

Effets des nanoparticules de dioxyde de titane sur les bactéries : de la cellule à la communauté

Stéphane Jomini

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Mention Ecotoxicité, Biodiversité, Ecosystèmes

EFFETS DES NANOPARTICULES DE DIOXYDE DE TITANE SUR LES BACTERIES: DE LA CELLULE A LA COMMUNAUTE

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Introduction

L'augmentation de la population mondiale et l'industrialisation croissante génèrent de plus en plus de pollution environnementale et de risques pour la santé humaine. Les avancées scientifiques ont permis la mise au point de nouvelles technologies telles que les nanotechnologies qui utilisent des nanoparticules manufacturées différentes des nanoparticules naturelles (produites suite à des feux de forêt, aérosols marins, éruptions volcaniques, érosion des sols...) (Buzea et al., 2007; Nowack and Bucheli, 2007). Les nanoparticules sont définies comme étant des particules dont au moins une dimension est < 100 nm (The Royal Society and the Royal Academy of Engineering, 2004) et les nanomatériaux comme « un matériau naturel, formé accidentellement ou manufacturé contenant des particules libres, sous forme d'agrégats ou sous forme d'agglomérats, dont au moins 50 % des particules, dans la répartition numérique par taille, présentent une ou plusieurs dimensions externes se situant entre 1 nm et 100 nm. Dans des cas spécifiques, lorsque cela se justifie pour des raisons tenant à la protection de l'environnement, à la santé publique, à la sécurité ou à la compétitivité, le seuil de 50 % fixé pour la répartition numérique par taille peut être remplacé par un seuil compris entre 1 % et 50% » (European Commission, 2011).

Les nanoparticules/nanomatériaux manufacturés présentent de nouvelles propriétés physico-chimiques et sont de nos jours largement utilisés dans le domaine industriel et dans des produits de consommation courante (Keller et al., 2013). Bien que les avantages à utiliser ces matériaux soient considérables et d'un intérêt financier certain, de nombreuses voix s'élèvent pour que l'évaluation de l'impact environnemental des ces nanoparticules/nanomatériaux soit réalisée rapidement. En effet, en raison d'une utilisation à grande échelle et des rejets qui lui sont inhérents, des quantités non négligeables sont amenées à être déversées dans l'environnement. Ainsi, l'OCDE a dressé une liste des nanoparticules à tester immédiatement (OECD, 2008a) afin d'en déterminer les effets néfastes potentiels. En effet, malgré le nombre d'études conséquent s'intéressant aux effets des nanoparticules, de nombreuses données manquent pour comprendre au mieux les impacts potentiels de ces nanoparticules sur l'environnement et sur les organismes qui en dépendent.

Les travaux de cette thèse s'inscrivent dans ce contexte d'évaluation. L'objectif a été d'évaluer l'impact des nanoparticules de dioxyde de titane sur les bactéries qui sont des acteurs fondamentaux des cycles biogéochimiques. Ces nanoparticules présentent des propriétés semiconductrices qui leurs permettent d'absorber les UV et leurs confèrent des capacités photocatalytiques. De plus, elles présentent un pouvoir blanchissant et opacifiant ce qui conduit à leur utilisation dans des peintures, des ciments, des verres auto-nettoyants ou

alors pour la dépollution et le traitement des eaux usées (Gottschalk et al., 2009). De ce fait, leur dispersion dans l'environnement et la contamination des écosystèmes qui en résulterait constitue une préoccupation de première importance (Moore, 2006; Wiesner et al., 2006). Peu d'études s'intéressent aux effets de ces nanoparticules sans illumination, ne considérant ainsi que l'effet nanoparticulaire. Ainsi, au cours de cette thèse, nous nous sommes uniquement intéressés aux effets à l'obscurité des nanoparticules de dioxyde de titane sur les bactéries, en souches pures ou en communautés.

Les travaux mis en œuvre visent à répondre aux interrogations suscitées par deux axes de recherche :

- Quels sont les interactions entre bactéries et nanoparticules
- Quels sont les effets délétères de ces interactions sur les bactéries

Pour ce faire, de la microbiologie classique a été associée à une approche moléculaire, toutes deux complétées par une caractérisation physico-chimique et microscopique (TEM, SEM, AFM) des nanoparticules testées.

Ce manuscrit de thèse est ainsi structuré en quatre chapitres :

Le premier chapitre constitue une revue bibliographique divisée en trois sous-parties.

- La première présente la notion de viabilité cellulaire, les moyens à notre disposition pour la mesurer et une brève revue sur la toxicité/génotoxicité des nanoparticules vis-à-vis des organismes bactériens.
- La seconde expose les différents mécanismes d'action des nanoparticules vis-à-vis des bactéries à l'échelle cellulaire, avec une progression spatiale d'analyses allant du milieu environnant jusqu'au cytoplasme et l'ADN de la cellule bactérienne.
- La dernière partie détaille les effets des nanoparticules vis-à-vis des populations et des communautés bactériennes.

Le deuxième chapitre de ce manuscrit s'intéresse aux interactions opérant entre les bactéries et les nanoparticules et à l'impact de ces interactions sur la toxicité des nanoparticules. Ainsi, lorsque ces travaux ont été initiés, les études de toxicité présentaient des résultats contradictoires sans que l'on comprenne pourquoi de telles variations pouvaient survenir (Hu et al., 2009; Huang et al., 2000; Jiang et al., 2009; Simon-Deckers et al., 2009). En s'intéressant plus particulièrement aux effets des interactions électrostatiques entre bactéries et nanoparticules, la première étude de ce chapitre visera à expliciter l'importance des électrolytes en solution sur les interactions électrostatiques pouvant s'établir entre bactéries et nanoparticules et à leur rôle dans la modulation de la toxicité des TiO_2-NPs vis-à-

