determine the statistical significant differences between the treatments ($\alpha = 0.05$).

Results

Characterisation of TiO_2 nanoparticle suspensions

The TEM revealed that TiO_2 nanoparticles were homogeneous in shape and size, with an aspect ratio of up to 1:5 between the diameter and length, forming elongated, spheroidal shapes. The transmission-electron diffraction pattern showed the TiO_2 to be in its anatase phase. BET analyses revealed the surface area to be between 190 and 290 m^2/g and the average particle size to be 15 nm.

Table I. Average percentage composition of membrane fatty acid samples from *T. thermophila* exposed to TiO_2 particles at 25° C after 4 h. The data are presented as total sums of various fatty acids of lipid extracted from the three independent cultures. Percentages are expressed as means ± standard error (SE). B, bulk concentration; NP, nanoparticle concentration; FA, fatty acid.

Particle type	Bulk-TiO_2 (B)						Nano-TiO_2 (NP)					
Particle concentration (μg/ml)	0	0.1	1	10	100	1000	0	0.1	1	10	100	1000
Straight chain saturated FA (%)	37.2 ± 0.9	36.2 ± 0.4	37.1 ± 0.8	38.3 ± 0.1	36.0 ± 0.6	37.4 ± 0.4	31.0 ± 0.4	31.0 ± 0.5	30.8 ± 0.8	31.3 ± 0.1	31.5 ± 0.5	32.4 ± 0.2
Unsaturated FA (%)	49.6 ± 1.0	50.1 ± 0.4	50.0 ± 1.1	48.3 ± 0.3	50.6 ± 0.8	49.4 ± 0.4	52.0 ± 0.4	51.6 ± 0.6	52.5 ± 0.6	51.7 ± 0.6	51.5 ± 0.7	50.1 ± 0.4
Monounsaturated FA (1×) (%)	22.4 ± 0.6	21.3 ± 1.3	21.2 ± 0.8	22.6 ± 1.5	21.4 ± 0.5	23.7 ± 1.2	22.3 ± 0.5	22.3 ± 0.5	21.7 ± 1.0	22.7 ± 0.3	21.8 ± 0.6	22.1 ± 0.3
Polyunsaturated FA (2×) (%)	8.3 ± 0.2	8.6 ± 0.2	8.5 ± 0.2	8.0 ± 0.1	8.6 ± 0.2	8.1 ± 0.1	8.8 ± 0.0	8.3 ± 0.5	8.7 ± 0.1	8.7 ± 0.2	8.7 ± 0.1	8.4 ± 0.1
Polyunsaturated FA (3×) (%)	18.9 ± 1.4	20.3 ± 1.3	20.2 ± 1.7	17.7 ± 1.2	20.6 ± 1.1	17.7 ± 1.3	21.0 ± 0.9	21.1 ± 0.3	22.0 ± 1.0	20.3 ± 0.7	21.1 ± 0.4	19.6 ± 0.6
Saturated iso and anteiso branched FA (%)	5.6 ± 0.3	5.4 ± 0.1	5.6 ± 0.2	5.9 ± 0.1	5.4 ± 0.1	5.8 ± 0.2	7.3 ± 0.4	7.5 ± 0.1	7.6 ± 0.1	7.7 ± 0.2	7.5 ± 0.2	7.8 ± 0.3
Saturated iso FA (%)	5.0 ± 0.3	4.8 ± 0.1	5.0 ± 0.2	5.2 ± 0.1	4.8 ± 0.1	5.1 ± 0.1	6.5 ± 0.1	6.4 ± 0.1	6.5 ± 0.0	6.6 ± 0.2	6.5 ± 0.1	6.7 ± 0.3
Saturated anteiso FA (%)	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.4	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
Average number of C-atoms in membrane FA	14.7	14.9	15.0	14.9	14.9	15.0	14.7	14.7	14.9	15.0	14.7	14.6

Dynamic light scattering analysis showed the average value of the hydrodynamic radius *R*h, of TiO_2 nanoparticles suspended in test medium to be 820 nm. The average size of bulk-TiO_2 could not be measured accurately with this approach because of the presence of larger agglomerates.

Zeta potentials of TiO_2 nanoparticle suspension (1000 μg/ml) were measured in the same medium used to expose cells, at pH 7.4. The value recorded was -15, which is equivalent to a suspension of incipient stability (Figure 2).

Cellular fatty ACID composition

No significant differences have been found in membrane fatty acid profiles of *T. thermophila* after exposure to different concentrations of nano- or bulk-TiO_2 at 25°C after 4 h (Table I). This suggests that TiO_2 particles have no effect on *T. thermophila* cell membranes.

Lipid peroxidation

There were no differences in lipid peroxidation of analysed *T. thermophila* samples after 0 and 4 h of incubation at 25°C with nanoparticles, when compared with control cells. The average content of malondialdehyde in the control samples was 140 ± 23 nM of MDA per milligram of protein.

Reactive oxygen species production

In comparison with control cells, no ROS production was detected after 4 h of incubation with TiO_2 particles of any size at a concentration of 0.1 μg/ml; however, at 100 μg/ml bulk-TiO_2, but not nano-TiO_2, significant elevation of intracellular ROS formation was detected (Figure 3A).

To explore whether TiO_2 nanoparticles (0.1 and 100 μg/ml) induced ROS formation not only at the end of exposure but also during the experiment, we measured the kinetics of their formation in *T. thermophila* cells in different time frames during 4 h of exposure (Figure 3B). Comparison between treated groups and controls tested by two-tailed Student's t-test and $p < 0.05$ showed no statistically significant changes in ROS formation.

Comet assay

Statistical analysis of the results obtained with a Comet assay after alkaline lysis indicated significant damage of DNA in *T. thermophila* in both *in vivo* and *in vitro* treatments with TiO_2 in comparison with control. This was independent of both the size and the concentration of particles (Figure 4).

Statistical analysis of results of a Comet assay obtained with embedded nuclei (*acellular* exposure) also showed significant DNA damage at all TiO_2 exposure concentrations and sizes used, except for 100 μg/ml nano-TiO_2 concentration. Statistically significant differences, calculated using Duncan's multiple comparison test, between DNA damage in the two exposure concentrations of particles have been observed in *acellular* Comet assays. A possible explanation for this is that nano- and bulk particles in suspensions aggregate more at higher concentrations (100 μg/ml) and this may hinder penetration into the gels.

Statistical analysis of Comet assays by neutral lysis showed that in cells treated with TiO_2, the average DNA tail length does not significantly differ from that in control cells,

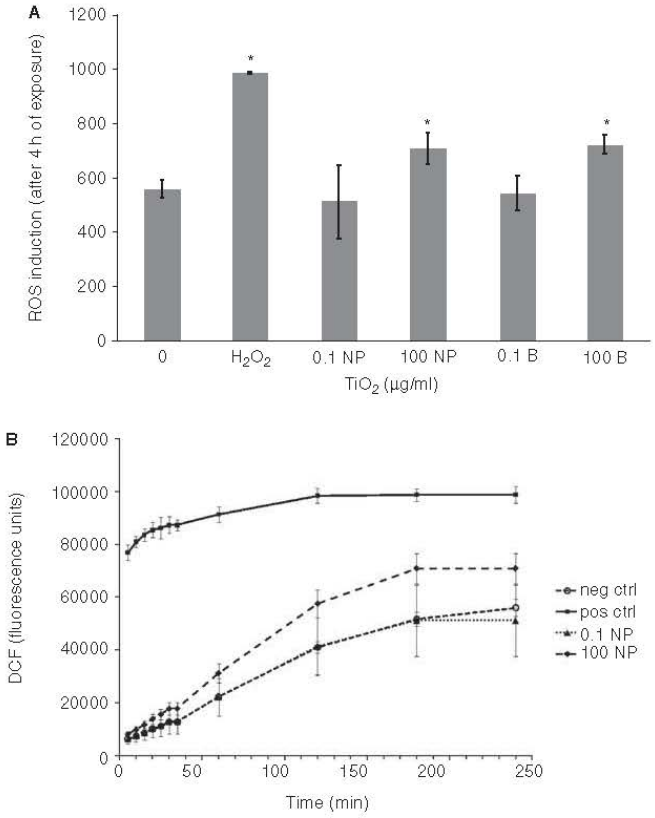

Figure 3. Induction of reactive oxygen species (ROS) formation in *T. thermophila* cells. (A) treated with H_2O_2 (250 µM= pos ctrl), nano-TiO_2 particles (NP, 0.0 µg TiO2/ml = neg ctrl, 0.1 and 100 µg/ml), bulk-TiO_2 particles (0.1 and 100 µg/ml) and presented as a relative increase in DCF fluorescence after 4 h of exposure to TiO_2 particles. Each bar is represented as a mean ± standard error (SE) of three independent experiments. (B) Kinetics of ROS formation during exposure for 4 h to TiO_2 NPs (0.1 and 100 µg/ml). Each point represents the mean of six replicates ± standard error (SE).

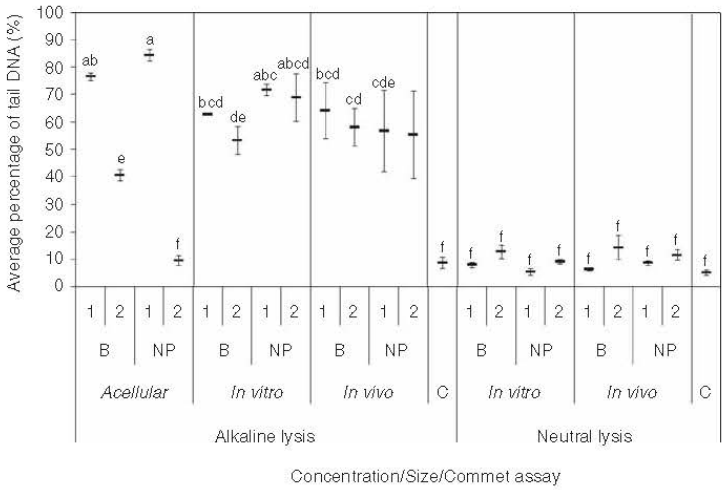

Figure 4. Results of the Comet assay experiment are presented as an average percentage of tail DNA (assessed in 60 cells). *T. thermophila* was treated with nanoparticles (NP) or bulk TiO_2 particles (B) at two different concentrations (1 - 0.1 µg/ml and 2 - 100 µg/ml). Control group was not treated with TiO_2 and is indicated as "C". Three different exposure scenarios (acellular, *in vitro, in vivo*) were applied and two different protocols of Comet assay (alkaline lysis and neutral lysis). There is no statistically significant difference between averages indicated with the same letter (a,b,..., f).

indicating no double-strand breaks occur as a result of exposure to TiO_2 (Figure 4). Double strand breaks did not occur in bulk- or in nano-TiO_2 treated cells. Concentration and exposure type, namely in *in vivo* or *in vitro* experiments, failed to produce double strand breaks in DNA. When cells were treated with 100 µM MMS, a reference positive control for double-strand breaks, a statistically significant level of DNA damage was recorded.

Simultaneous performance of alkaline lysis and neutral lysis in this study indicates that single strand breaks are the main category of DNA damage caused by TiO_2 particles. No double-strand breaks were observed (Figure 4). The results of the *acellular* exposure to TiO_2 revealed the capacity of TiO_2 particles to produce extensive single-strand breaks when interacting with embedded nuclei and imply that when TiO_2 particles are present in the medium during in a Comet assay, they can interfere with DNA and give rise to false-positive results and overestimates of actual genotoxicity.

Discussion

We report experimental evidence of TiO_2 particle interactions with DNA during the Comet assay that resulted in a positive test result. We studied the DNA damage sustained by *T. thermophila* incubated with TiO_2 bulk- and nanoparticles and assessed by a Comet assay, and we analysed cellular responses, including lipid peroxidation, ROS formation and membrane fatty acid profiles. The DNA was exposed to particles in three different exposure scenarios in order to assess whether nanoparticles could directly interact with DNA during the course of the assay – and thus produce a false-positive result or an overestimate of the actual genotoxicity.

The ability of TiO_2-NPs to damage DNA has been shown in many studies (Gurr et al. 2005; Wang et al. 1998; Trouiller et al. 2009; see (Karlsson 2010); see (Sathya et al. 2010)), but it has generally been rationalised as a consequence of oxidative stress. Our results showed that only TiO_2 bulk particles at 100 µg/ml cause significant ROS production, a result never observed with nanoparticles. Other cellular markers such as membrane fatty acid profiles and lipid peroxidation, which could be also regarded as markers of cytotoxicity, remained unchanged compared with control cells. ROS elevation by bulk-TiO_2 particles (100 µg/ml) does not imply a higher degree of DNA damage and these results clearly indicate that oxidative stress is not a cause of the genotoxicity, which was detected in our Comet assay study. Consequently, the recorded genotoxicity must be either independent of oxidative stress or a false-positive result. Since literature data failed to report direct primary genotoxicity but rather genotoxicity driven by oxidative stress, the Comet assay results would appear to be false positives. There is only one published study in which, judging by an alkaline Comet assay, no genotoxicity was observed with nanoparticulate TiO_2 (Bhattacharya et al. 2009). We hypothesise that in this case, complete removal of the nanoparticles from the test system was achieved and the particles were not endocytosed to any significant degree. Potential causes of false-positive results include particles that may remain in the test medium or particles that are present in food vacuoles or have been endocytosed. That intracellular particles can gain access to DNA after lysis in the course of a Comet assay has been discussed by Stone et al. (2009) and by Karlsson (2010).

Our results agree with those in other reports. A review by Landsiedel et al. (2009) reported results of nanomaterial genotoxicity tests, which were dependent on the tests themselves. In an assessment of the genotoxicity of nanoparticles, in six studies, the Ames test showed no genotoxicity, and this was associated with a barrier to penetration by the nanomaterials through the bacterial cell wall. By contrast, of 14 *in vitro* micronucleus assays, 12 produced evidence of genotoxicity, and in the Comet assay, 14 of 19 studies showed nanomaterials to be highly genotoxic (Landsiedel et al. 2009). A partial explanation for these inconsistencies among the tests may be the fact that the Comet assay is the most sensitive of the assays, but since different concentrations of nanoparticles were applied in the studies, this suggestion cannot be a complete explanation and other factors, such as direct interaction of NPs with DNA during the tests, should be considered.

Based on the results of our study presented here, we suggest that when the Comet assay is selected for assessment of genotoxicity of nanoparticles, pretesting of potential of nanoparticles to interact with DNA *post festum* must be carried out. One means by which such interactions could be detected is use of the *acellular* Comet test. In addition, before settling on the Comet assay, it is important to know whether to expect substantial amounts of intracellular nanoparticles that could interact with DNA while the test is proceeding.

Suspected genotoxicity should be confirmed by an independent assay or, at a minimum, with biomarkers indicating DNA repair, for example, mRNA expression of tumour suppressor gene p53 and its downstream regulated responsive genes (Petkovic et al. 2011b), DNA deletions (Trouiller et al. 2009), inflammation (Trouiller et al. 2009, Grassian et al. 2007), or indications of oxidative stress status such as lipid peroxidation, or elevated levels of ROS (Gurr et al. 2005; Kang et al. 2008).

In isolation, the results of Comet assays are unreliable as a measure of nanoparticles' genotoxicity due to the possibility of false positives. In the future, the test protocol needs modifications in terms of exclusion or control of particle–assay interactions and combination with other oxidative stress markers. Only with such refinements will the Comet assay remain a test capable of reliably confirming or disproving genotoxicity.

Conclusions

(1) Genotoxicity of TiO_2 nanoparticles was demonstrated when *T. thermophila* cells were incubated with nano-TiO_2 or bulk-TiO_2 in a suspension (*in vivo* exposure), or embedded in gels (*in vitro* exposure) or when only embedded nuclei (*acellular* exposure) were exposed to nanoparticles (Figure 1). Since positive Comet assay results were not accompanied by cytotoxicity markers such as lipid peroxidation, ROS formation or changes in composition of cell membranes, our Comet assay results appear to represent a false positive.

(2) We suggest that in the future, pretesting of particle DNA interactions should be conducted in an *acellular* Comet assay and only the Comet assay results consistent with this pretesting should be accepted.
(3) Data obtained from a Comet assay method alone are inadequate to support an assertion of an enhancement of the genotoxic potential of NPs. The genotoxic potential of NPs as obtained by a Comet assay should be accepted only when combined with evidence adduced by properly selected oxidative stress biomarkers.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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2.1.3 Učinki inženirsko proizvedenih nanodelcev na celično strukturo in na rast kvasovke *Saccharomyces cerevisiae*

The effects of engineered nanoparticles on the cellular structure and growth of *Saccharomyces cerevisiae*

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V tej študiji smo ugotavljali učinke različnih nanodelcev na celično viabilnost in celično ultrastrukturo. Uporabili smo modelni mikroorganizem kvasovko *Saccharomyces cerevisiae*, ki smo jo izpostavili različnim koncentracijam naslednjih nanodelcev in delcev večjih od 100 nm: TiO_2 (1–3 nm), ZnO (<100 nm), CuO (<50 nm) in nanodelcem Ag (10 nm) ter ogljikovih nanocevk (SWCNT). Citotoksičnost in genotoksičnost smo ugotavljali s testom GreenScreen. Spremembe celičnih ultrastruktur smo ocenjevali z transmisijsko elektronsko mikroskopijo. Naša raziskava je pokazala, da so nanodelci CuO zelo citotoksični, saj se je celična gostota zmanjšala za 80% pri 9 cm^2/ml, in v celicah so nastale maščobne kapljice. Pri celicah izpostavljenih nanodelcem Ag (19 cm^2/ml) in TiO_2 (147 cm^2/ml) so bili opazni temni skupki v celičnih vakuolah, celični steni in v veziklih, celična gostota se je zmanjšala za 40 oziroma 30%. Nanodelci ZnO (8 cm^2/ml) so povzročili povečanje celičnih vakuol, vendar niso bili citotoksični. Ogljikove nanocevke niso bile citotoksične in tudi sprememb v ultrastrukturi niso povzročile. Uporabljena testa genotoksičnosti sta dala nasprotujoče si rezultate genotoksičnosti, v pričujočem delu obravnavamo možne razloge za to razhajanje. Predlagamo, da klasične teste citotoksičnosti pri študiji nanodelcev dopolnimo še z metodami, ki merijo fiziološko stanje celic, kot je npr. transmisijska elektronska mikroskopija.

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The effects of engineered nanoparticles on the cellular structure and growth of *Saccharomyces cerevisiae*

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Abstract

In order to study the effects of nanoparticles (NPs) with different physicochemical properties on cellular viability and structure, *Saccharomyces cerevisiae* were exposed to different concentrations of TiO_2-NPs (1–3 nm), ZnO-NPs (<100 nm), CuO-NPs (<50 nm), their bulk forms, Ag-NPs (10 nm) and single-walled carbon nanotubes (SWCNTs). The GreenScreen assay was used to measure cyto- and genotoxicity, and transmission electron microscopy (TEM) used to assess ultrastructure. CuO-NPs were highly cytotoxic, reducing the cell density by 80% at 9 cm^2/ml, and inducing lipid droplet formation. Cells exposed to Ag-NPs (19 cm^2/ml) and TiO_2-NPs (147 cm^2/ml) contained dark deposits in intracellular vacuoles, the cell wall and vesicles, and reduced cell density (40 and 30%, respectively). ZnO-NPs (8 cm^2/ml) caused an increase in the size of intracellular vacuoles, despite not being cytotoxic. SWCNTs did not cause cytotoxicity or significant alterations in ultrastructure, despite high oxidative potential. Two genotoxicity assays, GreenScreen and the comet assay, produced different results and the authors discuss the reasons for this discrepancy. Classical assays of toxicity may not be the most suitable for studying the effects of NPs in cellular systems, and the simultaneous assessment of other measures of the state of cells, such as TEM are highly recommended.

Keywords: nanoparticles, genotoxicity, cytotoxicity, cellular structure, transmission electron microscopy, lipid droplets, vacuoles

Introduction

In the growing field of nanotoxicology, there is a demand for low-cost, high-throughput *in vitro* assays for particle risk assessment. The biological effects of nanoparticles (NPs) on cellular function have been explored in several prokaryotic or eukaryotic models, but data remain limited. *Saccharomyces cerevisiae* shares essential conserved cellular and biochemical mechanisms with other eukaryotes, including humans (Meaume & Weber; Walczak et al. 2012; Zhang et al. 2013), and its short generation time and easy culture conditions makes it an ideal model system for nanotoxicology. However, data on the effects of different NPs on *S. cerevisiae* cell viability are limited (Kasemets et al. 2009). Here, the authors explore the cytotoxic, genotoxic and ultrastructural effects of titanium (IV) oxide NPs (TiO_2-NPs), zinc oxide NPs (ZnO-NPs), copper oxide NPs (CuO-NPs), silver NPs (Ag-NPs) and single-walled carbon nanotubes (SWCNTs) on *S. cerevisiae*, since these NPs are widely used and intended for broad practical and real-world application.

TiO_2-NPs are extensively studied due to their unique photocatalytic properties, and capacity to be modified by biologically active molecules such as oligonucleotides (Zhang et al. 2013). Ultra-small TiO_2-NPs have been shown to enter the cell passively either by diffusion or adhesive interactions, rather than actively via endocytosis, and therefore require further evaluation (Warheit et al. 2007).

ZnO-NPs are widely used in paints, as drug carriers, medical materials and UV-blocking materials (Sultana et al. 2012; Zhang et al. 2012a,b). ZnO-NPs (50–70 nm) and ZnO-bulk forms have been shown to cause approximately 80% growth inhibition of *S. cerevisiae* possibly due to solubilised Zn ions (Kasemets et al. 2009). A similar mechanism has been suggested to explain the cell death observed in cancer cell lines (Buerki-Thurnherr et al. 2012).

CuO-NPs are also extensively used in science and technology, such as in gas sensors, magnetic phase transitions and catalysis (Chen et al. 2012a). Both nano- and bulk-

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form CuO have also been shown to be toxic to *S. cerevisiae* (Kasemets et al. 2009).

SWCNTs have aroused great interest due to their high conductance and potential use for miniaturised electronics, and as catalyst supports (Iijima 1991). Nevertheless, they may share similar carcinogenic properties to asbestos, as shown when administered intraperitoneally to mice (Kaiser et al. 2008). In a previous study on yeast cells, no toxic effects were detected after SWCNT internalisation (Phillips et al. 2012).

Finally, Ag ions and Ag-based compounds are known to be highly antibacterial, as well as being effective inhibitors of yeast growth and causing protein modifications (Kim et al. 2007; Walczak et al. 2012).

Here, the authors examine the effects of exposure of *S. cerevisiae* to NPs, focusing on their genotoxicity and the alterations they cause in cellular ultrastructure. Based on previous data, it was hypothesised that ion-releasing NPs would be expected to induce greater cytotoxicity than those NPs that do not release ions. To the best of the authors' knowledge, this is the first comparative analysis of the ultrastructural changes of yeast cells after NP exposure, and the first report on the potential internalisation and adsorption of particles at the yeast cell surface. This study provides a link between oxidative stress, primary and secondary particle characteristics and the biological response of *S. cerevisiae* to NPs. In addition, they illustrate that *S. cerevisiae* is a simple yet efficient model to study the biological effects of NPs in eukaryotic cells, and the suitability of these measured biological responses for assessing nanotoxicity.

Methods

Test materials

Ag-NPs colloidal solution, 0.1 mg/ml (10 nm), TiO_2-NPs rutile (1–3 nm), Plasmachem GmbH, (Münster, Germany). SWCNTs 519308, 1.2–1.5 nm × 2–5 µm, ZnO-NPs, 544906, <100 nm, TiO_2, 14027, ZnO, puriss, 96479, CuO-NPs, 544868, <50 nm, Sigma-Aldrich Co. (St. Louis, MO, USA). CuO, 33307, Alfa Aesar (Karlsruhe, Germany). Reagents used in the comet assay were purchased from Sigma-Aldrich Co., Merck (Darmstadt, Germany) or Biolife (Milano, Italia). Reagents for transmission electron microscopy (TEM) were purchased from Merck, TAAB (Berkshire, England), Ladd (Burlington, VT, USA) and Leica (Wien, Vienna, Austria).

NPs stock suspensions

The NPs stock suspension and bulk particles were freshly suspended in sterile deionised water (MilliQ, Millipore, Massachusetts, USA), sonicated in a water bath sonicator for 30 min and vortexed vigorously at maximum speed.

Zeta-potential and dynamic light scattering (DLS)

The measurements were performed in triplicates at 500 mg/l in sterile deionised water (with 5 mM NaCl for background low ionic strength) or the exposure medium provided in the GreenScreen assay. The measurements were assessed with a Malvern Zetasizer Nano series V5.03 (PSS0012-16 Malvern Instruments, Worcestershire, UK) and the analysis program dispersion technology software (Malvern Instruments). The polydispersity index (PDI) was also assessed.

Cell-free dichlorofluorescein assay

To study the oxidative potential of the NPs, a cell-free method with some modification was utilised (Foucaud et al. 2007). Briefly, 25 µl of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) at 2.2 mM was hydrolysed to DCFH at pH 7.0 with 100 µl of 0.01 N NaOH for each sample for 30 min in darkness. The reaction was stopped with phosphate buffered saline (PBS), 5 µl of horse radish peroxidase (20 U/ml) was added, incubated at 25°C for 120 min and the DCFH oxidation was measured at 485 nm/530 nm. The NPs concentrations analysed were 100, 31.25 and 15 mg/l (only 15 mg/l was measured for Ag-NPs).

Inductively coupled plasma-mass spectrometry

Inductively coupled plasma-mass spectrometry (ICP-MS) was performed to assess the portion of ions dissolved from NPs (31.25 mg/l) in the medium after the exposures. The samples were ultracentrifuged at 100,000 *g* for 1 h. ICP-MS was performed on the collected supernatant and non-centrifuged NPs which were diluted (10 and 500 times, respectively). A 10 ppb of rhodium was added as internal standard, the measurement were performed in triplicates on PerkinElmer NexION 300 ICP-MS and analysed with NexIon.

GreenScreen assay

The GreenScreen assay was performed according to the recommendation from the suppliers and two *S. cerevisiae* strains were provided, GenC01 and GenT01, where the promoter for a DNA repair gene (RAD54) has been copied and placed in front of a green fluorescent protein (GFP) gene, which produces increasing quantities of GFP upon chromosomal damage. The exposures to NPs were performed under shaking for 16 h at 25°C. The assay simultaneously reported the cyto- and genotoxicity of the compound. The cytotoxicity was measured as decrease in absorbance at 600 nm and genotoxicity measured the GFP production in GenT01 strains at 485–538 nm. The yeast cultures at OD600 = 0.200 units/ml (0.25×10^7 cells/ml) were exposed to equal volumes of NPs suspension (nine-, twofold dilutions, i.e. 31.25 – 0.01 mg/l). Methyl methanesulphonate (MMS) and 3–7% methanol were used as positive control for genotoxicity and cytotoxicity, respectively. Yeast cells grown without NPs, NPs suspensions and exposure medium were used as negative control. The toxicity was calculated with the software provided by the suppliers.

Comet assay

The alkaline comet assay was performed with major modifications of the original protocol (Peycheva et al. 2009). Briefly, *S. cerevisiae* cells were cultivated in liquid yeast peptone D-glucose (YPD) medium and exposure to the NPs, bulk form and MilliQ water was performed comparatively with the GreenScreen assay at 31.25 mg/l. After exposure, cells were washed with MilliQ water and re-

Table I. The physicochemical properties of NPs.

	Powder					Dispersed			
NPs	Purity %	Shape/crystal structure	Size (nm)	Molecular weight (kDa)	Specific surface area (m^2/g)	Solvent	Hydrodynamic size (nm)	PDI	Zeta-potential (mV)
ZnO	79.8	Hexagonal, wurtzite	<100	81.4	15–25	H_2O	612 ± 10.9	0.4 ± 0.0	-33.8 ± 10
						medium	5294 ± 3184	0.8 ± 0.2	-24 ± 4.7
CuO	77.3	Monoclinic crystals	<50	79.5	29	H_2O	1511 ± 468	0.3 ± 0.07	-13.4 ± 0.9
						medium	3475 ± 357	0.3 ± 0.06	-16.4 ± 0.9
TiO_2	99+	Rutile	1–3	79.9	~470	H_2O	99.20 ± 6.2	0.4 ± 0.06	40.7 ± 1.6
						medium	337e5 ± 190e5	0.3 ± 0.1	-17.8 ± 0.9
Ag	99+	Spherical	10	107.9	60	H_2O	49.3 ± 0.3	0.3	-42 ± 2.1
						medium	-		-
SWCNT	50–70	Tubes	1.5 × 2–5 μm			H_2O	3537	1.0	-11.8 ± 1.0
						medium	2231	0.4	-8.3 ± 1.2

The characterisation of NPs in sterile deionised water with 5 mM NaCl, and exposure medium. Zeta-potential values not reported (-) could not be measured by the Zetasizer system. Information about NPs properties in powder form were obtained from the respective manufacturing companies.
NP: nanoparticle; SWCNT: single-walled carbon nanotube.

suspended in S buffer (1 M sorbitol, 25 mM KH_2PO_4, pH 6.5) containing lyticase (36.9 mg/l) and centrifuged (200 *g*). Cells were then mixed with 1.6% low melting point agarose (LMPA) and spread over rough microscope slides coated with 0.5% agarose. The slides were covered again with 1% LMPA and exposed to PBS for 20 min on ice and then incubated in lysis solution (30 mM NaOH, 1 M NaCl, 0.1% (w/v)) laurylsarcosine (50 mM EDTA, pH 12) for 40 min. Then rinsed in electrophoresis buffer (30 mM NaOH. 10 mM EDTA, pH 12) and subjected to electrophoresis (10 min, 25 V and 300 mA). Thereafter, the gels were neutralised in 400 mM Tris-HCl pH 7.5 and stained with ethidium bromide (10 mg/l). Sixty random nuclear images of each slide were acquired with epifluorescent microscope (Olympus BX50), using BP 515–560 nm excitation and LP 590 nm barrier filters at 400× magnifications. Detected comets were scored by Komet 5.0 Kinetc Imaging-Software and the Olive tail moment (OTM) was calculated as: OTM = (tail mean - head mean) × % DNA tail/100 (Olive et al. 1990).

TEM analysis

The cells exposed to 31.25 mg/l were fixed (2% glutaraldehyde (GA) in 0.1 M sodium cacodylate buffer, 0.1 M sucrose, 3 mM $CaCl_2$, pH 7.4) at room temperature and maintained at 4°C o/n. The samples were washed with 0.1 M phosphate buffer, centrifuged, fixed with 2% osmium tetroxide (OsO_4) in 0.1 M phosphate buffer, pH 7.4 at 4°C for 2 h, dehydrated with ethanol and acetone, and embedded in LX-112. Ultrathin section were cut (Leica Ultracut UCT), contrasted with uranyl acetate followed by lead citrate, and examined in a Tecnai 12 Spirit Bio TWIN TEM (FEI Co., Eindhoven, The Netherlands), at 100 kV. Digital images were taken by using a Veleta camera (Veleta software, GmbH, Münster, Germany) (R Development Core Team 2011). Roughly 50 cells were analysed per sample.

Statistical analysis

The genotoxicity data were analysed with R program environment (R Development Core Team 2011). The OTM averages of the comet assay were compared using linear model for unbalanced block design with hierarchical sampling from three biological replicates. The data were transformed using square root transformation to satisfy assumption of normal distribution. Using both the GreenScreen analysis software, and GraphPad statistical analysis program (GraphPad Prism version 4 Harvey Motulsky), the cytotoxicity was presented as lowest effective dose (LEC) (mg/l) and percentage of relative cell density (RCD) ± standard error of the mean (SEM%) from four independent experiments and two technical replications inside each biological replication. One-way analysis of variance (ANOVA) and *post hoc* analysis (Duncan's multiple comparison tests) was used to determine statistical significance of the differences between values of treatment and control groups. Statistical significance was accepted at $p \leq 0.05$.

Results

Physicochemical properties of NPs

The results of the physicochemical characterisation of NPs are summarised in Table I. Information about the properties of the NPs in powder form was obtained from the manufacturer. The NPs had a significantly larger hydrodynamic size in medium, suggesting aggregation and/or agglomeration. The PDI values confirmed that the NPs were polydispersed (PDI > 0.2). Based on the zeta-potential measurements, ZnO-NPs and TiO_2-NPs dispersed in water were both qualified as stable suspensions, with values greater than ±30 mV (Malvern Instruments). The zeta-potential of TiO_2-NP suspensions showed a significant change of surface charge from +40.7 mV in H_2O to -17.8 mV in medium.

Oxidative stress induced by NPs

The oxidative potential of NPs was studied using cell-free dichlorofluorescein assay (Lu et al. 2008). The results are shown in Figure 1. SWCNTs, CuO-NPs as well as ZnO-bulk and CuO-bulk caused significant oxidation of DCFH-DA. TiO_2-NPs, ZnO-NPs and Ag-NPs did not induce measurable oxidative stress at any of the tested concentrations.

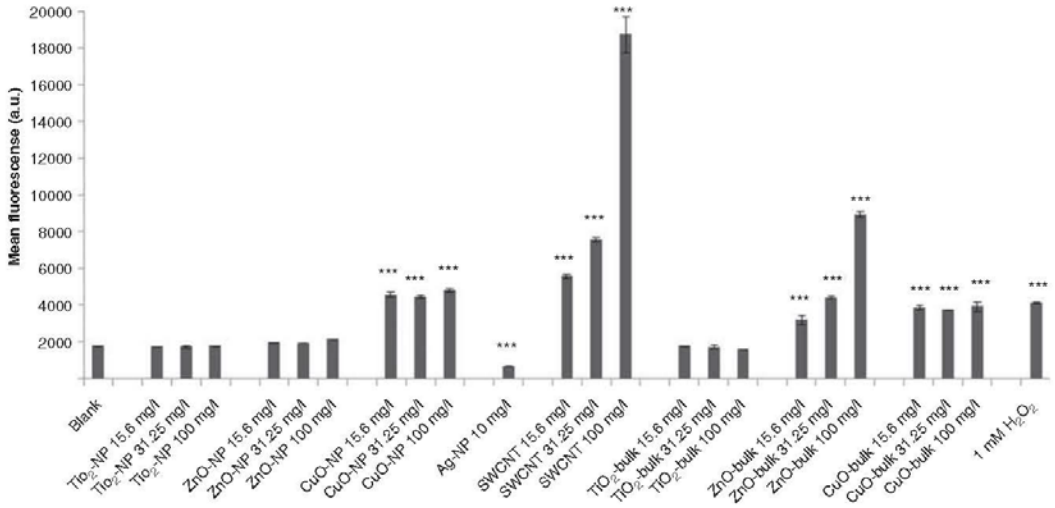

Figure 1. Oxidative potential assay. Fluorescence intensity (arbitrary units (a.u.)) of the NPs and the bulk-sized particles after incubation with DCFH for 2 h at room temperature. Values are the mean ± SEM from three experiments. For each treatment, three concentrations were used: 15, 31.25 and 100 mg/l, i.e. TiO_2-NPs (470, 147 and 73 cm^2/ml), ZnO (25, 8 and 4 cm^2/ml), CuO-NPs (29, 9 and 4 cm^2/ml) and Ag (9 cm^2/ml). Significance vs. blank control: ***$p < 0.001$.

Ion release by NP suspensions

Figure 2 summarises the concentration of ion isotopes, representing the total amount of ions measured by the ICP-MS, in the centrifuged and non-centrifuged NP suspensions. ICP-MS was not performed for SWCNTs, due to the technical limitations of the instrument. CuO-NPs and ZnO-NPs had the highest potential to release ions, at about 10 and 15 ppm, respectively. Very few ions were detected in the non-centrifuged TiO_2-NPs and Ag-NPs suspensions, maybe as a result of the attachment of NPs to the ICP-MS tubes. Nevertheless, the negligible amount of Ti and Ag ions detected from the centrifuged suspensions indicated a

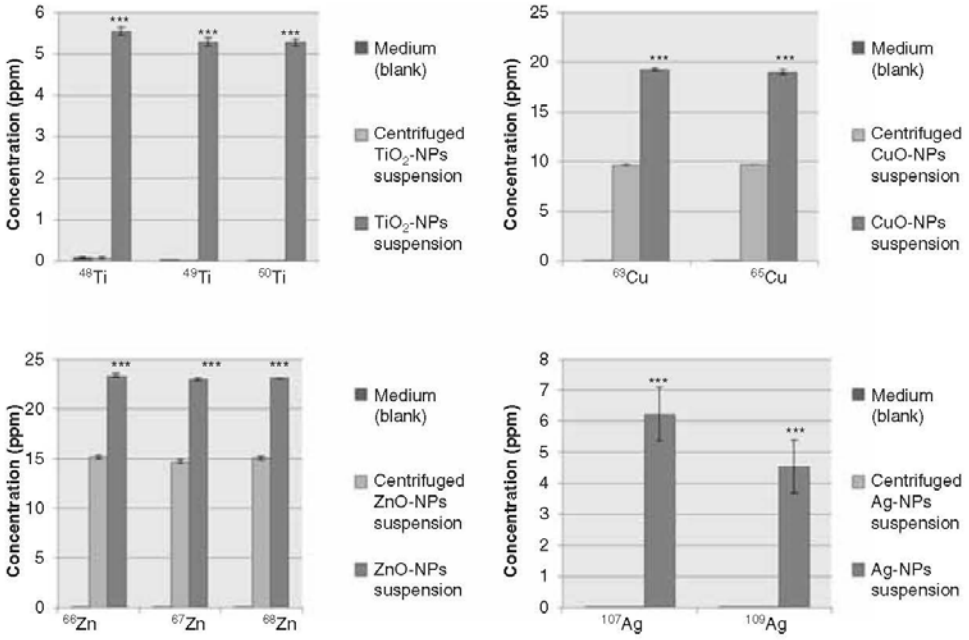

Figure 2. ICP-MS analyses of NPs. ICP-MS analysis of NPs suspended in exposure medium (at 31.25 mg/l) before and after ultracentrifugation at 100,000 *g* for 1 h. Cell culture exposure medium without NPs was used as blank. Significance between groups: ***$p < 0.001$.

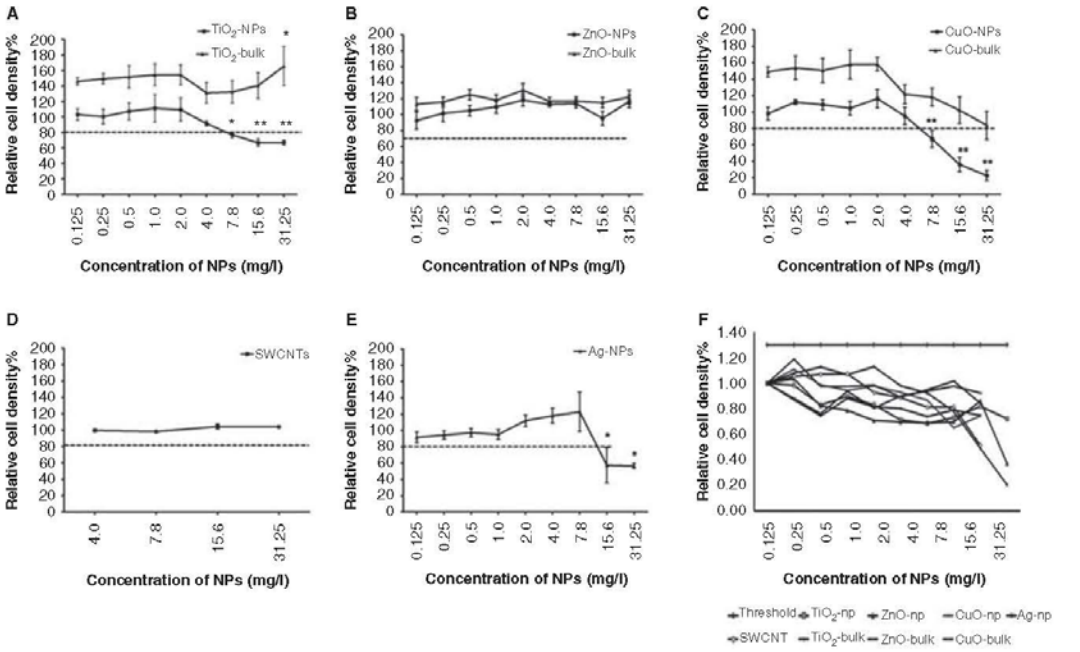

Figure 3. The GreenScreen assay data. The RCD ± SEM of the cells upon exposure to nine concentrations of NPs after 16 h of exposure performed in quadruplicates (A–E). The dotted line in graphs A–E represents the threshold for cytotoxicity at 80% cell density. The genotoxicity result based on the GreenScreen assay gathered from 3 independent experiments. (F). The dotted line represents the threshold for fluorescent induction. $^{*}p < 0.01$. $^{**}p < 0.001$. RCD ± SEM%: percentage of relative cell density ± standard error of the mean values.

lack of ion release from TiO_2-NPs and Ag-NPs under these experimental conditions.