vis d'une souche modèle *Escherichia coli* MG1655. En mettant en évidence le rôle primordial des interactions électrostatiques sur l'apparition d'effets toxiques, cette étude a également conduit à s'interroger sur le rôle spécifique des déterminants biophysiques de la surface bactérienne dans l'établissement de ces interactions. En effet, la membrane externe des bactéries Gram négatives est une structure chargée négativement, présentant des groupes fonctionnels (carboxyles, hydroxyles, phosphate et amides) à l'origine d'interactions électrostatiques au sein de l'environnement (Jiang et al., 2010; Omoike and Chorover, 2004; Parikh and Chorover, 2006). Ainsi, la seconde étude de ce chapitre s'intéressera à des mutants bactériens présentant des délétions géniques conduisant à des modifications significatives au niveau de leurs surfaces (longueur/densité des lipopolysaccharides (LPS) et des fimbriae, présence/absence de flagelle). L'objectif de cette seconde étude est de déterminer l'importance de ces variations phénotypiques de surface et le rôle de cette interphase sur l'établissement des interactions entre les bactéries et les TiO_2-NPs et donc de comprendre comment ces interactions peuvent moduler l'effet toxique des nanoparticules.

Le troisième chapitre concerne la détection des effets potentiellement mutagènes des nanoparticules de dioxyde de titane. Nous nous sommes focalisés sur le rôle primordial du milieu d'étude lors de la mesure d'un potentiel génotoxique. En se basant sur les hypothèses de travail des deux précédentes études(Jomini et al., 2012; Pagnout et al., 2012), nous avons formulé l'hypothèse que le milieu d'exposition des bactéries aux nanoparticules pouvait soit favoriser soit inhiber les interactions entre les deux et par conséquent influencer les effets toxiques, voir génotoxiques. Ainsi, la détection des effets mutagènes potentiels du TiO_2-NPs a été réalisée grâce au test bactérien de mutagénèse, le test Ames en fluctuation. La seconde étude de ce chapitre a eu pour objectif la rédaction d'un protocole guide permettant la réalisation du test d'Ames lors de l'étude du potentiel mutagène de nanoparticules.

Après avoir étudié les mécanismes de toxicité des nanoparticules vis-à-vis des bactéries, en mettant en évidence les paramètres déterminants dans l'établissement des interactions entraînant la toxicité et la génotoxicité des nanoparticules, le quatrième chapitre de cette thèse présente les résultats d'une étude s'intéressant aux communautés bactériennes, pour l'importance des fonctions écologiques qu'elles réalisent pour l'ensemble des écosystèmes . En effet, après avoir étudié le niveau cellulaire et populationnel, nous nous sommes intéressés aux effets des TiO_2-NPs sur une communauté bactérienne naturelle présente à la fois sous forme de biofilm précoce (bactéries sessiles) et sous forme libre dans la colonne d'eau provenant de la rivière Moselle. En sachant que de l'ordre de 99% des microorganismes vivent sous forme de biofilms fixés à des surfaces solides (Thiéry et al., 2012), il est

primordial d'obtenir des informations quant aux modifications touchant à la structure et à la diversité des communautés bactériennes.

Enfin, le manuscrit se termine par une conclusion générale qui présente les perspectives ayant été ouvertes par ce travail de recherche et les développements futurs à envisager.

Chapitre 1 : Synthèse bibliographique

Nanoparticle effects on bacteria

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Abstract

Bacteria colonized all biotope on earth and are fundamental for the functioning of ecosystems. They are at the basis of environmental food web and play a key role in biogeochemical cycles. The presence and use of nanoparticles in consumer products has increased over the year due to their specific physico-chemical properties. However, these utilizations led to the release of these nanoparticles into the environment, especially the aquatic compartment. As a result, the concentrations detected or predicted in the ecosystems are increasing exponentially. Thus, bacteria are exposed to increasing concentrations of nanoparticles arising from multiple sources. We review the current state of the knowledge on how to assess the bacterial state of live, the interactions occurring between nanoparticles and bacterial cells in ecosystem, the effects of these interactions on the survival of bacteria and what could be the later impact on bacterial communities.

We noticed that despite the large amount of data available on nanoparticle toxicity against bacteria, many studies report contradictory results, and key mechanisms are still lacking to fully understand the mechanisms of nanoparticles toxicity. Bacteria are at the basis of food web, we conclude that additional research focusing on food transfer of nanoparticles in higher organisms by dietary uptake are at first importance in a context of risk assessment of ecological impact of nanoparticles.

Introduction

Bacteria are unicellular, prokaryotic and ubiquitous microorganisms living as single free cells, associated in complex matrixes or associations such as biofilms, microcolonies, aggregates, filaments or as partners of other organisms. Bacteria colonize all biotope on earth, from the simplest to the most complex, from the most welcoming to the most extreme. They are fundamental for the functioning of ecosystems where they are at the basis of environmental food web: as a resource for other biological compartment such as protozoa, rotifers and daphnia, as primary producers and where they are a major actor of biogeochemical cycles. In fact bacteria contribute to the degradation, mobilization and flows of inorganic and organic matter in soils, in freshwater and marine environments. They also play a key role in many industrial processes such as water-treatment plants.

Scientific advances have enabled the development of new technologies such as nanotechnologies using manufactured nanoparticles in contrast to nanoparticles produced naturally. Nanoparticles (NPs) are defined as particles with at least one dimension <100 nm (The Royal Society and the Royal Academy of Engineering, 2004) and nanomaterials as "a natural material, incidental or manufactured containing particles, in aggregate form or in the form agglomerate including at least 50 % of the particles in the number size distribution, have one or more outer dimensions of between 1 nm and 100 nm. In specific cases, when justified for reasons of environmental protection, public health, safety or competitiveness, the threshold of 50 % set for the number size distribution can be replaced by a level between 1% and 50% "(European Commission, 2011).