Cell viability

The results from the GreenScreen assay are shown in Figure 3A–E and Table II. The dotted line in graphs A–E represents the threshold for cytotoxicity at 80% cell density. At 31.25 mg/l, CuO-NPs caused an almost 80% decrease in RCD, followed by Ag-NPs and TiO_2-NPs, representing a significant decrease compared with controls ($p < 0.01$). ZnO-NPs and SWCNTs and the respective bulk forms did not cause appreciable cytotoxicity, although CuO-bulk was close to the cytotoxicity threshold at 31.25 mg/l, which indicate potential for toxicity at higher doses.

Genotoxicity

Figure 3F shows the fluorescence produced by the GenT01 yeast cells due to DNA damage in the GreenScreen assay. NPs did not induce measurable genotoxic effects using this method. The degree of DNA damage caused by NPs and

Table II. Cyto- and genotoxicity of the NPs and their bulk form based on the GreenScreen and comet assay.

		GreenScreen assay				Comet assay	
Sample	Dose range corresponding to 31.25–0.12 mg/l (cm^2/ml)	Cytotoxicity	LEC (a) µg/ml (b) cm^2/ml	RCD ± SEM%	Genotoxicity	Genotoxicity	OTM ± SE
SWCNT	-	Negative	-	104 ± 1.8	Negative	Positive	21.0 ± 0.7
Ag-NP	19–0.07	Positive	(a) 15.6 (b) 0.9	56.4 ± 2.9	Negative	Positive	16.7 ± 0.7
TiO_2-NP	147–0.6	Positive	(a) 7.8 (b) 3.8	66.9 ± 3.0	Negative	Positive	16.6 ± 0.5
CuO-NP	9–0.04	Positive	(a) 7.8 (b) 0.2	17.6 ± 3.6	Negative	Positive	11.7 ± 0.5
ZnO-NP	8–0.03	Negative	-	116.4 ± 7.8	Negative	Positive	9.2 ± 0.5
TiO_2-bulk	-	Negative	-	165.6 ± 25.3	Negative	Positive	10.7 ± 0.5
CuO-bulk	-	Negative	-	83.8 ± 17.1	Negative	Positive	9.5 ± 0.5
ZnO-bulk	-	Negative	-	121.7 ± 8.8	Negative	Positive	8.1 ± 0.5
ddH_2O	-	Negative	-	100	Negative	Negative	5.5 ± 0.4

The percentage of cell survival at the highest concentration of NPs and their bulk form (i.e. 31.25 mg/l) is presented as RCD ± SEM%. The comet assay column shows results of Duncan's multiple comparisons test.
RCD ± SEM%: percentage of relative cell density ± standard error of the mean values, OTM: average olive tail moment. LEC: lowest effective dose; NP: nanoparticle; SWCNT: single-walled carbon nanotube.

Table III. Summary of the effects of the NPs on the ultrastructure of the cells based on the TEM images (Figures 4,5,6).

	Ultrastructural effects						
NPs	Nucleus	Vacuole	LDs	Cell wall	Dark deposits	NPs adsorbed on cells	Figures
Control	Well-defined spherical shape	Electron dense, defined spherical shape	Few LDs per cell cross-section	Uniformly thick, more electron dense external layer	In cytosol especially in vacuole and nucleus	-	4A1–A2
SWCNTs	No change	Autophagy visible	Moderate increase	In places folded, thicker and undulating appearance	No change	No visible adsorption	4B1–B2
TiO_2	No change	Different shape and content as in control	No change	In places undulating appearance	Increase, especially in vacuoles	NPs cover the entire external surface of cells	5A1–A2
ZnO	Effect on shape	Severe increase in size, granular appearance of content	No change	No change	Visible in vesicles	Adsorbed to cell wall in places; not dense	5B1–B2
CuO	Effect on shape	Disruption, LDs visible	Severe increase	In places folded, thicker and undulating appearance	Visible in vesicles	Attached to cell wall as thin outer layer	6A1–A2
Ag	Effect on shape	Could not be distinguished from other vesicles	Increased	Electron very dense external layer. Folded, thicker and undulating appearance Alterations of plasma membrane	Visible inside the cell wall and as larger particles in vesicles	NPs cover the external surface of cells in some others NPs are adsorbed to cell wall	6B1–B6 6

LDs: lipid droplets; NPs: nanoparticles; SWCNT: single-walled carbon nanotube.

bulk particles (31 mg/l) measured using the comet assay is shown Table II. In general, a significant amount of DNA damage was detected in NP-exposed cells when compared with controls (Table II). SWCNTs demonstrated the highest amount of DNA damage (31 mg/l after 16 h of exposure), followed by Ag-NPs and TiO_2-NPs, CuO-NPs, CuO-bulk and ZnO-NPs, with ZnO-bulk being the least genotoxic. NPs caused significantly higher genotoxicity than their respective bulk forms.

Ultrastructural changes in NP-exposed cells

The most prominent ultrastructural features seen in control and NP-treated yeast cells are summarised in Table III. In yeast control cells, the nucleus was round, the cytoplasm contained large vacuoles filled with homogenous granules, lipid droplets (LDs) were observed, and the cell wall was of uniform thickness with a more electron-dense external layer. Yeast cells exposed to ZnO-NPs, CuO-NPs and Ag-NPs displayed the most significant ultrastructural changes, including enlargement of the vacuoles, increased numbers of LDs and disruption of intracellular components, respectively (Figures 4–6).

Discussion

In this study, the authors assessed the cytotoxicity, genotoxicity and ultrastructural changes induced in *S. cerevisiae* by a range of different NPs and their bulk forms. Ag-NPs, TiO_2-NPs and CuO-NPs were cytotoxic, while other particles did not reduce RCD after 16 h of incubation at concentrations up to 31.25 mg/l. However, all the NPs caused some ultrastructural changes, which were more pronounced in the cells exposed to Ag-NPs, ZnO-NPs and CuO-NPs.

The authors first characterised the NPs in two suspensions (water and exposure medium) by measuring the zeta-potential, DLS, the cell-free dichlorofluorescein assay and ICP-MS. NPs in suspension did not retain their "nano-size", with significantly larger hydrodynamic sizes present in medium, suggesting either aggregation and/or agglomeration. Most studies show correlations between NP toxicity and their intrinsic reactive oxygen species (ROS) generation capacity which are thought to damage cellular structures (Xia et al. 2006; Nel et al. 2006). The oxidative potential of TiO_2-NPs, ZnO-NPs, CuO-NPs and Ag-NPs has been shown previously (Guo et al. 2011; Kumar et al. 2011; Bondarenko et al. 2012; Chen et al. 2012b), but there are also contradictory data. In this study, only SWCNTs showed significant oxidative capability, confirming some of the previous published findings (Das & Giasuddin 2012; Wang et al. 2012a; Cicchetti et al. 2011).

ICP-MS analysis was performed to estimate the fraction of dissolved ions generated from the particles in a test suspension after ultracentrifugation. Apart from dissolved ions, this fraction also included elements from those NPs which could not settle during centrifugation, due to their ultra-small mass resulting in them remaining in the supernatant. In case of TiO_2-NPs and Ag-NPs, Ti or Ag ions were not detected in the supernatant, while substantial numbers of Cu and Zn ions were present in the supernatant after ultracentrifugation of CuO-NPs and ZnO-NPs.

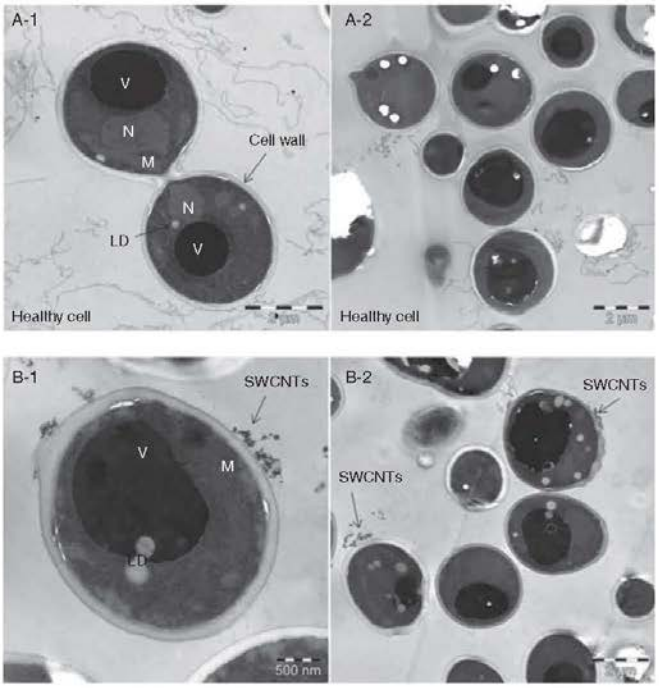

Figure 4. Control cells, i.e. yeast cell grown without NPs. Electron dense spherical vacuole (V), nucleus (N), rod-shaped mitochondrion (M) and lipid droplets (LD) are visible (A1–2). Cell exposed to SWCNTs at 31.25 mg/l for 16 h. (B1–2). No clear ultrastructural effects were visible.

Therefore, in the authors' experiments, the ultrastructural effects observed in cells exposed to TiO_2 and Ag-NPs were more likely to be related to the presence of NPs, rather than to dissolved ions, while effects observed in cells exposed to ZnO-NPs and CuO-NPs could be related to the ions released by these NPs.

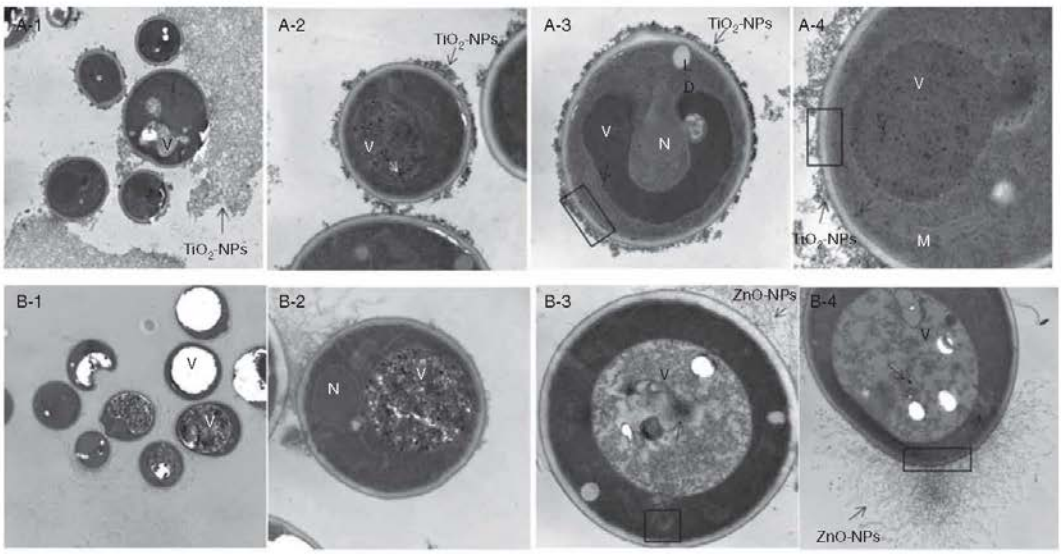

Figure 5. Cells exposed to TiO_2-NPs 31.25 mg/l (147 cm^2/ml) for 16 h. (A1–4). Arrows showing dense black deposits inside the vacuoles and in the cell membrane (marked with box). Cells exposed to ZnO-NPs 31.25 mg/l (7.8–4.7 cm^2/ml) for 16 h (B1–4). Significant effects: observations of significant enlargement on the vacuoles containing disrupted components, LDs and some dense black deposits (B3–4). Black deposits in vesicles (marked with arrows) and possible entries of ZnO-NPs (marked with box) by endocytosis (B4).

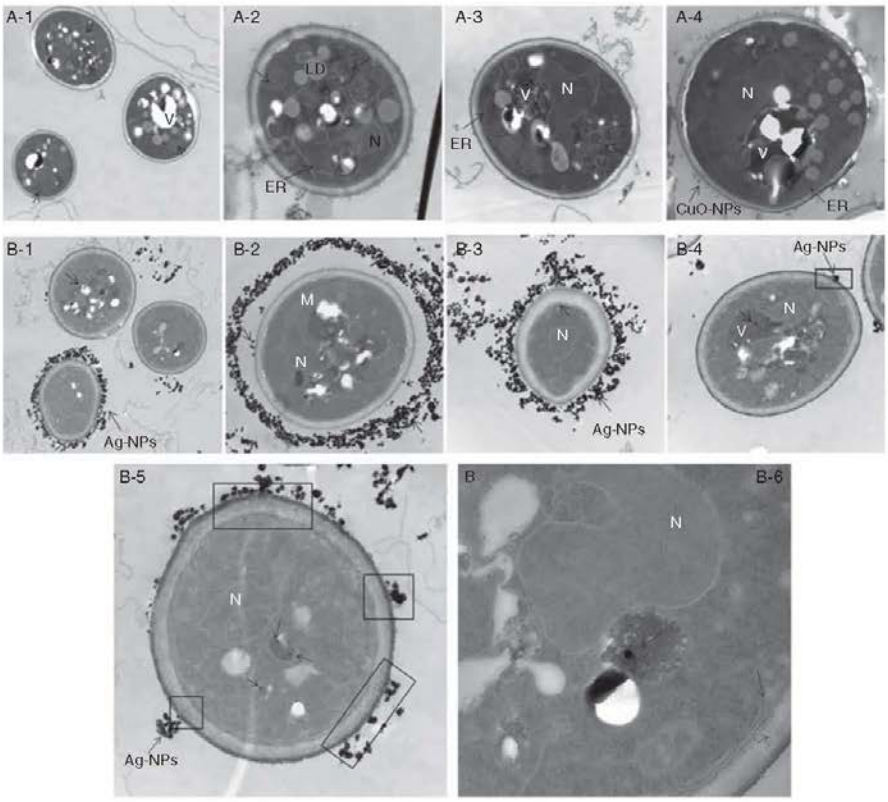

Figure 6. Cells exposed to CuO-NPs 31.25 mg/l (9.1 cm^2/ml) for 16 h (A1–4). The increase in the number LDs, the disruption or lack of vacuoles, as well as increase in the amount of small spherical organelles (marked with arrows) (A2–3). The LDs were in close contact with the ER as well as being involved with the vacuoles (A4). There is no evidence of CuO-NPs uptake inside the cells. Cells exposed to Ag-NPs 31.25 mg/l (18.8 cm^2/ml) for 16 h (B1–4). Alterations in intracellular components, the vacuoles, plasma membrane, cell nuclei and attenuation of the cytosol are visible (B1). Ag-NPs surround the cells and are adsorbed on the cell wall (B2). Shrinking of the cytosol and disruption of the cell membrane (B3). Adsorption of Ag-NPs on cell surface and presence of black deposits inside the vacuoles (B4). Ag-NPs adsorbed on the cell wall (marked with arrow). Disruption and undulating appearance at the membrane level visible (marked with box). Electron dense black deposits are visible inside vesicular organelles (marked with arrow) (B5). Close up of effect of Ag-NPs on the cells (B6).

Although there are several established methods for evaluating cytotoxicity, interactions between reagents and NPs can occur, such as with the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay, where CNTs can interfere with the MTT compound (Wörle-Knirsch et al. 2006). Therefore, the authors selected the GreenScreen assay to evaluate cytotoxicity, which is a reporter assay suitable for use with yeast cells, capable of reporting both cyto- and genotoxicity. SWCNTs were not shown to be cytotoxic, despite high oxidative potential. This is consistent with a previous study of yeast cells exposed to CNTs, in which significant differences in the growth rate and viability were not detected (Phillips et al. 2012). Other studies measuring the cytotoxicity of SWCNTs report results ranging from marked cytotoxicity (Davoren et al. 2007; Zhang et al. 2013; Murray et al. 2009) to complete lack of cytotoxicity (Leeuw et al. 2007; Kam et al. 2005; Kam & Dai 2005). However, these studies used a range of different exposure conditions, organisms, purity and concentrations of CNTs (Yehia et al. 2007), and the methodology applied, making direct comparison difficult. The lack of cytotoxicity of SWCNTs seen in this study might be explained by a relatively low exposure concentration and aggregation, and therefore an inability to penetrate the yeast cell wall.

Exposure to ultra-small TiO_2-NPs (1–3 nm) resulted in yeast cytotoxicity, whereas in other species and at other sizes of NP the effects have been shown to be very diverse. In *Arabidopsis thaliana*, exposure does not affect cell viability and morphology, but does result in microtubule network disruption (Kurepa et al. 2010). In hamster embryo fibroblasts, TiO_2-NPs (<20 nm) induce micronuclei and apoptosis (Rahman et al. 2002; Jin et al. 2008). Since the TiO_2-NPs used in this study showed no demonstrable oxidative potential or ion release, the observed cytotoxicity may depend on adverse effects caused by internalisation of these NPs. TiO_2-bulk particles, on the other hand, were not cytotoxic, agreeing well with previous findings (Kasemets et al. 2009).

Both nano-sized and bulk ZnO were not cytotoxic at concentrations of 31.25 mg/l. The cytotoxicity of ZnO-NPs to fungi is known to be concentration- and size-

dependent, with the minimal fungicidal concentration of ZnO-NPs estimated to be 100 mg/l (Lipovsky et al. 2011). It has been shown that ZnO-NPs (81 mg/l) perturb cytosolic zinc concentrations and zinc homeostasis (Kao et al. 2012). In previous studies of ZnO-NPs and bulk-treated *S. cerevisiae*, concentration-dependent effects on growth (24-h EC_{50} 131–158 mg/l) have been observed (Kasemets et al. 2009). In this study, the lack of cytotoxicity of ZnO-NPs could be due to primary particle size, low exposure concentrations and the fact that concentrations of Zn ions dissolved according to ICP-MS (15 mg/l) remained much lower than the levels known to induce cytotoxicity.

Yeast treated with CuO-NPs resulted in a significant decrease in RCD at 31.25 mg/l and the highest cytotoxicity among the NPs tested. Bulk CuO was the only bulk material which was potentially cytotoxic to yeast. Previous studies have also shown almost complete inhibition of growth of yeast at similar concentration ranges of NPs, and at 100 times higher concentration for the CuO-bulk form (Kasemets et al. 2009). CuO-NP toxicity has been postulated to be due to their oxidative potential (Karlsson et al. 2008) and generation of soluble Cu ions (Cohen et al. 2013; Studer et al. 2010). This study confirms that CuO-NPs have significant oxidative potential, and also ion dissolution (10 mg/l), consistent with this mechanism of cytotoxicity.

Ag-NPs were less cytotoxic than TiO_2-NPs and CuO-NPs, but some cytotoxic effects were observed in agreement with the antifungal activity of Ag-NPs previously described (Panáček et al. 2009) It is believed that the internalisation of NPs by scavenger receptors, followed by trafficking to the cytoplasm and release of Ag ions, is the most likely cytotoxicity mechanism of Ag-NPs (Singh & Ramarao 2012). Considering that the ICP-MS analysis detected only negligible amounts of Ag ion release, Ag-NP cytotoxicity might depend more on their uptake and adverse effects on cellular ultrastructure.

The authors assessed genotoxicity using two well-established assays, which generated different results. The GreenScreen assay did not show any measurable genotoxicity, while the comet assay indicated marked genotoxicity for all the NPs tested. There are many studies which have also demonstrated genotoxicity of these NPs using the comet assay (Kumar et al. 2011; Trouiller et al. 2009; Asharani et al. 2008; Kisin et al. 2007; Wang et al. 2012b). Therefore, on the basis of the literature, the recent data and in light of these results, the authors suggest that the results cannot be fully interpreted unless the degree of interaction between test medium, NPs and DNA is known. They recently proved, using *Tetrahymena thermophila* as a model organism and TiO_2 NPs, that false-positive results can occur in comet assay (Rajapakse et al. 2012). Furthermore, it is not possible to directly compare the effect of different concentrations of NPs on *S. cerevisiae* between different studies, due to different exposure durations and biological responses measured. On the basis of the data and the literature, the primary NPs characteristics such as particle size and oxidising potential are not directly related to the biological effects observed.

Finally, TEM was used in order to study the effect of NPs on the ultrastructure of the cells. SWCNTs did not have a significant impact on cellular ultrastructure, although some effects on cell wall morphology, autophagic vacuoles and adsorption to the cell exterior were seen. In previous studies, yeast exposed to low concentration of SWCNTs (0.25 and 50 mg/l) do not cause detectable morphological changes; however, at 100 mg/l, partial loss of cell contents and middle wall deformation was observed (Phillips et al. 2012). The lack of intracellular localisation of SWCNTs, and an increased number of surfactant-storing lamellar bodies, have also been observed in a 24 h exposure of A549 cells (Davoren et al. 2007) to SWCNTs. The findings suggest that cytotoxicity assessed by conventional toxicity parameters, such as cell viability, were not correlated with biological effects.

The TEM images obtained in this study showed an increase in electron-dense dark deposits inside the cells, especially within the vacuoles of cells exposed to TiO_2-NPs. Whether these deposits are internalised NPs, or the consequence of other metabolic events related to TiO_2-NP exposure, remain to be established. The authors also observed significant adsorption of TiO_2-NPs on the external surface of cells, which has not previously been reported in yeast. However in plants, the adsorption of particles onto the surface has been shown to disturb cellular metabolism (Slomberg & Schoenfisch 2012). The ultra-small TiO_2-NPs have been shown to be taken up in the plant model system *A. thaliana* (Kurepa et al. 2010).

In cells exposed to ZnO-NPs, severe effects on the vacuoles, and enlargement and alteration of internal components were visible. Although studies on *S. cerevisiae* transport systems for vacuolar zinc sequestration indicate that yeast can tolerate high concentrations of exogenous zinc (Devirgiliis et al. 2004; Eide 2006), the release of solubilised ions by ZnO-NPs contributes greatly to their toxicity (Kasemets et al. 2009; Ji et al. 2012).

The most significant increase in the amount of LDs was observed in cells exposed to CuO-NPs. In yeast, the accumulation and induction of LDs is reported to be associated with responses to environmental stress factors, such as limited nitrogen availability or osmotic stress (Murphy 2001; Ducharme & Bickel 2008). The link between Cu ions and lipid metabolism has been explained by altered expression of genes controlling cholesterol and lipid biosynthesis, which leads to the increased formation of LDs (Huster & Lutsenko 2007; Kennedy et al. 2009).

Ag-NPs also caused alterations in intracellular components, especially the vacuoles, plasma membrane, cell nuclei and attenuation of the cytosol. Recently, chromatin destruction and condensed DNA due to Ag-NP exposure has been reported (Das & Giasuddin 2012). Cell membrane damage due to Ag-NPs has also been evaluated, which showed membrane collapse and cell wall detachment in *S. cerevisiae*. In prokaryotes, Ag-NPs have been found attached to the cell surface, in the bacterial membrane (Morones et al. 2005), as well as causing disruption and penetration of the *Escherichia coli* plasma membrane (Raffin et al. 2008). However, the electron-dense deposits found associated with the cell wall and in the cytoplasm have been characterised as Ag ionic, not Ag-NP deposition (Despax et al. 2011) The insignificant amount of Ag ions detected by ICP-MS in the experiments

indicates that Ag deposits observed in the TEM images are most likely Ag-NPs.

The data provide evidence that frequently used cytotoxicity and genotoxicity analysis are not suited for NP studies, since NPs may disturb spectroscopic analysis due to their light scattering ability (such as possibly in the GreenScreen assay), adsorption to the surface of cells in suspension or cause post-exposure effects because they have not been removed from the medium during the test (such as in the comet assay). The authors' study supports the use of TEM for providing versatile and useful information on the interactions between NPs and the components of cells, and to generate plausible hypotheses on the possible biological effects. Therefore, classical assays of toxicity may not be the most suitable for studying the effects of NPs, and the simultaneous assessment of physiological measures of the state of cells or organisms are highly recommended.

Conclusions

TiO_2-NP, CuO-NP and Ag-NPs were cytotoxic after 16 h of exposure at 31.25 mg/l. All the tested NPs affected cell ultrastructure, the exact features being particle-dependent. They caused changes in the appearance of vacuoles, nucleus or cell wall or caused an increase in the number of LDs. NPs were adsorbed to the external cell surface, most significantly with TiO_2-NPs and Ag-NPs, which might have an indirect effect on cell metabolism. Genotoxicity data obtained using two different genotoxicity tests could not be accurately interpreted without a better understanding of the degree of interference between the test reagent, NPs and DNA after exposure.

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Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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OSTALO POVEZOVALNO ZNANSTVENO DELO

2.1.4 Proteomska analiza zgodnjega odziva mikroorganizma *Tetrahymena thermophila* ob izpostavitvi nanodelcem TiO_2

Particle-stimulated early response of unicellular eukaryotic microorganism *Tetrahymena thermophila* to TiO_2: a comparative proteomic study

K. Rajapakse, D. Drobne, D. Kastelec, C. Gallampois, A. Amelina, G. Danielsson, R. Marinsek-Logar, S. Cristobal

Objava poslana v revijo Particle and fibre toxicity

V naši študiji smo raziskovali zgodnji odziv mikroorganizma *T. thermophila*, ki smo ga izpostavili koncentracijam nanodelcev TiO_2 in delcev TiO_2 večjih od 100 nm (0.1 in 100 µg TiO_2/ml), ki so nimajo učinka toksičnosti. S proteomskim pristopom (2DGE + ESI-MS/MS) smo preučili citosolno celično frakcijo. V povprečju smo zabeležili 930 lis na gelu, pri tem je bilo pri gelih, kjer smo obravnavali citosol tretiranih celic 77 lis statistično značilno različno izraženih v primerjavi lisami v kontrolnih poskusih. Med 77 lisami smo na podlagi statistično značilnih sprememb v izraženosti izbrali 18 lis in jih identificirali. Izbrani proteini sodelujejo pri metabolizmu maščob in maščobnih kislin (Acil-CoA dehidrogenaza, trioze fosfat izomeraza), pri energetskem metabolizmu (protein družine enolaz, akonitatna hidrataza, trioze fosfat izomeraza), pri stresu slanosti (protein družine S-adenozilmetionin sintetaz, protein družine peptidaz M20/M25/M40, DnaK protein), pri razgradnji proteinov (protein družine proteasoma tipa A in B), pri metabolizmu purinov (protein purin nukleozid fosforilaza 1, protein družine specifične za inozin in gvanozin) in pri metabolizmu *per se* (protein družine fosfolipaz in karboksilaz, oksidoreduktaza, protein družine aldo/ketoreduktaz). Potrdili smo da je v primeru izpostavitve nanodelcem TiO_2 pri najnižji koncentraciji (0,1 µg nanodelcev TiO_2/ml) prišlo do nanospecifičnega učinka. Zaključujemo, da se zgodnji odziv pri praživali *T. thermophila* na izpostavitev delcem TiO_2, kaže s spremembo v metabolizmu lipidov in maščobnih kislin, v energetskem metabolizmu in v ionski regulaciji že pri nizkih izpostavitvenih koncentracijah delcev TiO_2.

Particle-stimulated early response of unicellular eukaryotic microorganism *Tetrahymena thermophila* to TiO_2: a comparative proteomic study

Rajapakse K, Drobne D., Kastelec D., Gallampois C., Amelina A., Danielsson G., Marinsek-Logar R., Cristobal S.

Abstract

Our study investigated the early response of eucaryotic protozoan *T. thermophila* to nano-TiO_2 or bulk TiO_2 particles at subtoxic concentrations (0.1 and 100 µg TiO_2 /ml) using proteomic analyses of cytosolic cell fraction. The early response of *T. thermophila* exposure to TiO_2 particles was related to alteration of lipid and fatty acid metabolism, energetic metabolism and ion regulation already at low exposure concentrations. The response was not dose-dependent, but in case of the lowest nano-TiO_2 concentration (0.1 µg TiO_2 /ml) it was size dependant. The response to lowest nano-TiO_2 concentration signifficantly differed from that at higher nano-TiO_2 or both bulk-TiO_2 concentrations. In this paper we propose a definition of an early response as a measurable physiological response where no cytotoxicity markers, e.g. oxidative stress, are observed.

List of abbreviations

2-DE: two-dimensional gel electrophoresis; CBB G-250: Coomassie Brilliant Blue G250.

Introduction

With advent of omic tools, the insight into the overall molecular mechanisms of interactions between chemicals and cells could be studied what may allow revealing some new mechanisms not anticipated before (Matranga and Corsi, 2012). Nanoproteomics has easily found its way into nanotoxicity, since precise mode of interaction between nanoparticles and cells is not clearified yet (Ray et al., 2010). It is expected from the omic tools to indicate some nano-specific biological effects.

Indeed, in case of exposure to TiO_2 nanoparticles (TiO_2-NPs), conventional *in vitro* and *in vivo* studies provided evidence of oxidative stress, cytotoxicity, inflammation and a series of toxic responses (Ferin et al., 1992) (Park et al., 2008) (Monteiller et al., 2007, Johnston et al., 2009). The proteomic analysis provided some additional insight into interractions between TiO_2 NPs and cells like downregulation of proteins related to antimicrobial activity, up-regulation of lipid and fatty acid metabolism related proteins, downregulation in mRNA processing proteins and histone isoforms ((Ge et al., 2011; (Gao et al., 2011)). (Ge et al., 2011) reported novel antioxidant defense pathway or mechanism mediated by MAPK/ERK activated Nrf-2 signaling after exposing BEAS-2B cells lines to TiO_2 NPs. Gao et al. (2011) also provided evidence that, proteomic analysis of early response in mice lymph nodes after TiO_2-NPs injection revealed a differential expression of proteins involved in immune response and nucleosome assembly (Gao et al., 2011). Most of these effects could not be elucidated using conventional non “omic” approach.

Here we present the complementary study to our previous work on effects of unicellular eucaryotic organism *Tetrahymena thermophila* to TiO_2-NPs (Rajapakse et al., 2012). That study provided evidence that neither, TiO_2-NPs nor bulk TiO_2 particles at concentrations up to 1000 µg/ml provoke adverse effect as assessed by ROS generation or lipid peroxidation. However, changes of membrane fatty acid profile were evident (Rajapakse et al. 2012).

The aim of study presented here is to elucidate biochemical pathways involved in early response of *T. thermophila* to TiO_2-NPs by proteomic tools. *T. thermophila* is a convenient

model unicellular organism to study different physiological pathwas under optimal or suboptimal conditions. Its genome has been sequenced (Tetrahymena Genome Database; www.ciliate.org). Its mitochondrial proteome (545 proteins) (Smith et al., 2007), phagosome proteome (73 proteins) (Jacobs et al., 2006) and ciliome proteome (223 cilia proteins) (Smith et al., 2005) are well studied. In addition, *T. thermophila* shares a higher degree of functional conservation with human genes than other microbial eucariotic microorganisms (Turkewitz et al., 2002).

In this study, we investigated cytosolic fraction of *T. thermophila* proteome where a great deal of proteins involved in energetic metabolism or fatty acid synthesis/regulation are expected. In addition, there are no reports on cytosolic proteome alterations alone as a result of nanoparticle exposure.

We hypothesise that exposure of *T. thermophila* to subtoxic concentrations of TiO_2-NPs or bulk TiO2 provokes different proteomic response. On the basis of results our previous research on cell membrane structure (Rajapakse et al., 2012) we expect alteratons in fatty acids and energetic metabolism. We disscuss the sensitivity and accuracy of proteomic tools in revealing the particle-cell interactions.

Methods and materials

Chemicals.

Unless otherwise specified, reagents were purchased from Sigma Aldrich Co (St. Louis, MO, USA), Merck (Darmstadt, Germany) or Biolife (Milan, Italy). TiO_2 -NPs with 99.7% purity were supplied in the form of a powder.

Bulk and nano-TiO_2 tested suspension.

Aqueous dispersions of NPs were put on carbon-coated grids, dried at room temperature, examined with a 200-keV field emission transmission-electron microscope (Philips CM 100; Koninklijke Philips Electronics, Eindhoven, The Netherlands), and analyzed by transmission-electron diffraction to identify the TiO_2 crystal phase.

Bulk TiO_2 and 15 nm TiO_2 -NPs were dispersed in poor medium (PM) (Rajapakse et al., 2012) which was adjusted with modifications after (Schultz, 1997), before treating the cell cultures. Bath sonication for 30 min was used to disperse particle agglomerates in stock solutions.

The suspensions of NPs (1000 µg/ml) were characterised by dynamic light scattering (DLS) using a 3D DLS-SLS (dynamic light scattering - static light scattering spectrometer: LS Instruments, Fribourg, Switzerland). The HeNe was used as the light source laser and was operating at a wavelength of 632.8 nm. Scattering was measured at an angle of 90^o.
Zeta potentials of TiO_2 nanoparticle suspensions (1000 µg/ml) were measured with ZetaPals, (Brookhaven Instrument Corporation) in the PM medium, which was used for the exposure to living cells.

Characterisation of of 0.1 µg/ml TiO_2-NPs was performed using DLS and measurements of zeta potential. The suspension was prepared and has undergone the same conditions as the suspension containing cells in the experiment would (TiO_2-NPs were dispesed in a poor medium (PM, described below) and shaken at 32°C for 24h).

***T. thermophila* growth and exposure conditions.**

Axenic cultures of *T. thermophila* from the Protoxkit FTM (MicroBioTests Inc.) were grown for 24 h in the dark at 32 °C in a semidefined-proteose-peptone based rich medium (RM) (Schultz, 1997). The cell density obtained after incubation in these culture conditions was approximately 10^5 cells/ml.

The cells were harvested by 3 min centrifugation at 60 rcf. Cells were washed and resuspended in a poor medium (PM), which consisted of the semidefined-proteose-peptone based medium used by Schultz (Schultz, 1997), but lacking yeast extract and bacteriological peptone. The pH of the medium was adjusted to 7.4 and temperature 32 °C for the entire experiment. All experiments were performed in 100 ml batch cultures that

were maintained in Erlenmeyer flasks and aerated by shaking at 90 rpm in an incubator in the dark.

After 1 h in the PM, we used centrifugation to concentrate the cells (3 min centrifugation at 60 rcf), followed by exposure of cells to suspension of bulk or nano-TiO_2. The final nominal concentration of particles in the medium, either bulk or nano-, was 0.1 and 100 µg/ml. *T. thermophila* cultures were incubated at 32 °C for 24 h. For each concentration of bulk or nano-TiO_2, we used five independent replicates. A set of five replicates without TiO_2, was set up as a control for each assay.

Two dimensional gel electrophoresis and image analysis.

After 24 h five replicate treatments with i) no added TiO_2 particles or ii) TiO_2 bulk or iii) TiO_2-NPs, 100 ml of cell suspension was harvested for the purpose of cell fractionation, which was slightly adapted as previously described by Guilleman and coworkers (Guillemin et al., 2005) (Fig 2). Briefly, deeply frozen cells were homogenised in ice-cold homogenization buffer containing 10 mM HEPES, 10mM NaCl, 1mM KH_2PO_4, 5mM $NaHCO_3$, 5mM EDTA, 1mM $CaCl_2$, 0.5 mM $MgCl_2$ and the following protease inhibitors: 0.2 mM PMSF, 2 µM leupeptin, 2 µM pepstatin, 1 mM Ɛ-aminocaproic acid. Thereafter, 50 µl of 2.5 M sucrose was added to restore isotonic conditions. The homogenates were centrifuged at 6300 x g for 10' to pellet the cell debris and nuclei. The supernatant was sedimented at 18 000 x g for 200' in a tabletop centrifuge. The supernatant represented the cytosolic fraction which was precipitated by 20% TCA and 100% cold acetone with 0.07% (v/v) β-mercaptoethanol. Proteins extracted by this method were solubilized in a solubilization buffer (7 M urea, 2 M thiourea, 4 % CHAPS (w/v), 0.5 % Triton X-100, 1 % β-mercaptoethanol (v/v), 0.2 % IPG buffer (3-10), 1 % DTT (w/v), modified from Rabilloud (1998). Afterwards, samples were alkylated with 30 m IAA for 15' in darkness and then mixed with rehydration solution containing 8 M urea, 2 % CHAPS (w/v), 15 mM DTT, 1% β-mercaptoethanol (v/v) and 0.2 IPG buffer (v/v) (3-10). Solubilized samples were applied onto 11 cm IPG strips, pH 3-10NL (BioRad, Hercules, CA). Protein concentrations were measured according to Bradford (Bradford, 1976) using bovine serum

albumin as a standard. The total protein applied per gel was 200 µg. Isoelectric focusing was performed on a Protean IEF Cell (BioRad) at 20°C using the following program: passive rehydration for 12 hours, rapid voltage slope for all the steps, step 1: 250 V for 15', step 2: 8000V for 2.5 hours and step 3: at 8000 V until it reached 35 000Vh. After this, the IPG strips were reduced (1% DTT (w/v)) and then were alkylated (4% IAA (w/v)) in equilibration buffer (6 M urea, 50 mM Tris pH 8.8, 30% glycerol (v/v), 2% SDS (w/v) and 0.002% CBB (w/v)). The second dimension was carried out on homogeneous 12.5% T Criterion precast gels (Bio-Rad, Hercules, CA), at 120 V for 2 h using a Criterion Dodeca Cell (Bio-Rad).

The protein spots in the gels were visualized by staining with Coomassie Brilliant Blue G250 (CBB G250), and the gel images were obtained using Image Scanner (GE Healthcare, Uppsala, Sweden). Image Master 2D Platinum 6.0 software (GE Healthcare) was used for matching and analysing of visualized spots among differential gels to compare the level of protein expression between cytoplasm from non-treated cultures of *T. thermophila* and nano- or bulk-TiO_2 treated cultures. Each spot intensity volume was processed by background substraction and total spot volume normalization, giving the spot volume percentage (Vol%). For the matching, two match sets were created grouping gels from nano- or bulk- TiO_2 size, five gels in each match set were organized into two match sets according to concentration. After completion of spot matching, the normalized spot intensity of each protein spot from individual gels was compared between groups using statistical analysis.

Statistical analysis.

The experiment with five tratments (control; nano-group: 0.1 µg TiO_2-NPs /ml and 100 µg TiO_2-NPs /ml; bulk group: 0.1 µg bulk-TiO_2/ml and 100 µg bulk-TiO_2/ml) was conducted in five replications (five gels for each treatment). Hierarchical clustering using Euclidean distance and Ward's method was used as main exploratory statistical analysis to find out groups of gels with similar protein expression. The heatmap with dendrograms was used for graphical presentation of the results, showing relative expression of spots for each gel. The gels and spots are on heatmap reordered according to two dendrograms given by

chosen clustering method. One-way ANOVA with Dunnet's multiple comparison tests with probability value $p < 0.05$ considered signifficant, was used to find out spots, by which treatments with nano and bulk TiO_2 in two different concentrations, statistically significant differ in their experession comparable to control.

Protein identification by nano-scale liquid chromatography-electrospray ionization tandem mass spectrometry (nLC-ESI-MS/MS) and database searching.