Nanoparticles/nanomaterials are nowadays widely used in industry and in consumer products (Keller et al., 2013). Although the advantages of using these materials are considerable and despite a certain financial interests, the environmental impact of nanoparticles/materials is questioned. In fact, by large-scale industrial uses, significant amounts are discards in environmental compartments (Alvarez et al., 2009; Boxall et al., 2007; Nowack et al., 2012; Wiesner et al., 2009). In this context, this review will attempt to establish the state of knowledge of the effects of nanoparticles/nanomaterials on bacteria, compiling methods of analysis and assessment available to highlight the mechanisms of nanoparticles/nanomaterials toxicity, from cellular level to communities and ecosystems.

How is it possible to assess the effects of nanoparticles exposure on bacterial cells?

1. How to define the state in which bacteria exist?

Since the discovery of bacterial life forms by Antoni van Leeuwenhhoek in 1684, abandoning the spontaneous generation hypothesis following the work of Pasteur and Koch, our knowledge of bacterial physiology and diversity has greatly improved. However, there are currently still controversies on subjects, such as cell viability and how to define it (Puspita et al., 2012; Roszak and Colwell, 1987). There is a clear and universal definition of viable bacteria nevertheless theories and definition concerning the environmental viable but non culturable bacteria (VBNC) and dead bacteria are controversial.

Viable cells are defined as having the ability to grow on specific agar-based medium and to form colony forming units (CFU) and also to grow in an optimal liquid medium leading to an apparent turbidity (Kell, 2000; Kort et al., 2008; Roszak and Colwell, 1987). Accurately, in this state, bacteria are in a dynamic state, metabolically and physiologically active, with fast adaptation to shifts in environmental parameters through a wide range of genotypic and phenotypic variations (Fakruddin et al., 2013; Tempest, 1978; Tempest et al., 1983; W Harder, 1983). It correspond to maintain intact DNA, enzyme synthesis, expressions and metabolic pathways adaptations to avoid possible blockages under growth-limiting nutrient, modulation of uptake rates for nutrients (amino acids, sugars…) available in excess, and all the modulation to maintain optimal growth (del Mar Lleo' et al., 1998; Morgan et al., 1993; Rahman et al., 1994; Roszak and Colwell, 1987; Roth et al., 1997).

At the opposite, dead cells are assumed to be unable to grow on specific agar-based medium, to form CFU and to proliferate in an observable way in liquid medium (Kell, 2000; Kort et al., 2008; Trevors, 2012). Dead or non-viable bacteria have no cellular activity or metabolism. Interaction between the cytosol and the outside of the cell no longer exists. The renewal of membrane lipids is no more effective impacting cell wall integrity leading to intracellular content leakage and cell lysis (Kort et al., 2008; Roszak and Colwell, 1987).

However, there is an intermediate stage for microorganisms remaining viable with an active metabolism even if they stop to divide and loss there culturability under laboratory conditions (Oliver, 1993; Votyakova et al., 1994), we talk about viable but non culturable bacteria (Sung et al., 2005). This situation was evocated after the work of Kogure and colleagues in 1979, who demonstrates the existence of a "metabolic activity" in a proportion of bacteria considered "dead" (Kogure et al., 1979). *Escherichia coli* and *Vibrio cholerae* were found to

enter a dormant-like state in response to starvation (Xu et al., 1982) and it was the first description of this state in a scientific article. Two years later, the term 'viable but non culturable' was used for the first time. Over these 30 years, more than 70 bacterial species, mostly gram-negative, have been found to be VBNC (Pinto et al., 2011).

The VBNC state its complex and is definition is still under severe discussion by the scientist community. VBNC bacteria can be defined as metabolically active bacterial cells that crossed a threshold and became unable to multiply in or on a medium normally supporting its growth (Oliver, 1995). Nevertheless, there is a very significant difference between the VBNC and the "starvation survival" state, wherein cells remain fully culturable despite a dramatic decrease in cell metabolism (Oliver et al., 1991). VBNC state corresponds to two different physiological states and describes bacteria that are:

- Potentially replicative, able to regain their ability to grow if specific conditions are effective, which leads to the process of "resuscitation" or "awakening" of the cells (McDougald et al., 1998). The bacterial ability to survive in hostile environments is essential for their persistence. A mechanism generally limited to gram-positive bacteria (such as Bacillus, Clostridium…) well-studied for long-term survival when environmental conditions are unfavorable is formation of spores. These spores are particularly resistant to heat, UV, chemicals or drying and they can maintain their viability for long periods, waiting to germination conditions. The question is how nonspore forming bacteria can resist adverse environments for long time. The VBNC state would constitute a survival strategy against harsh environmental conditions and undergo the same function that endospores (Alleron, 2008; Colwell, 2000; Stokell and Steck, 2001). These adverse environmental conditions triggering the VBNC state could be nutrient starvation, sharp changes in pH or salinity, extreme temperatures, osmotic stress and oxygen availability, exposure to heavy metals or food preservatives, white light, activation of lysogenic phages or suicide genes and decontaminating processes (Fakruddin et al., 2013).
- In a transition phase between life and death, where there is still signs of metabolic activity or respiration but degrading perpetually (Alleron, 2008; Yamamoto, 2000).