The protein identification was performed by nLC-ESI-MS/MS) analysis and *de novo* sequencing of the peptides produced by proteolytic digestion of excised spots from the 2-DE maps. Selection of spots is described in more detail under results. The selected spots were excised from the gels and treated as described in the UCSF In-Gel Digestion Protocol (http://ms-facility.ucsf.edu/ingel.html). In brief, the gel pieces were distained with 25 mM ammonium bicarbonate: acetonitrile (1:1, v/v) twice for 10 min and then dehydrated with acetonitrile for another 10 min before completely drying gel pieces with speed Vac. The samples were chemically reduced with 10 mM dithiothreitol (DTT) in 25 mM ammonium bicarbonate at 56 °C for 1 hour and then they were alkylated with 55 mM iodoacetamide in 25 mM ammonium bicarbonate, in the dark for 45 min. Afterwards, gel pieces were washed in 25 mM ammonium bicarbonate: acetonitrile (1:1, v/v) for 10 min and then dehydrated with acetonitrile for another 10 min before they were completely dried in a Speed Vac. Sequencing grade modified trypsin at 12.5 ng/µL in 50 mM ammonium bicarbonate was added to the gel pieces and they were left to swell on ice for 10 min on ice at 4°C, followed by overnight incubation at 37°C. The peptides were extracted and desaulted and concentrated using reversed-phase C18 solid phase extraction (TopTip, Glygen Corp., Columbia, USA) according to the manufacturer's protocol. Peptides were loaded on the TopTip, pre- conditioned with a washing solution consisting of 50% of acetonitrile followed with a binding solution consisting of 0.1% of formic acid. The peptides were eluted with the washing solution (2 times 35 µL), dried and then resuspended in 6µL 50% acetonitrile/0.1% formic acid for the LC-MS/MS analysis, performed with an on-line nano-flow HPLC system (EASY-nLC; Proxeon, Bruker Daltonics) coupled to a mass spectrometer HCTultra PTM Discovery System (Bruker

Daltonics) equipped with an electrospray ionization source. The peptides were eluted at a flow of 300 nL/min using C18 reversed-phase pre-column (20 mm×100 µm, 5 µm particle size, NanoSeparation, The Netherlands) and analytical column (100 mm×75 µm, 5µm particle size, NanoSeparation, The Netherlands). Separation was performed with a linear gradient of solvent (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile for 60 min as follow: 0–100% B in 40 min, then 100% B for 20 min. A volume of 4 µL was injected. The MS analysis was made by data dependent acquisition: MS full scan within the range 50-3000 m/z, followed by MS^2 precursor selection and exclusion for 1 min. MS^2 acquisition range was 200 to 1500m/z and the automated online tandem MS fragmentation (MS^2) of peptide ions were performed using alternating collision induced dissociation (CID) and electron transfer dissociation (ETD) of peptide ions. Data Analysis software (Bruker) was used to analyze the ESI/MS/MS spectra, Mascot (NCBI database) for protein identification search. For the Mascot search, NCBInr database was used with the following settings: (i) Taxonomy: other Alveolata, (ii) Enzyme: trypsin allowing up to 2 miss cleavages, (iii) fixed modification: carbamidomethyl (c), (iv) variable modifications: acetyl (N-term) and oxidation (M), (v) peptides tolerance: 0.8 Da, (vi) MS/MS tolerance: 0.8 Da, (vii) peptide charges: 1+ 2+ and 3 + for CID and 2+ 3+ and 4+ for ETD) and (viii) instrument: ESI-trap. The identified peptides are then assembled to identify proteins using a scoring scheme that measures their similarity and the one with the best score is assigned as the identified proteins. Peptides were considered reliable for identification if the MS/MS spectra had a MASCOT score above 35 and an expected value below 0.01 (Ingelsson and Vener, 2012).

Results

Sample separation using Two Dimensional Gel Electrophoresis.

To explore nano- and bulk-TiO_2 particle induced changes in the proteome of *T. Thermophila* exposed for 24 h at two different concentrations (0,1 µg/ml and 100 µg/ml), we performed quantitative proteomic analysis using 2-DE. By using Comassie brilliant blue staining, approximately 930 protein spots were detected on the 2-DE gels. Figure 1 shows representative 2-DE images of one (out of five replicates) of *T.thermophila* in a

control group and a group exposed to TiO_2 particles (0.1 µg nano-TiO_2/ml, 100 µg nano-TiO_2/ml, 0.1 µg bulk-TiO_2/ml, 100 µg bulk-TiO_2/ml).

Results of statistical analysis.

On average, 600 spots were detected in at least four out of five replications (gels) per treatment. Cluster analysis on 25 gels with the data for 600 spots (Fig. 2) showed two groups of gels: in one group are gels/cultures? treated with 0,1 µg nano-TiO_2/ml and in the second group are gels from all other treatments.

266 spots were detected in five control gels as well as in at least one other treatment. Statistical analysis based on one-way ANOVA revealed 94 spots that were differentially expressed at 0.05 signifficance level, and further analysis with Dunnet test showed 77 spots differentially expressed in comparison to the control gels. Heatmap analysis was performed on these spots (Fig. 3) and gave very similar results as cluster analysis on 600 spots: one group of gels with 0.1 µg nano-TiO_2/ml treated cultures and second group of gels from all other treatments.

Among 77 spots selected as described abowe, we selected 18 spots for identification. Our selection of spots for identification was based on two characteristics of spots:
a) differential expression in comparison to control in all four TiO_2 treatments (0,1 µg/ml and 100 µg/ml nano or bulk TiO_2)
b) statistically significanttly diffent expression in at least one of these TiO_2 treatments when compared with controls.
We have identified two additional proteins that were differentially expressed only in nano-TiO_2 treatments but not in bulk-TiO2 treatments in comparison to control gels. Also here, at least in one group, the protein expression level was statistically difffenet in comaprisonto controls.

The expression levels of all identified proteins are shown in Fig 4.

Identified proteins.

We identified 16 proteins where the expression level was changed in all four TiO_2 treatments as describend in previous paragraph, and statistically significantly differently in at least one TiO_2 treatment. In addition, we identified two proteins that were differentially changed in nano-TiO_2 treatments only.

The identified proteins are related to lipid and fatty acid metabolism (Acyl-CoA dehydrogenase, triosephosphate isomerase), glucose metabolism (enolase family protein, aconitate hydratase, triosephosphate isomerase), Fe^{2+} homeostasis (aconitate hydratase), salt stress (S-adenosylmethionine synthetase family protein, Peptidase family M20/M25/M40 protein, DnaK protein), protein degradation (Proteasome A-type and B-type family protein), purine metabolism (purine nucleoside phosphorylase I, inosine and guanosine-specific family protein) and metabolism *per se* (Phospholipase/Carboxylesterase family protein, oxidoreductase - aldo/keto reductase family protein) (Table 2).

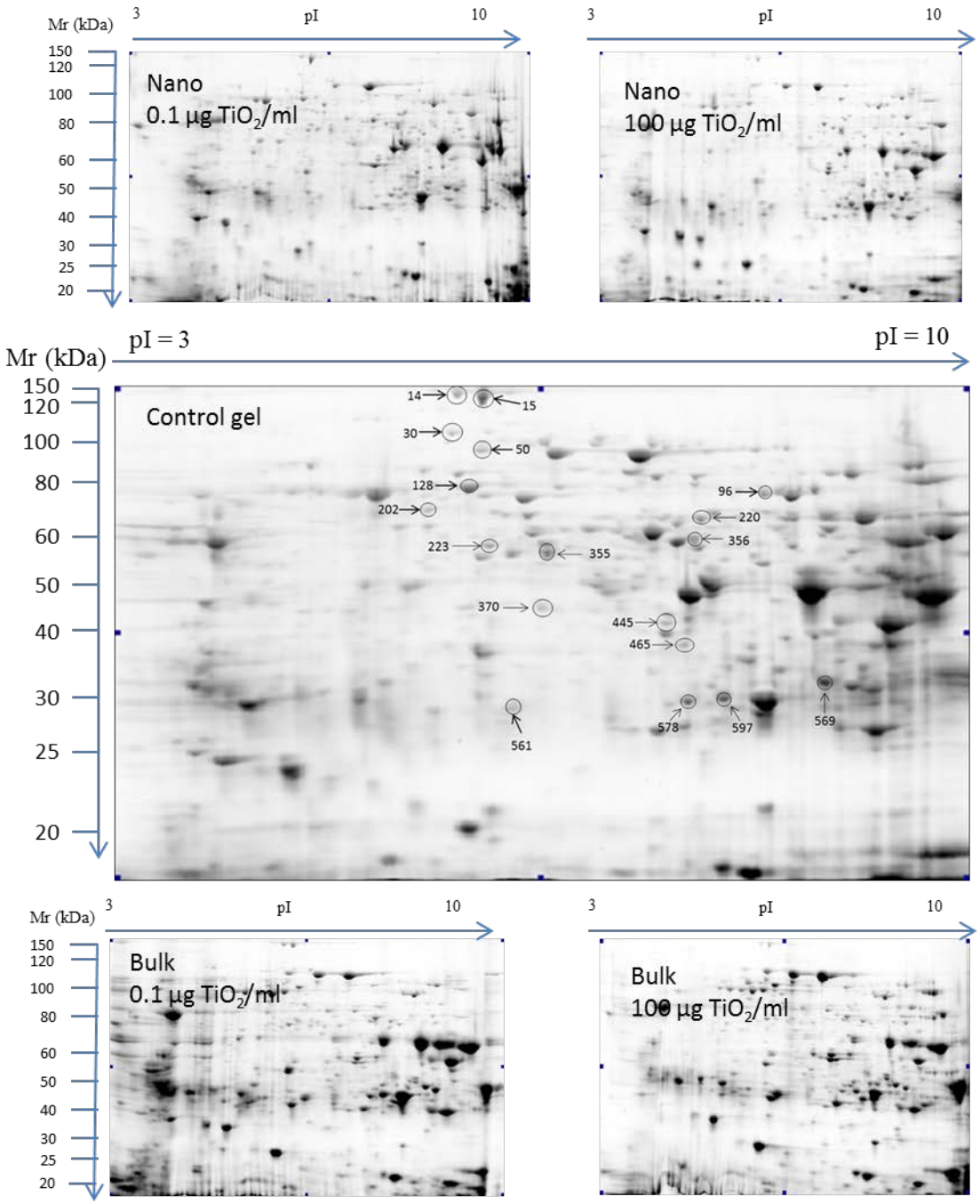

Fig. 1: 2-DE reference gel images of control and TiO_2 treated cultures (**Control gel** = control group; **nano-group:** 0.1 µg nano-TiO_2/ml; 100 µg nano-TiO_2/ml; **bulk group:** 0.1 µg bulk-TiO_2/ml; 100 µg bulk-TiO_2/ml)**.**

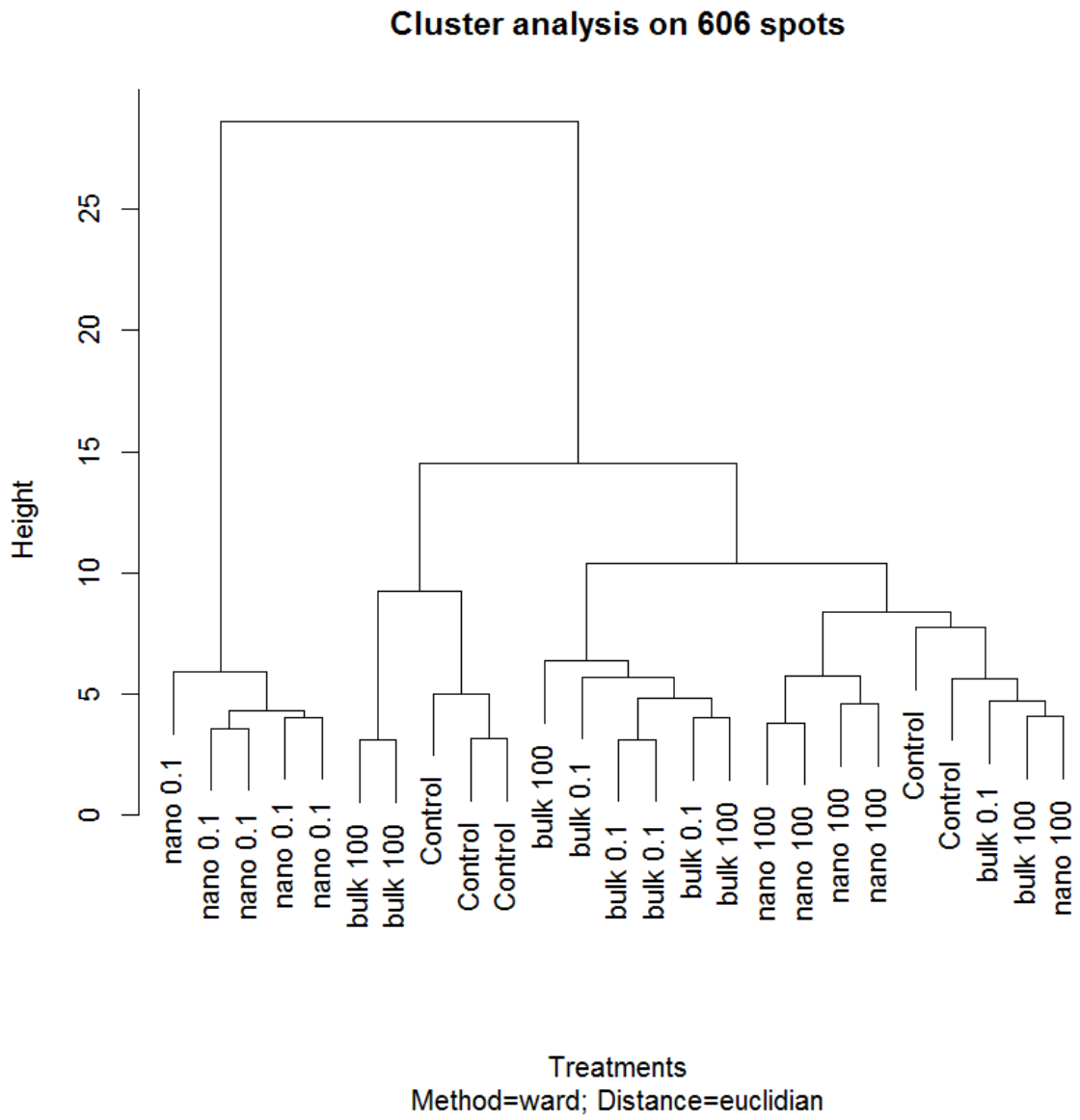

Fig. 2: Cluster analysis on 606 spots present in at least four out of five replicates (gels) per treatment. (Control= 0.0 µg TiO_2 particles/ml gels; nano 0.1 = 0.1 µg nano-TiO_2/ml gel; nano 100 = 100 µg nano-TiO_2/ml gel, bulk 0.1 = 0.1 µg bulk-TiO_2/ml gel, bulk 100 = 100 µg bulk-TiO_2/ml gel).

Table 1: Number of spots where protein expression levels (average relative percentage of protein) differed statistically significantly in expression compared to expression levels of spots in control gels (77 spots). NA=number of spots not expressed in treatment. Abbreviations: nano 0.1 = 0.1 µg nano-TiO_2/ml gel; nano 100 = 100 µg nano-TiO_2/ml gel, bulk 0.1 = 0.1 µg bulk-TiO_2/ml gel, bulk 100 = 100 µg bulk-TiO_2/ml gel).

Ratio Treatment/Control	bulk 0.1	bulk 100	nano 0.1	nano 100
less than -3	10	10	0	5
-3 to less than -2'	6	10	3	5
-2 to 2	37	29	19	50
more than 2 to 3	5	3	**27**	7
more than 3	3	6	**11**	4
Not expressed	16	19	17	6

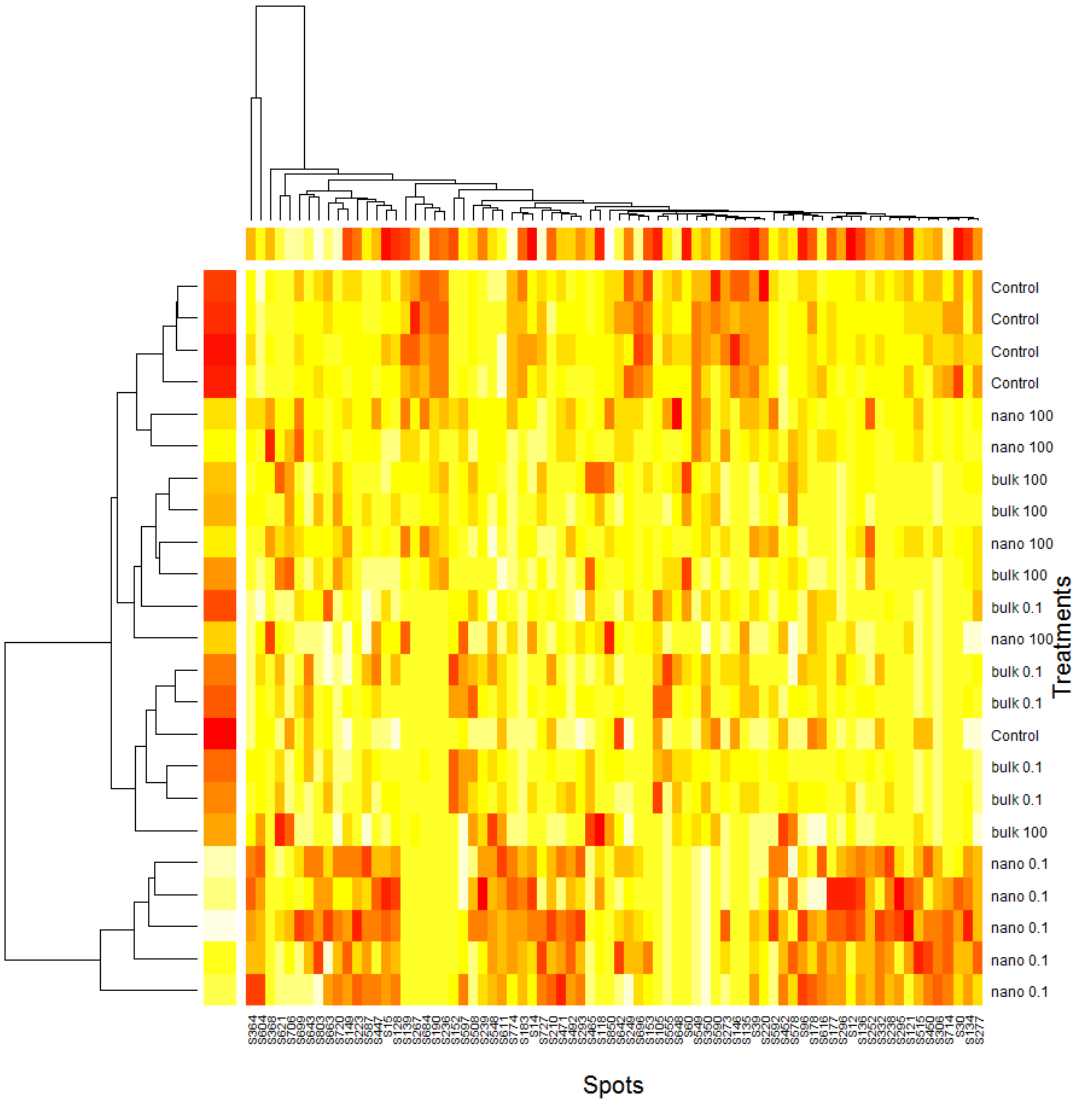

Fig. 3: Heatmap plot showing relative percentage of 77 signifficantly expressed spots in different treatments. One square in a heatmap equals every spot's relative percentage volume in a single gel, values are expressed in heat colors (from white to yellow, orange and red).

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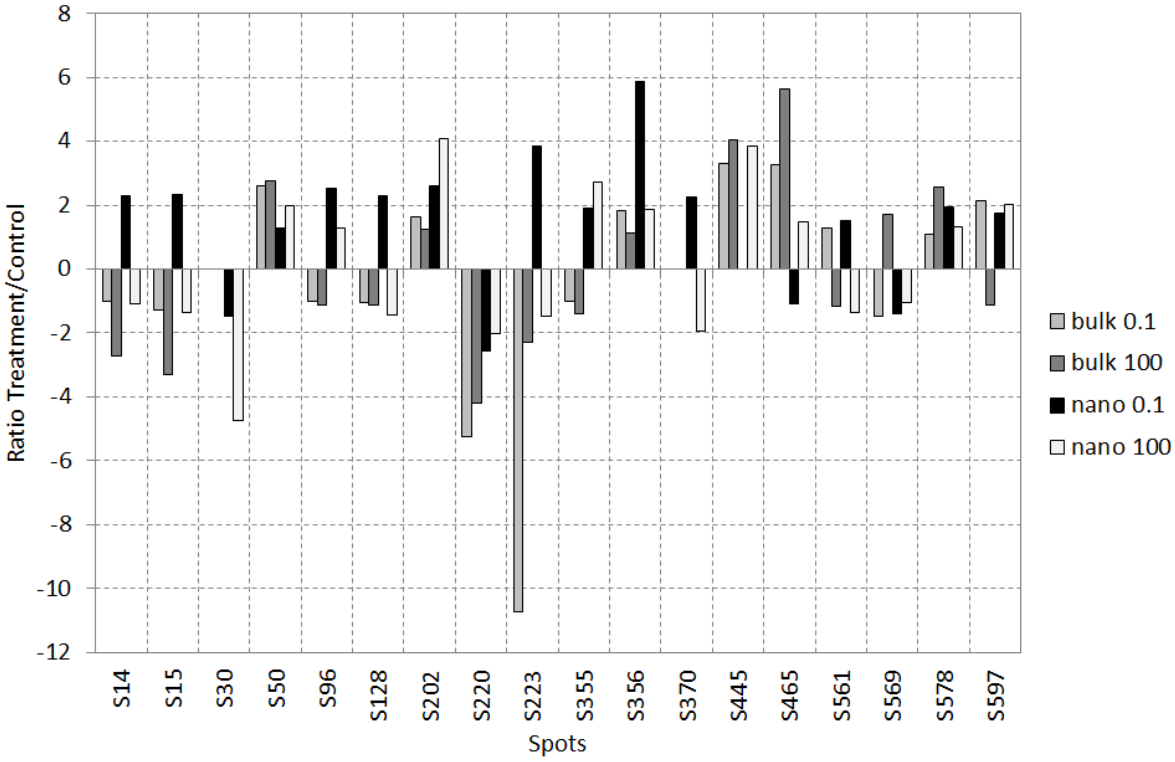

Fig. 4: Ratio between treatment and control protein expression in *T. thermophila* following exposure to TiO_2 particles (nano 0.1 = 0.1 µg nano-TiO_2/ml gel, nano 100 = 100 µg nano-TiO_2/ml gel, bulk 0.1 = 0.1 µg bulk-TiO_2/ml gel, bulk 100 = 100 µg bulk-TiO_2/ml gel). Protein expression levels were determined by relative volume using image analysis. Mean spot intensities on at least 4 out of 5 gels per treatment are shown.

Table 2: Identified differentially expressed proteins from *T. thermophila* cells treated with nano- or bulk-TiO_2 particles.

					Up-(↑), down-(↓) regulation or no change (0) in protein expression								
Spot nr	**Protein**	**Putative identification, NCBI sequence identifier**	**Protein function**	**Biological process**	**0.1 µg nano TiO_2 /ml**	**100 µg nano TiO2 /ml**	**0.1 µg bulk TiO2 /ml**	**100 µg bulk TiO2 /ml**	**MW (kDa)**	**Matched peptides**	**% coverage**	**Score**	**pI**
Lipid and fatty acid metabolism													
355	Acyl-CoA dehydrogenase, C-terminal domain containing protein	gi|118377002	The Acyl-CoA dehydrogenases are mitochondrial flavoenzymes that catalyse the initial step in fatty acid β-oxidation (GO:0016627).	energy production, synthesis of new fatty acids, fatty acid oxidation	↑	↑	0	0	45506	9 (8)	30	278	
356	Acyl-CoA dehydrogenase, C-terminal domain containing protein	gi|118377002	The Acyl-CoA dehydrogenases are mitochondrial flavoenzymes that catalyse the initial step in fatty acid β-oxidation (GO:0016627).	energy production, synthesis of new fatty acids, fatty acid oxidation	↑	↓	↓	↓	45506	7 (6)	24	211	
597	triosephosphate isomerase	gi|146162132	Fatty acid biosynthesis, gluconeogenesis, glycolysis, lipid synthesis, cytoplasm. Triosephosphate isomerase (TIM) is a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. The reaction is very efficient and requires neither cofactors nor metal ions.)	energy production	↑	↑	↑	↓	28210	13 (8)	38	294	
Glucose metabolism													
14	aconitate hydratase	gi|118367081	iron regulatory protein, Krebs cycle, cytoplasm	energy production	↑	↓	↓	↓	99387	19 (16)	23	451	
15	aconitate hydratase	gi|118367081	iron regulatory protein, Krebs cycle, cytoplasm	energy production	↑	↓	↓	↓	99387	30 (21)	31	607	
220	enolase family protein	gi|118362946	9th step in glycolysis	energy production	↓	↓	↓	↓	50329	2 (1)	7	135	
597	triosephosphate isomerase	gi|146162132	Fatty acid biosynthesis, gluconeogenesis, glycolysis, lipid synthesis, cytoplasm. Triosephosphate isomerase (TIM) is a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. The reaction is very efficient and requires neither cofactors nor metal ions.)	energy production	↑	↑	↑	↓	28210	13 (8)	38	294	
Fe^{2+} homeostasis													
14	aconitate hydratase	gi|118367081	iron regulatory protein, Krebs cycle, cytoplasm	energy production	↑	↓	↓	↓	99387	19 (16)	23	451	
15	aconitate hydratase	gi|118367081	iron regulatory protein, Krebs cycle, cytoplasm	energy production	↑	↓	↓	↓	99387	30 (21)	31	607	
Salt stress and Ca^{2}+ homeostasis													
30	dnaK protein	gi|118358030	Salt stress indication. Downregulation observed in case of salinity stress. (Lockwood and Somero, 2011)	salt stress response	↓	↓	0	0	71682	4 (4)	7	260	
445	S-adenosylmethionine synthetase family protein	gi|118380290	S-adenosylmethionine synthetase can be considered rate-limiting step of the methionine cycle.[2] S-adenosylmethionine is a methyl donor and allows DNA methylation.	cell growth, Ca+ homeostasis, SOS response	0	↑	↑	↑	44072	6 (5)	17	234	
128	Peptidase family M20/M25/M40 containing protein	gi|118364457	Includes a range of zinc metallopeptidases belonging to several families in the peptidase classification (Rawlings and Barrett, 1995)	stress response	↑	↓	↓	↓	52932	14 (11)	36	516	
Protein degradation													
569	Proteasome A-type and B-type family protein	gi|118358232	Proteasome A-type and B-type family protein (The 20S proteasome multisubunit proteolytic complex, is the central enzyme of nonlysosomal protein degradation in both the cytosol and nucleus.)	protein degradation	↓	↓	↓	↑	27828	16 (12)	60	492	
Purine metabolism													
561	purine nucleoside phosphorylase I, inosine and guanosine-specific family protein	gi|118375576	Family I consists of a group of plant stress-inducible proteins. The experimental results indicate PNP is an important route for the reduction of arsenate to arsenite in mammalian systems.	nucleoside metabolic process	↑	↓	↑	↓	30125	4 (3)	16	146	
Metabolism *per se*													
578	Phospholipase/Carboxylesterase family protein	gi|118396232	In molecular biology, the alpha/beta hydrolase fold is common to a number of hydrolytic enzymes of widely differing phylogenetic origin and catalytic function. This family consists of both phospholipases and carboxylesterases with broad substrate specificity, and is structurally related to alpha/beta hydrolases PF00561 [2].	metabolism	↑	↑	↑	↑	33302	6 (5)	20	201	
370	oxidoreductase, aldo/keto reductase family protein	gi|118397046	Aldo-keto reductases are a superfamily of soluble NAD(P)H oxidoreductases whose chief purpose is to reduce aldehydes and ketones to primary and secondary alcohols.	metabolism	↑	↓	0	0	39813	7 (6)	24	169	
Hypothetical protein													
50	hypothetical protein TTHERM_00086780	gi|118358970	/	/	↑	↑	↑	↑	71762	2 (2)	4	130	
96	hypothetical protein TTHERM_00864870	gi|146183651	/	/	↑	↑	↓	↓	60218	8 (6)	20	278	
202	hypothetical protein TTHERM_00086780	gi|118358970	/	/	↑	↑	↑	↑	71762	3 (3)	6	146	
223	hypothetical protein TTHERM_01002870	gi|229594034	/	/	↑	↓	↓	↓	48661	12 (9)	39	409	
465	hypothetical protein TTHERM_01002870	gi|229594034	/	/	↓	↑	↑	↑	48661	2 (1)	7	97	

Discussion

We investigated the early response of eukaryotic protozoan *T. thermophila* exposed to subtoxic concetrations of TiO_2-NPs or bulk TiO_2 particles in media and in food vacuoles, by means of proteomic tools. At selected concentrations, no elevation of ROS content or lipid peroxidation occurred (Rajapakse et al. 2012). In the work reported here we have used the cytosolic proteome and not the entire cell proteome. On average, we detected 930 spots per gel (treatment) among which all together 77 spots significantly differred in expression levels from those in control samples. We selected 16 protein spots out of the 77 where the expression levels were most significantly up- or down regulated in comparison to control (Fig. 4, Table 2). In addition, we identified two proteins out of these 77 that were differentially expressed only in TiO_2-NP treatments. The identified proteins are related to lipid and fatty acid metabolism (Acyl-CoA dehydrogenase, triosephosphate isomerase), glucose metabolism (enolase family protein, aconitate hydratase, triosephosphate isomerase), Fe^{2+} homeostasis (aconitate hydratase), salt stress (S-adenosylmethionine synthetase family protein, Peptidase family M20/M25/M40 protein, DnaK protein), protein degradation (Proteasome A-type and B-type family protein), purine metabolism (purine nucleoside phosphorylase I, inosine and guanosine-specific family protein) and metabolism *per se* (Phospholipase/Carboxylesterase family protein, oxidoreductase - aldo/keto reductase family protein) (Table 2). Interestingly, the proteome of *T. thermophila* exposed to low concentrations of nano-TiO_2 (0.1 µg nano-TiO_2/ml) differred from all other treatments (higher concentration of nano-TiO_2 and both concentations of bulk TiO_2) (Fig. 2, Fig. 3). The identified proteins were distributed into five groups according to their function.

In the first group of proteins which amount was significantly altered due to NP exposure is involved in lipid and fatty acid metabolism. One of them, mitochondrial flavoenzyme Acyl-CoA dehydrogenase catalyses the initial step in fatty acid β-oxidation (GO:0016627). It was found to be upregulated in two different spots on the gels (Fig. 1, Fig. 4, Table 2). Although these two are mitochondrial proteins, their are presence in cytosolic fraction is

expected because synthesis of mitochondrial matrix enzymes takes place in cytosol (Hay et al., 1984). Alterations in lipid and fatty acid meatabolism is in line with our previous study where a signifficant change in long chain fatty acid profiles was observed after exposure of *T. thermophila* to TiO_2 particles (Rajapakse et al., 2012). We explain this change as a consequence of fatty acid synthesis and degradation. Changes in lipid and fatty acid metabolism were also described by Gao et al., (2011) and Tilton et al., (2013) as an early response to TiO_2-NPs.

Another protein involved in the lipid and fatty acid metabolism, which was upregulated in nano TiO_2 exposed cultures, is the triosephosphate isomerase. This enzyme plays an important role in several metabolic pathways and is essential for efficient energy production (http://www.ebi.ac.uk/interpro/entry/IPR000652).

The second group of proteins where the level of expression was significantly altered due to NP exposure is composed of three glucose metabolism related proteins (enolase family protein, aconitate hydratase, triosephosphate isomerase). Either increase or decrease of these enzymes, however, did not lead to altered ATP content (Rajapakse et al., 2012). In our study, enolase family protein was down-regulated in all treatments (Table 2), which is in agreement with a study by Yang et al., (2010). These authors also reported changes of energy metabolism-associated proteins such as enolase 1 and phosphoglycerate mutase due to nano-SiO_2 exposure. The other protein in the glucose metabolism proteins group, aconitate hydratase was identified in two different spots where it was also down-regulated in all treatments (Fig. 1, Fig. 4, Table 2). Apart of involvement in glucose metabolism, aconitate hydratase has also a non-catalytic function in iron homeostasis (http://www.ebi.ac.uk/interpro/entry/IPR006249). The only identified protein related to energy production that was upregulated was triosephosphate isomerase, a glycolytic enzyme. This enzyme plays an important role in several metabolic pathways and is essential for efficient energy production (http://www.ebi.ac.uk/interpro/entry/IPR000652). Our results are partially in agreement with those obtained for carbon based NPs (CNP) (Blazer-Yost et al., 2011). These authors reported a general up-regulation of proteins involved in glycolysis/gluconeogenesis pathways. In contrast, our proteomic study only

found triosephosphate isomerase to be up-regulated, while other proteins that belong to this pathway were down-regulated.

Another group of identified cytosolic proteins whose expression levels changed significantly after exposure to TiO_2 NPs or bulk TiO_2 is a versatile group of proteins with diverse functions, however they all are involved in ion-oregulation. These are S-adenosylmethionine synthetase family protein, peptidase family M20/M25/M40 protein and DnaK protein (Fig. 1, Table 2). One of the physiological functions of S-adenosylmethionine synthetase family protein in plants is the adaptation to salt stress (Espartero et al., 1994). This enzyme is also linked to polyamines, known to affect DNA bending and transition from B to Z DNA, causing frameshifts and other infidelities at the RNA level, and modulate signal transduction (Wallace et al., 2003) (Wallace and Fraser, 2003) (Thomas and Thomas, 2003). The second protein of this group, peptidase family M20/M25/M40 protein includes a range of zinc metallopeptidases belonging to several families in the peptidase classification (Rawlings and Barrett, 1995) (Table 2). These proteins are described to be associated also with water deficit stress in *Arabidopsis thaliana* (Bray, 2002), with Ca^{2+} ion homeostasis and inflammatory response (Dua et al., 2011). DnaK protein, a member of Heat shock protein 70 family (Hsp 70), was found down-regulated in our study. This is in agreement with previous report on salinity stress (Lockwood and Somero, 2011), as well as with metal oxide engeneered NPs uptake into the immune cells of the sea urchin *Paracentrotus lividus* (Falugi et al., 2012). Further, Werner and Hinton (Werner and Hinton, 1999), concluded that the down-regulation as well as up-regulation of hsp70 proteins are indicators of stress in *Potamocorbula amurensis*. In medical research, the downregulation of mthsp70/GRP75 (mortalin, amitochondrial stress protein) was linked to Parkinsons Disease brains as well as to a cellular Parkinsons Disease model (Jin et al., 2006). In addition, some other authores reported up-regulation of Hsp70 family, after exposure of different organisms to different NPs which was accompanied by oxidative stress (Ahamed et al., 2010) (Siddiqi et al., 2012).

Next group of differentially expressed proteins identified in our study is associated with protein degradation. Proteasome A-type and B-type family protein (Fig. 1, Table 2) was signifficantly downregulated after nanoTiO_2 exposure. Since in higher eukaryotic cells

proteasomes are localised mainly in nuclear envelope - endoplasmic reticulum network compartment (Enenkel et al., 1998) and since the amounts of nuclear proteasomes are increased in mitotic cells (Amsterdam et al., 1993) (Palmer et al., 1996) (Klein et al., 1990), the presence of proteasome proteins in cytosolic fractions is only possible during mitosis, when the nuclear envelope breaks down. Thus, we explain the downregulation of proteasomal proteins in our study by decreased mitosis in TiO_2 treated *T. thermophila* cultures. Our findings are in agreement with a previous study on TiO_2 NPs, that reported an early cellular response effects on cell growth (Ge et al., 2011). Another nanoparticle related study reported a down-regulation of preoteasome β1 (Witzmann and Monteiro-Riviere, 2006).

To summarise, the proteomic study of cytosolic cell fraction showed that early response of *T. thermophila* to subtoxic concentrations of TiO_2 particles includes (a) alterations in lipid and fatty acid metabolism, (b) energetic metabolism and (c) ion regulation.

(a) In the study presented, we confirmed alterations in lipid and fatty acid metabolism and absence of oxidative stress as previously identified by (Rajapakse et al., 2012) using conventional cyto toxicity biomarkes and lipid profiling.

(b) By using proteomic study of cytosolic cell fraction we have also confirmed the disruption of energy budget under suboptimal conditions what is a well know phenomenon. Organisms exposed to suboptimal environments incur a cost of dealing with stress. The total amount of energy available for maintenance, growth and reproduction has to be allocated. This may have important population and ecosystem consequences (Smolders et al., 2004)

(c) In line with many other studies on nanoparticle effects, our proteomic study also shows ion-regulatory dysfunction including calcium homeostasis (Cao et al., 2013) (Huang et al., 2010) (Guo et al., 2013). For example, Simon et al. (Simon et al., 2011) clearly established the role of calcium homeostasis alteration in response to the presence of TiO_2-NPs.

We evidenced proteomic responses to both, TiO_2-NPs as well as bulk TiO_2 at both tested concentrations. The response to TiO_2-NPs at lower concentration (0.1 µg TiO_2-NPs /ml) significantly differed from that at higher concentrations of nano-TiO_2 (100 µg TiO_2-NPs /ml) and both bulk-TiO_2. The response of *T. termophila* to higher exposure concentration of TiO_2-NPs is similar to effects of bulk-TiO_2 particles at both concentrations. This indicates that the effect is dependant on size and density of agglomerates and not only on primary particle size suggesting surface adsorption and mechanical interactions between particles and cell surfaces (Phot. T.h. supplement). Ingested particles were not crucial for effect (Rajapakse et al. 2012).

Conclusions:

1. We confirmed the hypothesis that phagocytosis and surface exposure of *T. thermophila* of TiO_2-NPs or bulk-TiO_2 at two different subtoxic concentrations provokes different proteomic response. The significantly different protein expression pattern was observed in cells exposed to TiO_2-NPs at the low concentration when compared to other the three groups of cells.

2. As hypothesised, the early response includes alterations in fatty acids and energetic metabolism evidenced in all exposure groups. In addition, an alteration in ion regulation was also observed in all four groups.

3. Since the cells exposed to TiO_2-NPs at lowest concentration responded differently as the three other groups exposed to TiO_2 (nano or bulk), we relate the effect more to mechanical interactions between cell membrane and agglomerates than to ingested particles. Namely, low density of aggregates of smaller size had significantly different effects as high density aggregates of larger ones.

5. Contrary to expectations, also bulk-TiO_2 affected cells where the response in similar to that of higher concentration of TiO_2-NPs.

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3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

3.1.1 Vpliv nanodelcev TiO_2 na membrane praživali *T. thermophila*

Rezultati študije (Rajapakse in sod., 2012) so pokazali, da se ob izpostavitvi mikroorganizma *T. thermophila* nanodelcem TiO_2 in delcem TiO_2 večjih od 100 nm pri širokem razponu koncentracij (med 0,1 , 10, 1000 µg TiO_2 /ml) značilno spremenijo profili dolgoverižnih maščobnih kislin. Pri tem pa nismo zaznali niti sprememb v koncentraciji reaktivnih kisikovih zvrsti niti lipidne peroksidacije. Na podlagi teh ugotovitev in ker spremembe profila dolgoverižnih maščobnih kislin ne povezujemo s toksičnostjo, zaključujemo, da gre za aklimacijo organizma na neugodne razmere, v našem primeru je to izpostavitev delcem TiO_2 v mediju. V poskusu smo opazili pokanje celic mikroorganizma *T. thermophila*, pri približno 5% populacije, ki je neodvisno od velikosti ali koncentracije delcev. V kontrolnih poskusih pokanja celic nismo opazili.

V naši študiji smo opazovali odziv celic na delce TiO_2 preden se zgodi toksični odziv. Nanodelci TiO_2 in delci TiO_2 večji od 100 nm so povzročili statistično značilno spremembo v razmerju nenasičenih in nasičenih dolgoverižnih maščobnih kislin in sicer, delež nasičenih dolgoverižnih maščobnih kislin se je povečal. Značilno se je zmanjšal delež *iso* in *anteiso* razvejanih maščobnih kislin v celičnih membranah mikroorganizma *T. thermophila*. Znano je, da na fluidnost membrane vpliva struktura in izomerizacija dolgoverižnih maščobnih kislin (Sinensky, 1973), takšno prilagoditev so dokazali tudi pri bakterijah, izpostavljenih ekstremnim okoljskim pogojem (Klein in sod., 1999; Satyanarayana in Johri, 2005). Naši rezultati so v skladu s študijami, kjer trdijo, da zmanjšanje deleža anteiso oblik dolgoverižnih maščobnih kislin vodi v zmanjšanje fluidnosti membrane (Satyanarayana in Johri, 2005).