The VBNC state is characterize by a reduce cell size or dwarfing (K Costa, 1999), extensive modifications of fatty acid composition in cytoplasmic membranes (Day and Oliver, 2004), a decrease in RNA content, increased antibiotic resistance due to lower metabolic activity (Oliver, 2010), condensation of the cytoplasm, reduce nutrient transport (Fakruddin et al., 2013), non capacity to multiply or reduce metabolic activity (Byrd, 2000; McDougald et al., 1998; Tangwatcharin et al., 2006), changes in outer-membrane protein profile (Muela et al.,

2008), continuous gene expression (Maalej et al., 2004). Thus, in *Campylobacter jejuni*, the VBNC state corresponds to a lower intracellular pH and a decrease in the concentration of K^+ (Tholozan et al., 1999). In addition to metabolic and physiological characteristics, non-culturable viable bacteria exhibit morphological and structural differences compared to cultivable forms. It was shown, in *Enterococcus faecalis*, a significant increase of the mechanical strength of the bacterial cell wall (Signoretto et al., 2000). This can be explained by changes in the structure of the cell wall corresponding to what have been seen in *Vibrio vulnificus* (Day and Oliver, 2004). These authors observed a modification in the fatty acids membrane composition. This observation was carried out under conditions of hypothermic stress; issued hypothesis is that this change in composition of the membrane allows better membrane fluidity. Recently, it was proposed that one parameter driving the aging of bacterial cells was replicative damages (Ackermann et al., 2003). It is supposed that the accumulation of these aging proteins may be only a part of a much more general phenomenon based on the accumulation and segregation of all damaged proteins during senescence of bacterial cells (Maisonneuve et al., 2008). This accumulation may be an other parameter for bacteria to reach the VBNC state. It is also important to note that VBNC bacterial cell may resuscitate when the stress to which they were confronted disappears or when the medium contains molecules (glutamate, pyruvate...) that can help the injured cells to recover (Ducret et al., 2014).

In the environment, it is supposed that more than 99% of the microorganisms will be in VBNC state (Bogosian et al., 1996; Staley and Konopka, 1985). It is an important fact to take into account to study in an accurately way the potential adverse effects of nanoparticles and nanomaterials on bacteria at the community level. A balance should be made between size of the population, viable fraction (proportion of cells with respiratory activity and membrane integrity) and the fraction that was actually impacted or killed by the test substance.

2. Tools and methods to evaluate bacterial viability and potential injury

To assess the potential effects of nanoparticles on bacteria three types of methods must be considered: classical or Pasteurial microbiology based on growth, direct methods using imaging or flow cytometry and the molecular methods.

A. Growth methods

The most commonly used technique is the plating on nutritive medium in order to follow the development of colony forming unit (CFU) by growth of bacterial cells. This medium

presents a particular composition in order to be suitable for growth of the bacterial strain of interest. Conventionally, test compound is incorporated into agar medium and potential reduction of CFU that can be observe is used to define thresholds inhibition. Exposure of microorganisms to a presumed toxic substance may also occur in liquid medium prior to deposition of various dilutions on agar plates. Colony count occurs after a period of several hours/days/weeks incubation at a precise temperature depending on the bacterial strain used (Zhukova et al., 2012). Comparison between exposed plates and control allow scientist to establish thresholds of growth inhibition. Results are expressed in terms of minimum inhibitory concentration (MIC) who gives the lowest concentration of an antimicrobial substance that will inhibit visible growth of a microorganism after overnight incubation, or of the minimum bactericidal concentration (MBC) which is defined as the lowest concentration of antimicrobial substance that will prevent the growth of an organism after subculture onto antibiotic-free media. Conventionally, it is the concentration that will kill at least 99.9% of a planktonic (MBCP) or biofilm (MBCb) bacterial population (Andrews, 2002; Choi et al., 2010; Harrison et al., 2007). This type of assay may present biases due to the inclusion of nanoparticles into the agar-based medium, reducing nanoparticles motilities and surface area in contact with the microorganisms.

The bacterial growth inhibition can also be determined in liquid medium, corresponding to the analysis of growth curve inhibition. The bacterial strain is exposed to the xenobiotic for a fixed time in an appropriate medium, maintained at a specific temperature under rotation to optimize the aeration of the system. When the bacterial cells are cultured with different concentrations of toxic substances, xenobiotic can inhibit the cell multiplication. The growth was then followed by a spectrophotometric method, generally by measuring the OD_{600} nm every hour or 30 minutes. The percentage of viable cells was determined by comparing the absorbance of control and exposed bacteria, generally when the solution reaches the stationary phase of the bacterial cells (Bringmann and Kuhn, 1977; Pagnout et al., 2012). This kind of determination can be realized in microplates, as done in the *Pseudomonas putida* test (ISO 10712, 1995; Juvonen et al., 2000). This technique allows maximum contact between nanoparticles and microorganisms leading to higher sensitivity than techniques using agar-based medium. Nevertheless, the states in which nanoparticles are present in suspension depend on the medium composition and could deeply modulate the sensitivity of the test.

Another simple technique used to assess the viability of bacterial cells is to use a microrespirometric assay. It consists to expose planktonic or biofilms bacterial cells in plate

to various dilutions of tests samples containing the substance of interest. In each well, a precise volume and concentration of bacteria was present, then the dilution of the sample to test is added and an oxygen fluorescence probes aliquot was added (for example: Redlight from Luxcel Biosciences). The well is then sealed with a drop of heavy mineral oil in order to prevent liquid evaporation and oxygen transfer from ambient air. The fluorescence of the cells is then followed for a determined time generally by using a microreader with specific excitation and emission filters to detect fluorescence. By using a calibration curve, it is possible to determine the number of metabolically active cells (Choi et al., 2010).

Another way to determine the toxicity of nanoparticles against bacterial cells, is to determinate the loss of cellular respiration. Specifically, after exposure of the test organisms to specific xenobiotic concentrations, cells were recuperated, centrifuged, the pellet was resuspended in sterile water or in buffer and used for respirometric assay. The assay is conducted in a specific chamber fitted with an oxygen electrode. The reaction needs to be initiated by an electron donor (sodium succinate or glucose), and the reduction of 2,3,5 triphenyltetrazolium chloride (TTC) to its reduced product, 2,3,5-triphenyltetrazolium formazan (TTF), occurred. After incubation at fixed temperature and in the dark, samples were centrifuged; the pellets were extracted with methanol. Cells were then removed by centrifugation and absorbance at 485 nm of the red supernatant was measured spectrophotometer. Concentrations of TTF formed were determined by comparison to a standard curve with freshly prepared TTF and the rate of O_2 or TTC reduction was measured (Maness et al., 1999; Smith and Pugh, 1979).

B. The imaging methods

Cell viability of bacterial organisms and impact of nanoparticles on these organisms may also be detected by the achievement of different imaging techniques.

a. Fluorimetric approach

[1] Live/Dead BacLight assay

This assay is the most commonly used commercial bacterial viability kit (LIVE/DEAD BacLight Bacterial Viability Kit; Molecule Probes Inc., Eugene OR). This assay employs two fluorescent nucleic acid-binding dyes, the SYTO 9 (with emission at 500 nm) which moves across the membrane cells readily and the Propidium Iodide (PI) (emission at 635 nm), to differentiate cells considered intact (generally assumed to be lived cells, stained in green) and

injured cells with a compromised bacterial membrane (generally assumed dead cells, stained in red). After exposing bacteria to nanoparticles, a combined volume with a specific concentration of SYTO 9 and PI was added to the test sample. Sample was then incubated for generally 15 minutes at room temperature and in the dark, dropped onto a microscope slide with a cover slip and then imaged. Analysis can occur with an epifluorescence microscope with a specific detector and filters, or a confocal laser scanning microscope (Boulos et al., 1999; Clift et al., 2011; Stokell and Steck, 2001).

This assay can be largely employed nevertheless, interference in staining and in visualization can occurs when high ionic force solution where used to exposed bacteria to nanoparticles (Pagnout et al., 2012).

[2] CTC/DAPI assay

The redox fluorescence dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), targets dehydrogenase activity and was reduced by an electron transport chain to an insoluble red fluorescent CTC formazan salt (after excitation at 450nm, emission at 630nm red fluorescence), accumulating intracellularly and is a proof of cellular respiratory activity (Cappelier et al., 1997; Choi et al., 2010; Yu et al., 1995). A counterstaining is generally realized with DAPI, 4'-6 diamino-2 phenylindole, which fluoresces in blue (excitation at 372 nm, emission at 456 nm) after specifically binding to DNA. This double staining allows to enumerate and differentiate on one filters living cells from dead cells. After exposing bacteria of interest to nanoparticles, a combined volume with a specific concentration of CTC/DAPI was add to the test sample. Sample was then incubated for generally 15-30 minutes at room temperature and in the dark, diluted and then filtered, covered with a mineral oil and analyzed by epifluorescence microscopy or a confocal laser scanning microscope.

It is important to note that this assay, just as the BacLight assay, cannot be used in the presence of fluorescent particles, as for example, nanosilver aggregates who fluoresce in red (Choi et al., 2010).

[3] Flow cytometry

Flow cytometry has become one of the most powerful tool to count and study bacteria living in aquatic environments, it allows to determine abundance, provides cellular information on individual cells (Casamayor et al., 2007; Gregori et al., 2001; Pan et al., 2005). The basis of a

flow cytometry analysis is a labeled suspension of individual cells which passes through a focused laser beam. Capillary forces cause the cells to pass the flowcell, where the labels are stimulated by the laser light. The emitted flurescent light from the fluorophores and the scattered-light are detected separately, the right angle light scatter is related to cell shape and structure and the forward angle light scatter is related to cell size (Trigui et al., 2011). When incubated with fluorescence dyes, this technique can provide information on cell structure heterogeneity or physiology. It is also possible to determine the nucleic acid content of single cells after specific staining with nucleic acid dyes, such as SYBERGreen (Button and Robertson, 2001; Button et al., 1996). With this technique, it is possible to realize several staining to evaluate the state of a bacterial population. Sohm et al. realized a staining with Syto 9, PI, bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DIBAC4(3)) and fluorescein diacetate (FDA) to visualized the impact of TiO2-NPS exposure on E. coli. Samples were incubated 10 to 30 min at room temperature depending on the dyes and analyzed after excitation at 488 nm. Syto9, DIBAC4(3), and FDA fluorescence was recorded at 530 nm to discriminate bacteria from the background and nanoparticle aggregates and PI was analyzed at 585 nm (Sohm et al., in preparation). It is possible to couple these analysis of flow cytometry to CAtalyzed Reporter Deposition-Fluorescence *In Situ* Hybridization (Zubkov et al., 2002) or Denaturing Gradient Gel Electrophoresis analysis (Longnecker et al., 2005) in order to assess the phylogenic diversity of bacterial community.

b. Electron microscopy techniques

Electron microscopy techniques are one of the most important techniques used to detect and observed directly nanoparticles and bacterial interactions (A. Kumar et al., 2011; Labille et al., 2010). Indeed, these techniques allow viewing NPs primary particles size and agglomerates, bacterial cells, bacterial appendages, spatial conformation and perspective appreciation in two dimensions.

The Transmission electron microscopy (TEM) can be applied to electron dense material able to absorb the electron beam visualized against the bright field background, but crystalline structure can be also observable due to the diffraction of the electrons beam from the pattern of electron back scattering (Handy et al., 2012). This technique is generally used to characterize nanoparticles/nanomaterials distribution and size and allows researcher to visualize potential adverse effect on bacterial cells (Maurer-Jones et al., 2013, p. -; Rodea-Palomares et al., 2011; Yuan et al., 2013). The image obtained need to be analyzed carefully.

Indeed, tests conducted by Brayner et al in 2006 with NP-ZnO have shown internalization of particles within the cell and accumulation in the bacterial wall (Brayner et al., 2006).
However, after analysis, it was found that these particles were from the recrystallization of zinc which was dissolved in the medium. Nanoparticles of zinc and ions resulting thus had not caused with cell membranes but had entered the cell by ion channels or ATP-dependent pumps (Brayner et al., 2006). The preparation of sample before TEM analysis needed dehydratation and Epon matrix inclusion. After that, it's necessary to realized thin section of the matrix. It's important to note that dehydratation steps needed when preparing the cells can lead to false results, as cutting the Epon matrix.