Spremembe v zgradbi membrane in posledično spremembe v njeni fluidnosti se pojavijo ob nastopu neugodnih okoljskih pogojev, kot je v primeru povišanja okoljske temperature (Sinensky, 1973; Ivancic in sod., 2009), hiperosmotskega stresa (Laroche in sod., 2001), prisotnosti delcev in UV svetlobe (Peng in sod., 2010) in tudi nanodelcev (Mortimer in sod., 2011). Številni avtorji poročajo, da je rigidnost membrane povezana z lipidno peroksidacijo (Alonso in sod., 1997). Na tem mestu je ključna razlika med aklimacijo in odzivom, ki je posledica suboptimalnih pogojev, prisotnost oksidativnega stresa. V pričujoči študiji (Rajapakse in sod., 2012) smo dokazali spremembe v profilu dolgoverižnih maščobnih kislin v odsotnosti lipidne peroksidacije. Zato trdimo, da gre pri spremembi profila dolgoverižnih maščobnih kislin ob izpostavitvi mikroorganizma *T.*

thermophila nanodelcem TiO_2 in delcem TiO_2 večjim od 100 nm v mediju za aklimacijo na suboptimalne pogoje.

Temperaturne pogoje (32°C) v naši študiji smo izbrali na podlagi literaturnih podatkov, kjer je zagotovljena optimalna rast mikroorganizma (Schultz, 1997; Pauli in Berger, 1997). Prav celične delitve mikroorganizma *T. thermophila* so nujno povezane z *de novo* sintezo dolgoverižnih maščobnih kislin, ki je predpogoj za dokazano aklimacijo. Velikost spremembe v profilih dolgoverižnih maščobnih kislin ni bila odvisna od koncentracije delcev (nanodelcev in delcev večjih od 100 nm) v širokem območju od 0,1 do 1000 µg TiO_2 /ml.

V znanstveni literaturi najdemo poročila o različnih mehanizmih interakcije nanodelcev TiO_2 s celično membrano. Hussain in sod. (Hussain in sod., 2005) poročajo o pritrjanju nanodelcev na celično membrano in njihovem vnosu v celico. Sayes in sod. (2008) so okrnjeno stabilnost membrane in zmanjšanje mitohondrijskega membranskega potenciala po izpostavitvi celic nanodelcem TiO_2 pripisali mehanizmu »puščajočih membran«, ki je verjeten obrambni odziv pri izpostavitvi celic velikim koncentracijam delcev. Drugi avtorji zmanjšanje stabilnosti in poškodbe membrane pripisujejo direktni interakciji med nanodelci in membrano (Banaszak, 2009) ali lipidni peroksidaciji, ki je posledica oksidativnega stresa, povzročenega z nanodelci (Gurr in sod., 2005; Wang in sod., 2009).

V naši študiji (Rajapakse in sod., 2012) smo nanodelce opazovali v prebavnih vakuolah, pri čemer smo opazili, da je število celic, ki vsebujejo prebavne vakuole s TiO_2 delci odvisno od koncentracije in trajanja izpostavitve *T. thermophila* mediju z delci TiO_2. Delež prebavnih vakuol v celicah pa ni imel povezave s spremembo profila dolgoverižnih maščobnih kislin ali s koncentracijo molekul ATP. Zaključili smo, da privzemanje delcev TiO_2 ne povzroča škode celo pri koncentraciji 1000 µg TiO_2 /ml in 48 - urni izpostavitvi. Mortimer in sod. (2010) so poročali o hitrejšem privzemu nanodelcev CuO v primerjavi z delci večjimi od 100 nm. To je v nasprotju z rezultati naše študije, kjer smo opazovali hitrejši privzem delcev večjih od 100 nm v primerjavi s časovnim potekom privzema nanodelcev. Prav tako nismo opazili popolnega polnjenja vakuol z delci, niti po 48 urah ne. Mortimer in sod. (2010) navaja popolno napolnitev celice s CuO nanodelci že po 4 urah izpostavitve mikroorganizma *T. thermophila*.

Opazovali smo tudi praznjenje vakuol napolnjenih z delci TiO_2 po 24 - urni izpostavitvi, kar lahko razložimo kot del prehranjevalnega cikla. Praznjenju vakuol sledi nov ciklus polnjenja. Poročila o popolnem praznjenju vakuol predhodno še niso bila objavljena. Povečevanje števila celic, ki so vsebovale vsaj eno prebavno vakuolo napolnjeno z delci TiO_2 pri določenem času, je bilo povezano z večanjem koncentracije delcev TiO_2 v mediju. Prisotnost nanodelcev v prebavnih vakuolah poleg telesne površine predstavlja pomembno dodatno izpostavitev mikroorganizma *T.thermophila* nanodelcem.

V naši študiji smo prilagodili medij, v katerem smo pražival *T. thermophila* izpostavili TiO_2 delcem, tako da smo odstranili vse sestavine (proteine), ki bi lahko vplivale na nanodelce, saj je znano, da na velikost nanodelcev v suspenziji močno vpliva prisotnosti organskih molekul (Murdock in sod., 2008). Kljub temu smo ustvarili zadovoljive energetske pogoje, da smo se izognili stradanju in kompeticiji mikroorganizma *T.thermophila* za hrano znotraj obravnavane populacije.

Na podlagi naše študije zaključujemo, da predvidene koncentracije nanodelcev TiO_2 v okolju (Mueller in Nowack, 2008; Tiede in sod., 2008), pomenijo majhno tveganje za pražival *T. thermophila*.

3.1.2 Ocena interakcij nanodelcev TiO_2 z DNA modelnega mikroorganizma *T. thermophila* in kvasovke *S. cerevisiae*

V naši študiji smo eksperimentalno potrdili interakcijo delcev TiO_2 z molekulami DNA med izvedbo kometnega testa. V raziskavi smo uporabili praživalT. *thermophila*, ki smo jo izpostavili nanodelcem TiO_2 in delcem TiO_2 večjih od 100 nm (Rajapakse in sod., 2013), in kvasovko *S. cerevisiae*, ki smo jo izpostavili nanodelcem (TiO_2, ZnO, CuO, Ag in ogljikovim nanocevkam (SWCNT)) in delcem večjim od 100 nm (TiO_2, ZnO, CuO, Ag) (Bayat in sod., 2013).

V študiji (Rajapakse in sod., 2013) smo genotoksičnost nanodelcev TiO_2 za praživalT. *termophila* ugotavljali na podlagi treh možnih scenarijev: a) *in vivo* izpostavitev (*T. thermophila* smo izpostavili suspenziji nanodelcem TiO_2 in delcem TiO_2 večjih od 100 nm; b) *in vitro* izpostavitev (*T. thermophila* smo vklopili v gel, ki smo ga izpostavili suspenziji nanodelcem TiO_2 in delcem TiO_2 večjih od 100 nm); c) acelična izpostavitev (jedra, vklopljena v gel smo smo izpostavili suspenziji nanodelcem TiO_2 in delcem TiO_2 večjih od 100 nm). Poleg študije poškodb DNA s kometnim testom, smo ugotavljali tudi koncentracijo reaktivnih kisikovih zvrsti, lipidno peroksidacijo in spremembe v profilih dolgoverižnih maščobnih kislin v membranah mikroorganizma *T. thermophila*.

Jedrno DNA smo delcem izpostavili na tri različne načine, da bi ugotovili ali direktna interakcija med nanodelci TiO_2 in DNA vodi v prelome DNA. Predvsem nas je zanimalo ali do poškodb DNA res prihaja med samo izvedbo kometnega testa, torej po že opravljeni izpostavitvi celic nanodelcem, kot so nakazovala nekatera predhodna znanstvena poročila. Ugotovili smo, da pride pri kometnem testu do lažnih pozitivnih rezultatov in precenjene genotoksičnosti nanodelcev. Številni avtorji so poročali o genotoksičnosti TiO_2 (Gurr in sod., 2005; Trouiller in sod., 2009; Karlsson, 2010; Sathya in sod., 2010), ki so jo večinoma pripisali oksidativnemu stresu. Naši rezultati so pokazali statistično značilno tvorbo reaktivnih kisikovih zvrsti le pri koncentraciji delcev večjih od 100 nm (pri koncentraciji 100 µg TiO_2 /ml), ne pa tudi pri izpostavitvi mikroorganizma *T. thermophila* nanodelcem. Ostali celični markerji, kot so spremembe v profilu dolgoverižnih maščobnih kislin in lipidna peroksidacija, so bili nespremenjeni v primerjavi s kontrolnimi celicami. Povečana koncentracija reaktivnih kisikovih zvrsti pri delcih TiO_2 večjih od 100 nm (100 µg/ml) ni bila povezana z večjimi poškodbami DNA, kar jasno kaže na to, da oksidativni stres ni glavni mehanizem genotoksičnosti v naši študiji. Izmerjena genotoksičnost je torej neodvisna od oksidativnega stresa ali pa je lažni pozitivni rezultat. Le redke študije, ki so za ugotavljanje genotoksičnosti uporabile kometni test, poročajo, da nanodelci TiO_2 niso povzročili prelomov DNA (Bhattacharya in sod., 2009). Predvidevamo, da so v omenjeni študiji uspeli popolnoma odstraniti nanodelce iz testnega sistema in da celice nanodelcev niso privzele ob izpostavitvi. Potencialno nevarnost za lažno pozitivne rezultate kometnega testa predstavljajo tudi delci, ki po izpostavitvi ostanejo v testnem mediju ali pa so vstopili

v celico z endocitozo. Po lizi celic, ki je del postopka pri kometnem testu, lahko taki delci pridejo v neposreden stik z genomsko DNA (Stone in sod., 2009; Karlsson, 2010).

Landsiedel in sod. (2009) so poročali, da so v številnih študijah rezultati o genotoksičnosti nanomaterialov, povezani z izborom vrste testa genotoksičnosti. Pri šestih študijah je Amesov test pokazal, da nanodelci niso genotoksični, oziroma avtorji zaključujejo, da je negativen rezultat posledica neprehodnosti bakterijske celične stene za nanodelce. V nasprotju z omenjenimi ugotovitvami pa so pri 12 od 14 študij, kjer so ugotavljali prisotnost mikrojeder, potrdili genotoksičnosti nanodelcev, pri ugotavljanju prelomov dvojne vijačnice s kometnim testom pa pri 14 od 19 študij (Landsiedel in sod., 2009). Delno nasprotujoče si rezultate lahko razložimo z veliko občutljivostjo kometnega testa, v primerjavi z drugimi testi. Pomembna razlika je tudi v vrsti celic, ki jih lahko testiramo, pri kometnem testu lahko uporabljamo le evkariontske celice, pri Ames testu in nekaterih drugih pa prokariontske celice.

Na podlagi rezultatov naše študije menimo, da je pri študiji genotoksičnosti nanodelcev v primeru izbora kometnega testa ključno predvideti interakcije nanodelcev z DNA po izpostavitvi celic nanodelcem, ki lahko povzročijo lažne pozitivne rezultate. Eden od načinov za preizkušanje ali interakcije nanodelcev z DNA povzročajo prelome, je acelularni kometni test. Pri tem smo v naši študiji pred izpostavitvijo celic nanodelcem TiO_2, z alkalno lizo odstranili vse celične komponente razen jeder, ki so ostala vklopljena v gel. Stekelca na katerih so bila jedra vklopljena v gel smo izpostavili suspenziji delcev TiO_2. Na ta način smo lahko testirali predpostavko, ali nanodelci TiO_2 neposredno povzročajo prelome dvojne vijačnice.

Potrebno je tudi vedeti ali lahko pričakujemo privzem nanodelcev v izbranih celičnih kulturah, torej kakšna je verjetnost da bodo nanodelci prisotni v notranjosti celice, zaradi česar lahko med izvedbo kometnega testa interagirajo s celično DNA.

Predlagamo, da je genotoksičnost nanodelcev (in tudi delcev večjih od 100 nm) ovrednoteno s kometnim testom potrebno potrditi ali ovreči z neodvisnim testom, ali vsaj z biomarkerji, ki kažejo na izražanje popravljalnih mehanizmov DNA, npr. prisotnost mRNA gena p53 in drugih genov povezanih s p53 (Petkovic in sod., 2011b), delecije DNA (Trouiller in sod., 2009), na vnetje (Trouiller in sod., 2009; Grassian in sod., 2007) ali kazalce oksidativnega stresa kot sta lipidna peroksidacija in povečanje koncentracije reaktivnih kisikovih zvrsti (Gurr in sod., 2005; Kang in sod., 2008).

Menimo, da so rezultati kometnega testa z nanodelci (in tudi delci večjimi od 100 nm) brez dodatnih merilcev genotoksičnosti nezanesljivi, saj obstaja verjetnost lažno pozitivnih rezultatov. Test potrebuje prilagoditve, s katerimi bomo kontrolirali ali izločili možnost direktnih interakcij med nanodekci in DNA in potrebno ga je izvesti v kombinaciji s testi,

ki merijo oksidativni stres. Z omenjenimi prilagoditvami bo kometni test primeren za ocenjevanje genotoksičnosti nanodelcev.

Z namenom primerjati in ovrednotiti kvaliteto in občutljivost dveh različnih testov genotoksičnosti smo v razširjeni vzporedni študiji uporabili nekatere v industriji najpogosteje uporabljane nanodelce pri še enemu evkariontskemu modelnemu organizmu. Ocenjevali smo genotoksičnost različnih nanodelcev (TiO_2, ZnO, CuO, Ag in ogljikovih nanocevk (SWCNT)) in delcev večjih od 100 nm (TiO_2, ZnO, CuO, Ag) na kvasovko *S. cerevisiae,* pri tem pa smo uporabili test GreenScreen in kometni test. Rezultati obeh testov so si nasprotovali. Test GreenScreen ni pokazal genotoksičnosti, medtem ko je kometni test pokazal izrazito genotoksičnost za vse testirane nanodelce (Bayat in sod., 2013).

Številni avtorji so predhodno potrdili genotoksičnost obravnavanih nanodelcev z uporabo kometnega testa (Kumar in sod., 2011; Trouiller in sod., 2009; AshaRani in sod., 2009; Kisin in sod., 2007; Wang in sod., 2012). Na podlagi literature, naših predhodnih študij (Rajapakse in sod., 2013) in rezultatov dobljenih v tej študiji zaključujemo, da ni mogoče zanesljivo intrepretirati dobljenih rezultatov, razen v primeru, ko poznamo stopnjo interakcije nanodelcev s sestavinami testa in DNA (Bayat in sod., 2013). Pomembno je torej predvideti možnost pojava lažno pozitivnih rezultatov. Primerjava različnih študij na kvasovki je zaradi uporabe različnih pogojev izpostavitve in različnih velikosti nanodelcev, ter meritev različnih bioloških odzivov težavna.

3.1.3 Vpliv nanodelcev TiO_2 na izražanje citosolnih proteinov pri praživali *T. thermophila*

V naši študiji (neobjavljeno Rajapakse in sod., 2013) smo raziskovali zgodnji odziv mikroorganizma *T. thermophila*, ki smo ga izpostavili koncentracijam nanodelcev TiO_2 in delcev TiO_2 večjih od 100 nm (0.1 in 100 µg TiO_2/ml), ki ne povzročijo opaznih kvarnih učinkov. S proteomskim pristopom (2DGE + ESI-MS/MS) smo preučili citosolno celično frakcijo. V povprečju smo zabeležili 930 lis na gelu, pri tem je bilo pri gelih, kjer smo obravnavali citosol tretiranih celic 77 lis statistično značilno različno izraženih v primerjavi z lisami v kontrolnih poskusih. Med 77 lisami smo na podlagi statistično značilnih sprememb v izraženosti v skupine, glede na njihovo vlogo v celici. Izbrani proteini sodelujejo pri metabolizmu maščob in maščobnih kislin (Acil-CoA dehidrogenaza, trioze fosfat izomeraza), pri energetskem metabolizmu (protein družine enolaz, akonitatna hidrataza, trioze fosfat izomeraza), pri stresu slanosti (protein družine S-adenozilmetionin sintetaz, protein družine peptidaz M20/M25/M40, DnaK protein), pri razgradnji proteinov (protein družine proteasoma tipa A in B), pri metabolizmu purinov (protein purin nukleozid fosforilaza 1, protein družine specifične za inozin in gvanozin) in pri metabolizmu *per se* (protein družine fosfolipaz in karboksilaz, oksidoreduktaza, protein družine aldo/ketoreduktaz). Potrdili smo da je v primeru izpostavitve nanodelcem TiO_2 pri najnižji koncentraciji (0,1 µg nanodelcev TiO_2/ml) prišlo do nanospecifičnega učinka. Na podlagi klastrske analize 606 izraženih proteinov, prisotnih na vsaj 4 gelih (od 5) v posamezni izpostavitvi, je bil vzorec v spremembi izražanja proteinov zadosten, da kulture tretirane z 0,1 µg nanodelcev TiO_2/ml značilno ločimo od vseh ostalih skupin v poskusu (0.1 µg/ml delcev TiO_2 večjih od 100 nm in 100 µg/ml nanodelcev TiO_2 in delcev TiO_2 večjih od 100 nm).

V prvi skupini proteinov, pri katerih se je izražanje spremenilo statistično značilno po izpostavitvi nanodelcem TiO_2, sta bila dva proteina, ki sta vpletena v **metabolizem lipidov in maščobnih kislin**. Povečalo se je izražanje mitohondrijskega flavoencima, Acil-CoA dehidrogenaze, ki katalizira prvo reakcijo pri β-oksidaciji maščobnih kislin. Encim smo našli v dveh različnih lisah, najverjetneje gre za encima Acil-CoA dehidrogenazi z različnimi posttranslacijskimi modifikacijami. Znano je da sta to mitohondrijska encima, vendar pa sinteza vseh proteinov mitohondrijskega matriksa poteka v citosolu (Hay in sod., 1984). Naši rezultati so v skladu z našimi predhodnimi študijami, kjer smo po izpostavitvi praživali *T. thermophila* delcem TiO_2 pokazali statistično značilne spremembe v profilih dolgoverižnih maščobnih kislin (Rajapakse in sod., 2012), ki pa je nedvomno posledica sinteze in razgradnje maščobnih kislin. To je tudi v skladu z nekatero nedavno objavljeno znanstveno literaturo, kjer so prav tako poročali o spremembah v metabolizmu lipidov in maščobnih kislin in jih pripisali zgodnjemu odzivu na nanodelce TiO_2 (Gao in sod., 2011; Tilton in sod., 2013).

Naslednji protein, ki ga povezujemo z metabolizmom lipidov in maščobnih kislin je trioze fosfat izomeraza. Izražanje tega encima je bilo v naši študiji povečano pri vseh kulturah T. thermophila, izpostavljenih delcem TiO_2. Trioze fosfat izomeraza sodeluje v številnih metabolnih poteh in je ključen encim pri zagotavljanju energije v celici (http://www.ebi.ac.uk/interpro/entry/IPR000652).

Druga skupina proteinov, pri katerih se je izražanje spremenilo statistično značilno po izpostavitvi nanodelcem TiO_2, spada med proteine, ki so vpleteni v **metabolizem glukoze** (protein družine enolaz, akonitatna hidrataza, trioze fosfat izomeraza). Ne glede na vrsto spremembe v izražanju proteina, le-ta ni imela vpliva na vsebnost ATP pri izpostavljenih kulturah delcem TiO_2 v primerjavi s kontrolnimi kulturami (Rajapakse in sod., 2012). V naši študiji se je zmanjšalo izražanje proteina iz družine enolaz pri vseh izpostavitvah delcem TiO_2, to je v skladu s študijo, ki so jo opravili Yang in sod. (2009). Poročali so o spremembah proteinov vključenih v energetski metabolizem po izpostavitvi celic nanodelcem SiO_2.