Scanning electron microscopy (SEM) is also used for imaging the nano-size domains but with less magnification capability than the TEM (two or three orders of magnitude). SEM is particularly useful for studying complex environmental and biological matrices, interaction between NPs and bacterial cells, for surfaces morphology analysis and for allowing the creation of a three dimensional representation. Samples need to be fixed, dried and dehydrated before observation can occur (Handy et al., 2012; Mu et al., 2011; Sondi and Salopek-Sondi, 2004). It is possible to couple scanning electron microscopy to a cryogenic step. This kind of electron microscopy analysis is used for nanoparticles agglomerate morphology, and is also used to study bacterial interactions with mineral and nanoparticles (Chenu and Jaunet, 1992; Horst et al., 2010). In order to directly image bacteria in their environment, it's now possible to use environmental scanning electron microscopy (ESEM) that allow to image sample in liquid form but to our knowledge there are very few study using this king of electron microscopy.

To go further than just observation, electron microscopy is often combined with sensors to realize X-ray detection, such as energy dispersive X-ray (EDX) spectroscopy or X-ray diffraction (XRD). These modules are important in the spatial co-location of nanoparticles elements and biological domains identification present in the samples. It allows the differentiation between NPs surface and matrix constituents when they are complexed. The identification of particles is realized by its X-ray diffraction pattern. EDX and related methods are very effective for the heavier metals (e.g. Cd, Ti, Ag, Cu, Zn, and Fe). Similar to EDX, micro-X-ray fluorescence (μ-XRF) can be coupled with X-ray absorption spectroscopy (XAS) in order to be more accurate. The two forms of XAS, to know X-ray absorption near-edge spectroscopy (XANES) and extended X-ray absorption fine structure (EXAFS) are highly

concentration dependent and EXAFS requires higher concentrations of elements in modelisation (Handy et al., 2012; Yuan et al., 2013).

In order to directly image bacteria in their environment, it's now possible to use environmental scanning electron microscopy (ESEM) that allow to image sample in liquid form but to our knowledge there are very few study using this king of electron microscopy.

c. AFM

Atomic force microscopy (AFM) has been widely used for the investigation of natural and manufactured NPs. Indeed, due to the possibility of imaging samples in different environments (different types of solutions, air) (Baalousha and Lead, 2012; Balnois et al., 1999; Gibson et al., 2007; W. Zhang et al., 2012) and because of its high spatial resolution, the AFM is one of the most interesting and widely used tool in nanoparticles and bacterial interactions analysis and characterization. The interest of this tool is to present a large enough resolution and magnification to allow the simultaneous analysis of nanoparticles and bacteria physico-chemical parameters. AFM is capable to generate force-distance curves that can be used to determine the hardness and elasticity of bacterial cells (Sullivan et al., 2007), as well as their adhesiveness (W. Zhang et al., 2012). In addition, this tool allows to directly measure at the cellular level the effects of nanoparticles (Baalousha and Lead, 2012; W. Zhang et al., 2012).

When using the AFM it is possible to choose three different modes:

The contact mode, where the tip is always in contact with the sample surface. In this mode of analysis, we must pay attention to the adsorption of nanoparticles on bacterial cell surface as it is possible to tear of the particles with the movement of the tip as it moves (Baalousha and Lead, 2012). Moreover, tip-sample interaction may induce artifacts due to particle/molecule distortion, re-deposition after particle displacement.

The non-contact mode, where the tip and the cantilever are at several nanometers above the sample surface. On this mode, the cantilever realized oscillation above the sample at a specific frequency in order to measure surface charges and forces. One variation of this mode is the electrical mode of AFM, known as Kelvin probe force microscopy (KPFM). This variation can be used to map and quantify the local surface potential of the bacterial cells. Moreover, force-distance curves can be made in order to measure cell surface hardness, adhesiveness or elasticity (W. Zhang et al., 2012).

The tapping mode, the tip touches the surface of the sample and the cantilever had a specific up and down oscillation near its resonance frequency.

When especially looking at the biases of this technique, adhesion forces between AFM tip and NPs can cause tip contamination, leading in reduced image resolution, limited reproducibility and incorrect morphology and size analysis.

D. The molecular analysis of diversity

To have a better look to microbial ecology, the study of microbial community structure is very important (Holben and Harris, 1995). Knowing that culturable techniques are selective and unrepresentative (Wagner et al., 1993) molecular approach was preferred. Thus, to assess environmental microbial community structure nucleic-acid based methodologies are widely used and are largely based on taxonomic markers (small subunit of the rRNA, etc). Molecular techniques using DNA are widely used in microbiology but need a very high efficiency and quality DNA extraction to fully success analysis. The goal is to maintain a qualitative representation of the studying bacterial community without introducing any biases for the PCR step which is a critical step.

a. Genetic fingerprint techniques

[1] T-RFLP

After DNA extraction and PCR amplification with specific fluorescent PCR primers, the amplificated Genes encoding for the 16S rRNA were purificated and then digested by a specific restriction enzyme generating fluorescent fragments with length polymorphisms (the terminal fragment is fluorescent). These fragments are then separated by electrophoresis gel, and the gel was then analyzed by measuring variation in fluorescence and size of all the fragments. This technique is used to compare community fingerprint to a database for identification or can also be used in order to follow modification occurring after pollution or nanoparticles exposure (Ge et al., 2011; Liu et al., 1997; Militon, 2007). This technique has limitation in the number of species that can be followed; it's most of the time, restricted to the 50 most abundant organisms and have a sensitivity limits of 0.5% of the total rDNA amplified for species detection (Liu et al., 1997).

[2] DGGE

Regarding other methods, PCR-DGGE is fast, cheap, and could be used to compare microbial community structure modifications in natural water (Casserly and Erijman, 2003; Kaksonen et al., 2004; Liu et al., 2002; Rowan et al., 2003). A simple way to monitor bacterial community

modifications after an exposition to nanoparticles is to use the analysis of the highly conserved gene (Bradford et al., 2009; Handy et al., 2012; Tong et al., 2007), 16S rRNA, ubiquitous in all microorganisms (Olsen et al., 1986; Pace, 1997; Watanabe, 2001; Woese, 1987). They can be obtained after DNA extraction and after performing a denaturing gradient gel electrophoresis technique (DGGE). Indeed, this approach is used since 1993 (Muyzer et al., 1993) in molecular microbial ecology (Konstantinov et al., 2003; Muyzer, 1999; Plant et al., 2003; Ranjard et al., 2001; Rowan et al., 2003) and especially to focus on changes in the microbial community (Araya et al., 2003; Clegg et al., 2003; Cummings et al., 2003; Kleikemper et al., 2002; Schönfeld et al., 2003; Watanabe et al., 2000). Generally, the amplified 16S rRNA were separated on polyacrylamide gels with a denaturing gradient according to the difference in G-C proportion in the sequences. Due to a GC clamp, DNA is unable to totally denatured, leading it to stop at a precise place in the denaturing gel. The gels were then for 16 h, stained with a dye fixing DNA and imaged with scanner. The DGGE profiles are then normalized and a comparison of the band based was realized. To obtain further information, sequenced clone libraries of amplified 16S rRNA can be made and compared to database.

b. Hybridation techniques

Since a very little proportion of naturally occurring microorganisms are able to be cultivated in laboratories, molecular analysis techniques became the most powerful tools for microbial community analysis (Gérard et al., 2005). In order to obtain quantitative and qualitative representation of a bacterial community while avoiding the amplification step, it is possible to perform fluorescence *in situ* hybridization (FISH). This technique uses a synthetic oligonucleotide probe labeled with a fluorochrome which targets highly conserved regions of microbial rRNA and enables to study bacterial responses to exposure of a given substance. To allow the penetration of the probe inside the cells, bacteria must be permealized and fixed and RNA degradation must be prevented. Probe specificity and hybridization conditions are variable and adaptable to measure the relative abundance of specific taxonomic species to very large taxonomic groups (Amann et al., 1995). FISH combines the precision of molecular approach and microscopy visualization. Due to this, this technique is dependent on the fluorescence microscopy resolution limits. To overcome these limitations, the catalyzed reporter deposition technique was developed, also called CARD-FISH. The CARD-FISH amplify fluorescent signal by using a oligonucleotide-linked catalytic enzyme. By doing this, it's possible to increase the detection rate, to improve the signal compared to the noise

background and allow the detection of undetectable groups by classical FISH (Ferrari et al., 2006; Militon, 2007; Schmidt et al., 2012b) but it's possible to couple this technique with electron microscopy technique to obtain higher resolution results (Gérard et al., 2005; Schmidt et al., 2012a). This technique could also be coupled with flow cytometry in order to analyze much higher bacterial cells and to sort them.

3. The toxicity and genotoxicity data available

The in vitro evaluation of pollutants genotoxicity on microorganisms was made possible by the creation of different genotoxicity tests. To our knowledge, the first test set up at a large scale is the Ames test (Ames et al., 1973, 1972). Briefly, the test consist to uses several strains of *Salmonella typhimurium* auxotroph for histidine. These strains were selected based on their mutation sensitivity due to an increased cell wall permeability to large molecules, a mutation in the bacterial DNA excise and repair system leading to the inability to repair damaged/mutated sections (uvrB mutation), a multicopy plasmid that contain error-prone DNA repair systems and R-factor plasmids. The assay is based on reverse mutations caused by exposure to mutagenic compounds that can reactivate the ability of mutated bacterial strains to synthesize histidine allowing them to grow in the absence of this essential amino acid. For more information you can see (Ames et al., 1973, 1972; Jomini et al., 2012; Maron and Ames, 1983; Mortelmans and Zeiger, 2000; Pagnout et al., 2014).

From this genotoxicity test, many other tests were created, based on the induction of the SOS system, such as the bioluminescence test (Ulitzur et al., 1980), the Mutatox® genotoxicity test (AZUR Environmental, (Kwan et al., 1990) and the LUMIStox assay (Ribo and Kaiser, 1987). The Salmonella umu test is also a widely used genotoxic assay, faster but less sensitive compared to the Ames test (Ptitsyn et al., 1997; Robbens et al., 2010). Another important genotoxicity test is the SOS Chromotest, set up by Quillardet and colleague (Quillardet et al., 1982a, 1982b). This test is designed to monitor the expression of the lacZ gene placed under the control of sulA (sfiA is the old name), one regulon of the latest step in the SOS repair system, after operating a fusion between sulA and the lacZ operon (Robbens et al., 2010). This modification led to the expression of the lacZ when PQ37 *E. coli* is subjected to DNA damages.

The Ames test and the SOS chromotest are developed here because to our knowledge, they are the two main in vitro genotoxicity tests commonly used for the detection of mutagenic and genotoxic potential of nanoparticles on bacteria. There are only few studies with the SOS

chromotest, (Nam et al., 2013). At the opposite, the Ames test is largely used due to its low cost and time effective results. Nevertheless, results are very controversy. Indeed, when assessing the ability of a nanomaterial to have genotoxic impact on bacterial cells, the Ames test present a negative response while these same nanoparticles express detectable genotoxic effects when genotoxicity tests are realized on eukaryotic cells (Doak et al., 2012). It correspond to what can be observed for the titanium dioxide, where several studies showed no mutagenicity (Pan et al., 2010; Warheit et al., 2007) or very weak mutagenicity (A. Kumar et al., 2011) whereas NP-TiO_2 has been found to have positive genotoxic responses in other in vitro cellular test systems as the micronucleus assay or the comet assay (Balasubramanyam et al., 2009; Di Virgilio et al., 2010; Osman et al., 2010; Shi et al., 2010). In a previous study, we showed that a simple modification in the test protocol can be made in order to make the test suitable for nanoparticles (Jomini et al., 2012). Much remains to best assess the genotoxic/toxicity potential of nanoparticles and to find a battery of tests that would eliminate all barriers leading to highly variable results.