Naslednji protein, ki je vključen v metabolizem glukoze, akonitatna hidrataza, smo identificirali v dveh različnih lisah. Izražanje proteina se je zmanjšalo v obeh primerih in pri vseh izpostavitvah praživali *T. thermophila* delcem TiO_2. Poleg vpletenosti v glukozni metabolizem, ima akonitatna hidrataza tudi nekatalitsko vlogo pri homeostazi železa v celici (http://www.ebi.ac.uk/interpro/entry/IPR006249).

Edini identificirani protein, vključen v metabolizem glukoze s povečanim izražanjem je bila trioze fosfat izomeraza. Ta protein je vpleten v glikolizo. Ima pomembno vlogo pri številnih metabolnih poteh in je ključen pri energetskem metabolizmu (http://www.ebi.ac.uk/interpro/entry/IPR000652). Naši rezultati so delno ujemajo z ugotovitvami študije na ogljikovih nanodelcih (Blazer-Yost in sod., 2011), kjer so ugotovili povečano izražanje proteinov, ki so vpleteni v glikolizo in glukoneogenezo. V naši študiji je prišlo do povečanega izražanja le v primeru trioze fosfat izomeraze, pri ostalih encimih pa smo zabeležili zmanjšanje izražanja v primerjavi s kontrolnimi kulturami *T. thermophila*.

Naslednja skupina identificiranih citosolnih proteinov, pri kateri je po izpostavitvi delcem TiO_2 prišlo do spremenjenega izražanja, je glede na vloge proteinov v celici precej raznolika. Skupna lastnost vseh proteinov v tej skupini je ta, da so vsi povezani z **ionoregulacijo** (protein družine S-adenozilmetionin sintetaz, protein družine peptidaz M20/M25/M40, DnaK protein).

Ena od vlog proteina, ki pripada skupini proteinov povezanih z ionoregulacijo je S-adenozilmetionin sintetaza. Pri rastlinah ta encim sodeluje pri prilagoditvah na stres slanosti (Espartero in sod., 1994). Isti encim pa je povezan tudi s poliamini, ki vplivajo na

prenos signala v celici ter na pregibanje DNA in prehod dvojne vijačnice iz B v Z DNA, pri tem prihaja do premikov bralnega okvirja in drugih nepravilnosti (Thomas T. in Thomas T. J., 2003; Wallace in Fraser, 2003; Wallace in sod., 2003).

Naslednji protein iz skupine proteinov povezanih z ionoregulacijo je protein družine peptidaz M20/M25/M40, ki vključuje številne metalopeptidaze, ki pripadajo različnim družinam (Rawlings in Barrett, 1995). Te proteine povezujejo s stresom suše pri *Arabidopsis thaliana* (Bray, 2002), s homeostazo kalcijevih ionov in z vnetnostnim odzivom (Dua in sod., 2011).

DnaK protein, zadnji v skupini proteinov povezanih z ionoregulacijo, spada v družino stresnih proteinov z molekulsko maso 70 kDa. V naši študiji se je po izpostavitvi delcem TiO_2 izražanje DnaK proteina zmanjšalo v primerjavi s kontrolnimi kulturami. Podobno sta poročala Lockwood in Somero (Lockwood in Somero, 2011) pri stresu slanosti in tudi s študijo kjer so celice imunskega sistema privzemale nanodelce kovinskih oksidov pri morskem ježku (*Paracentrotus lividus*) (Falugi in sod., 2012). Nadalje, Werner in Hinton (1999) sta zaključila da zmanjšano in povečano izražanje stresnih proteinov z molekulsko maso 70 kDa kažeta na stress pri školjki *Potamocorbula amurensis*. V medicinskih raziskavah so ugotovili, da je zmanjšano izražanje mitohondrijskega stresnega proteina z molekulsko maso 70 kDa (mthsp70/GRP75) povezano s parkinsonovo boleznijo pri možganskih celicah in pri celičnem modelu Parkinsonove bolezni (Jin in sod., 2006). V nasprotju z našimi ugotovitvami, pa številni avtorji poročajo o povečanem izražanju družine stresnih proteinov z molekulsko maso 70 kDa po izpostavitvi različnih organizmov številnim nanodelcem (Ahamed in sod., 2010; Jeon in sod., 2010; Siddiqi in sod., 2012).

Naslednja skupina proteinov je povezana z **razgradnjo proteinov**. Zabeležili smo zmanjšano izražanje proteina, ki sodi v družino proteasoma tipa A in B, po izpostavitvi kultur nanodelcem TiO_2. Znano je, da so pri evkariontskih celicah proteasomi lokalizirani pretežno v jedru in v endoplazemskem retikulumu (Enenkel in sod., 1998). Količina jedrnih proteasomov se poveča pri celicah ki vstopajo v mitozo in se delijo (Amsterdam in sod., 1993; Klein in sod., 1990; Palmer in sod., 1996), zato je prisotnost proteinov proteasoma v citosolni frakciji možna le med delitvijo celice, kjer zaradi razpada jedrne ovojnice pride do sprostitve proteasomov v citosol. Zmanjšano izražanje proteinov proteasoma v naši študiji torej razlagamo z manjšo stopnjo celičnih delitev pri kulturah praživali *T. thermophila* po izpostavitvi nanodelcem TiO_2. Naše ugotovitve so v skladu z drugimi študijami na nanodelcih TiO_2, kjer so potrdili zgodnji odziv in spremembe v celičnem ciklu pri BEAS - 2B celicah (Ge in sod., 2011), ter zmanjšano izražanje proteasomskega proteina β1 pri humanih keratinocitih (Witzmann in Monteiro-Riviere, 2006).

Rezultati pridobljeni v naši proteomski študiji so v skladu z eno od naših predhodnih študij, kjer smo ugotovili, da po izpostavitvi kultur *T. thermophila* delcem TiO_2 ne pride do oksidativnega stresa, niti do sprememb v vsebnosti ATP (Rajapakse in sod., 2012).

Zaključujemo, da se zgodnji odziv pri praživali *T. thermophila* na izpostavitev delcem TiO_2, kaže s spremembo v metabolizmu lipidov in maščobnih kislin, v energetskem metabolizmu in v ionski regulaciji že pri nizkih izpostavitvenih koncentracijah delcev TiO_2. Odziv na najnižjo koncentracijo nanodelcev TiO_2 se je statistično značilno razlikoval od višje uporabljene koncentracije nanodelcev TiO_2 in od obeh koncentracij delcev TiO_2 večjih od 100 nm.

Predvidevamo da imajo celice številne mehanizme s pomočjo katerih se spopadajo s suboptimalnimi pogoji, vključno z izpostavitvijo nanodelcem, pri kateri pride do sprememb v izražanju številnih metabolnih encimov. Očitno po izpostavitvi nanodelcem TiO_2 pride tudi do sprememb v energetskem ravnovesju celic. Spremembe ionskega ravnovesja pa lahko vodijo k resnejšim (kvarnim) učinkom, zato si to področje raziskav zasluži večjo pozornost in usmerjeno raziskovanje pri študiji interakcije med nanodelci in celicami.

3.2 SKLEPI

3.2.1 Splošni sklepi doktorske disertacije:

Interakcije nanodelcev z živimi sistemi lahko raziskujemo z različnimi biološkimi testi, s katerimi ovrednotimo učinke nanodelcev na celično membrano (lipidna peroksidacija, koncentracija ROS, profili dolgoverižnih maščobnih kislin, polnjenje prebavnih vakuol, pokanje celic), proteom (proteomska orodja) in DNA (Kometni test, GreenScreeen test).

V naših študijah smo le delno potrdili hipotezo o različnih učinkih različno velikih delcev (nanodelcev TiO_2 in delcev TiO_2 večjih od 100 nm) na praživalo *Tetrahymena thermophila*:

- Pri študiji sprememb v strukturi **membran** praživali *T. thermophila* po izpostavitvi nanodelcem TiO_2 oziroma delcem TiO_2 večjim od 100 nm, velikost delcev ni imela vpliva na velikost ali tip spremembe v profilih dolgoverižnih maščobnih kislin.
- V študiji **genotoksičnosti** delcev TiO_2 pri praživali *T. thermophila* in kvasovki *S. cerevisiae* nismo niti potrdili niti ovrgli zastavljene hipoteze.
- V **proteomski** študiji smo dokazali, da je velikost delcev TiO_2 vplivala na odziv modelnega mikroorganizma *T. thermophila* na nanodelce TiO_2 pri najnižji izpostavitveni koncentraciji (0,1 µg/ml).

Sklepi študije interakcij med delci TiO_2 (nanodelci TiO_2 in delci TiO_2 večjimi od 100 nm) s celično membrano:

Nanodelci TiO_2 in delci TiO_2 večji od 100 nm so povzročili aklimacijo mikroorganizma *T. thermophila*, ki se je odrazila s spremembo profila dolgoverižnih maščobnih kislin v smeri povečanja rigidnosti membran in z odsotnostjo reaktivnih kisikovih zvrsti in oksidativnega stresa.

Mikroorganizem *T. thermophila* je privzemal delce TiO_2 (nanodelce TiO_2 in delce TiO_2 večje od 100 nm) v prebavne vakuole v odvisnosti od trajanja izpostavitve, koncentracije in velikosti delcev, ki mu je sledilo praznjenje vakuol.

3.2.2 Sklepi študije interakcij med delci TiO_2 (nanodelci TiO_2 in delci TiO_2 večjimi od 100 nm) in DNA:

Ob neposredni interakciji nanodelcev TiO_2 z DNA, je prišlo do poškodb DNA v celicah modelnega mikroorganizma *T. thermophila*, vendar pa genotoksičnih vplivov ob izpostavitvi praživali *T. thermophila* delcem TiO_2 in ob izpostavitvi kvasovke *S. cerevisiae* nekaterim drugim nanodelcem (ZnO, CuO, Ag in ogljikovim nanocevkam (SWCNT)) in

delcem večjim od 100 nm (ZnO, CuO, Ag), nismo dokazali, čeprav so rezultati kometnega testa navidezno potrdili genotoksičnost vseh testiranih delcev.

Pri izbiri kometnega testa za ugotavljanje genotoksičnosti je potrebno predhodno preizkusiti ali interakcije med DNA in nanodelci povzročajo prelome DNA z acelularnim kometnim testom in rezultate upoštevati pri interpretaciji rezultatov kometnega testa. Genotoksičnost ovrednoteno s kometnim testom je potrebno potrditi ali ovreči z neodvisnim testom, ali vsaj z biomarkerji, ki kažejo na izražanje popravljalnih mehanizmov DNA, da bi izključili možnost lažno pozitivnih rezultatov kometnega testa.

3.2.3 Sklepi študije interakcij med delci TiO_2 (nanodelci TiO_2 in delci TiO_2 večjimi od 100 nm) in celico, ki so se odrazili na spremenjenem izražanju citosolnih proteinov:

Nanodelci TiO_2 so povzročili spremenjeno izražanje citosolnih proteinov pri mikroorganizmu *T. thermophila.*

Zabeležen zgodnji odziv se je pokazal s spremembo v metabolizmu lipidov in maščobnih kislin, v energetskem metabolizmu in v ionski regulaciji že pri nizkih izpostavitvenih koncentracijah delcev TiO_2.

Izpostavitev praživali *T. thermophila* nanodelcem TiO_2 je povzročila spremembe fiziološkega stanja, stres in nanospecifični odziv.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

V doktorski nalogi smo z uporabo poskusnega evkariontskega mikroorganizma vrste *Tetrahymena thermophila* (*Protozoa, Ciliata*) proučevali interakcije pogosto uporabljanih nanodelcev TiO_2 s celico. Pri eni od študij smo poleg delcev TiO_2, uporabili tudi mikroorganizem *Saccharomyces cerevisiae* (*Ascomycetes, Saccharomyces*) in nekatere druge pogosto uporabljane nanodelce (ZnO, CuO, Ag, ogljikove nanocevke (SWCNT)), oziroma delce večje od 100 nm (ZnO, CuO, Ag). Interakcije nanodelcev z živim sistemom smo spremljali z različnimi biološkimi testi (lipidna peroksidacija, koncentracija ATP, koncentracija ROS, profili dolgoverižnih maščobnih kislin, spremembe citosolnega proteoma, polnjenje prebavnih vakuol, pokanje celic, prelomi DNA). V nalogi smo predvideli, da bodo različne velikosti delcev TiO_2 različno vplivale na modelni organizem.

Ugotovili smo, da testirani nanodelci nimajo strupenostnega učinka na modelni organizem in se razen v primeru odziva proteoma ne razlikujejo od delcev TiO_2 večjih od 100 nm. Delci TiO_2 povzročijo spremembe v profilih dolgoverižnih maščobnih kislin, ki nakazujejo povečanje rigidnosti membrane, nismo pa ugotovili sprememb v koncentraciji ATP molekul, nastanka reaktivnih kisikovih zvrsti ali lipidne peroksidacije. Spremembe v membranah smo razložili kot aklimacijo na neugodne okoljske razmere in ne kot toksični odziv. Uporabljena testa genotoksičnosti sta dala nasprotujoče si rezultate genotoksičnosti, v pričujočem delu obravnavamo možne razloge za to razhajanje, v primeru kometnega testa pa smo eksperimentalno dokazali tudi možnost lažnih pozitivnih rezultatov. Predlagamo, da je genotoksičnost ovrednoteno s kometnim testom potrebno potrditi ali ovreči z neodvisnim testom, ali vsaj z biomarkerji, ki kažejo na izražanje popravljalnih mehanizmov DNA, npr. prisotnost mRNA gena p53 in drugih genov povezanih s p53, delecije DNA, na vnetje ali kazalce oksidativnega stresa kot sta lipidna peroksidacija in povečanje koncentracije reaktivnih kisikovih zvrsti.

Zaključili smo, da se v celicah mikroorganizma *T. thermophila* kopičijo nanodelci TiO_2 v prebavnih vakuolah, to pa je odvisno od velikosti delcev, koncentracije delcev in časovne izpostavitve delcem. Ugotovili smo da ob izpostavitvi nanodelcem TiO_2 pride do pokanja pri 5% celic v populaciji.

Rezultati analize citosolnega proteoma po izpostavitvi delcem TiO_2 so pokazali spremembe fiziološkega stanja, stresa in nanospecifični odziv. Spremembe v izražanju proteinov povezanih z metabolizmom maščobnih kislin so dopolnile ugotovitve pri študiji o spremenjenih profilih dolgoverižnih maščobnih kislin. Zaključujemo, da se zgodnji odziv pri praživali *T. thermophila* na izpostavitev delcem TiO_2, kaže s spremembo v

metabolizmu lipidov in maščobnih kislin, v energetskem metabolizmu in v ionski regulaciji že pri nizkih izpostavitvenih koncentracijah delcev TiO_2. Delci TiO_2 vplivajo na modifikacijo membran, spremembe v citosolnem proteomu modelnega mikroorganizma, in povzročajo prelome DNA ob neposrednem stiku. Delci TiO_2 imajo lahko nano-specifične učinke in lahko v modelnem organizmu povzročijo biološke odzive, ki še ne kažejo na strupenost, vendar ne moremo trditi, da so ti delci biološko inertni. Tovrstne specifične odzive organizma je v nadaljnjih študijah potrebno preveriti s sub-toksičnimi in sub-stresnimi koncentracijami nanodelcev.

4.2 SUMMARY

During the last decade the preparation and use of nanomaterials have increased extraordinarily, and information on their toxicity is urgently needed because specific characteristics of nanostructures can present previously unencountered forms of potential hazard.

In the work presented here we studied the bioactivity in a model eucaryotic microorganism *Tetrahymena thermophila* (*Protozoa, Ciliata*). We were interested in bioacivity of one of the most commonly used nanomaterials, examining the effect(s) of TiO_2 nanoparticles, which are found in many food, cosmetic and pharmaceutical products. In one of the studies presented in this work, we investigated genotoxicity on yeast *Saccharomyces cerevisiae* (*Ascomycetes, Saccharomyces*). In case of yeast we also studied some other nano- and bulk- particles, that were also chosen based on widespread use, namely nanoparticles: TiO_2, ZnO, CuO, Ag and SWCNT and bulk: TiO_2, ZnO, CuO, Ag.

We used different methods at distinct levels of biological organization to study the biological activity of nanomaterials. After exposure of protozoa to nano-TiO_2, classical toxicological parameters such as ATP levels, lipid peroxidaxion, particle ingestion, cell rupture were observed. Our main focus was to gain an insight in responses at three different levels of biological organization.

As the first interaction of nanoparticles with any living organism is the cell membrane, this was our first research scope. Using FAMEs analysis, we provided experimental evidence that changes in the membrane fatty acid profile of *T. thermophila* incubated with nano- or bulk TiO_2 particles are not accompanied by ROS generation or lipid peroxidation. We interpreted these changes as acclimation to unfavorable conditions and not as toxic effects. *T. thermophila* cells were exposed to TiO_2 particles at different concentrations for 24 h at 32 ◦C. Treatment of cultures with nano- and bulk TiO_2 particles resulted in changes of membrane fatty acid profile, indicating increased membrane rigidity, but no lipid peroxidation or ROS generation was detected. There were no differences in membrane composition when *T. thermophila* was exposed to nanosized or bulk-TiO_2 particles. We also observed reversible filling of food vacuoles, but this was different in case of nano- or bulk TiO_2 exposure. Our results suggested that interactions of particles and cell membranes are independent of oxidative stress.

At the DNA level, we employed a Comet assay. We have studied the genotoxicity of TiO_2 particles on a unicellular organism *T. thermophila*. Exposure to bulk- or nano-TiO_2 of free cells, cells embedded in gel or nuclei embedded in gel, all resulted in a positive Comet assay result but this outcome could not be confirmed by cytotoxicity measures such as lipid peroxidation, elevated reactive oxygen species or cell membrane composition. Published

reports state that in the absence of cytotoxicity, nano- and bulk-TiO_2 genotoxicity do not occur directly, and a possible explanation of our Comet assay results is that they are false positives resulting from post festum exposure interactions between particles and DNA. We suggest that before Comet assay is used for nanoparticle genotoxicity testing, evidence for the possibility of post festum exposure interactions should be considered. The acellular Comet test described in this report can be used for this purpose.

The genotoxicity was studied also on *S. cerevisiae*, exposed to different concentrations of nano-TiO_2 (1–3 nm), nano-ZnO (<100 nm), nano-CuO (<50 nm), their bulk forms, nano-Ag (10 nm) and single-walled carbon nanotubes (SWCNTs). Two genotoxicity assays, GreenScreen and the Comet assay were employed for comparisson reasons. The assays produced different results and we discussed the reasons for this discrepancy.

Little is known about interactions between nanoparticles and cells before oxidative stress occurs. A diverse setup of either exposure conditions or responses in proteomic studies have been attributed to the early response of cells to TiO_2 nanoparticles, however no common definition has been proposed yet. We propose a definition of an early response as a measurable physiological response where no cytotoxicity markers, e.g. oxidative stress, are observed. Based on previous results, we created a study design of the early response of *T. thermophila* to nano-TiO_2 or bulk TiO_2 particles at subtoxic concentrations (0.1 and 100 µg TiO_2 /ml) using proteomic analyses of cytosolic cell fraction. We conclude that the early response of *T. thermophila* exposure to TiO_2 particles is related to alteration of lipid and fatty acid metabolism, energetic metabolism and ion regulation already at low exposure concentrations. The response was not dose-dependent, but in case of the lowest nano-TiO_2 concentration it was size dependant. The response to lowest nano-TiO_2 concentration signifficantly differed from that at higher nano-TiO_2 or both bulk-TiO_2 concentrations.

The results of our work showed that during early response of *T. thermophila* to TiO_2 particles in suspension alteration of lipid and fatty acid metabolism, energetic metabolism and ion regulation already at low exposure concentrations occurs. The methods used in our studies are suitable for monitoring the bioactivity of nanomaterials at different levels of biological organization. The model organism used in our studies is suitable for testing biological effects of nanomaterials.

The results of our proteomic study showed, that TiO_2 nanoparticles could have nano-specific effects and can also cause nano-specific responses in a model organism. Such responses arguably cannot be referred to as toxic, but they clearly showed that once present inside an organism, the tested nanoparticles are not biologically inert. Such responses should in future be tested with sub-toxic and sub-stressful concentrations of nanoparticles.

For future work development of new protocols for assessment of nano-specific effects will be important.

5 VIRI

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