Regarding the toxicity of nanoparticles, Table I presents a non-exhaustive survey of the literature. With these data, one can observe a large variability in all published data. This variability is expressed in both the concentrations used, the duration of exposure of organisms to nanoparticles thereby leading to a high variability in the results obtained, the nature of the strains used, their growth phase, the presence of nutrients.. Thus, for example, silver nanoparticles exposure concentrations are ranging from 2µg/L to more than 38mg/L and time exposure are ranging from 1 to 36 hours. The disparity found in these results is closely linked to physico-chemical properties of nanoparticles (surface area, size, surface charges, structure, shape, density, coating, crystalline face, BET, purity, aggregation, solubility...) and interactions with microorganisms that result (Baalousha and Lead, 2012).

Table 1: Non exhaustive summary of nanoparticules toxicity against bacteria.

Nanoparticules (NP)	Organisms/Cells	Concentration	Time	Effects	References
Ag	*S. epiderminis*	> 0.1 or 1 %	36 hours	Growth inhibition	(Alt et al., 2004)
	S. aureus				
	E. coli	MIC > 3.3 nM	24 hours	Growth inhibition	(Kim et al., 2007)
	S. aureus	MIC > 33 nM			
	E. coli	> 6 mg/L	24 hours	99% CFU Growth inhibition	(Morones et al., 2005)
	E. coli	6-50 mg/L	24 hours	99% CFU Growth inhibition	(Pal et al., 2007)
	E. coli	> 30 µg/mL	24 hours	99% CFU Growth inhibition	(Sondi and Salopek-Sondi, 2004)
	E. coli	58.41 µg/mL	24 hours	90% Growth inhibition	(Yoon et al., 2007)
	B. subtilis	32.12 µg/mL			
	P. fluorescens	0.002 mg/L	3 hours	Growth inhibition	(Fabrega, 2009)
	Marinobacter sp	5 mM	24 hours	80% Growth inhibition	(Sinha et al., 2011)
	Ordinary heterotrophic organisms	0.13 mg/L	4 hours	33 % Biomass inhibition	(Garcia et al., 2012)
	Autotrophic bacteria	1 mg/L	/	Growth inhibition	(Choi et al., 2008)
	E. coli		24 hours	IC_{50}=4.0µM	
	E. coli	MBC= 10 mg/L	3 hours	Bactericidal effect	(Choi et al., 2010)
	Biofilm	MBC= 38 mg/L			
	Activated sludge	1 mg/L	12 hours	41% nitrification inhibition Shift and loss of nitrifying bacteria	(Liang et al., 2010)
	N. europae	/	3 hours	IC_{50}: 0.36-2.9 mg/L	(Yuan et al., 2013)
	Flectobacillus sp	1 mg Ag/ L Ag-NPs	1 hour	100 % Growth inihibition	(Sheng and Liu, 2011)
	CFB group bacterium				
	C. normanense				
	Aeromonas sp				
	Enterobacter sp				
	E. coli	MIC= 40-180 µg/mL	24 hours	Bactericidal effect	(Ruparelia et al., 2008)
	B. subtilis	MIC= 40 µg/mL			
	S. aureus	MIC= 120 µg/mL			
Ag/Ag+	*E. coli*	MBC= 2.4 mg/L	3 hours	Bactericidal effect	(Choi et al., 2010)

	Biofilm	MBC= 9.5 mg/L			
Fe	*Serratia* JL03000	10-100 mg/L	1 hour	Growth inhibition	(J. Liu et al., 2013)
	E. coli	45 mg/L	1 hour	3 order of magnitude of CFU Growth inhibition	(Lee et al., 2008)
Cu	*E. coli*	MIC= 140-280 µg/mL	24 hours	Bactericidal effect	(Ruparelia et al., 2008)
	B. subtilis	MIC= 20 µg/mL			
	S. aureus	MIC= 140 µg/mL			
ZVCN	*E. coli*	312.5 ppm	30 minutes	100% Growth inhibition	(Rispoli et al., 2010)
CuO	*E. coli*	2 mg/L	6 hours	99% Growth inhibition	(Zhao et al., 2013)
	E. coli		30 minutes	LC_{50}: 4.63 mg/L	(Dasari et al., 2013)
	E. coli	33.49 µg/mL	24 hours	90% Growth inhibition	(Yoon et al., 2007)
	B. subtilis	28.20 µg/mL			
	E. coli	200 mg/L	2 hours	82 % Growth inhibition	(Hu et al., 2009)
	E. coli	/	24 hours	EC_{50}: 25.7-31.9 mg/L	(Baek and An, 2011)
	B. subtilis			EC_{50}: 51.6-72.4 mg/L	
	S. aureus			EC_{50}: 62.5-96.5 mg/L	
Cu/Sepiolite	*E. coli*	1wt%	24 hours	99.9 % Growth inihibition	(Esteban-Cubillo et al., 2006)
	S. aureus				
MgO	*E. coli*	Paint 5%wt	24 hours	> 93 % Bactericidal effect	(Huang et al., 2005)
	S. aureus				
	B. subtilis	1 mg/mL	4 hours	> 95 %Bactericidal effect	(Makhluf et al., 2005)
	S. aureus			Depend on size particles	
NiO	*E. coli*	/	24 hours	EC_{50}: 147.6-173.9 mg/L	(Baek and An, 2011)
	B. subtilis			EC_{50}: 97.7-152.3 mg/L	
	S. aureus			EC_{50}: 105.4-139.0 mg/L	
ZnO	*E. coli*	1.7mM	12 hours	90 % Growth inhibition	(Brayner et al., 2006)
	E. coli	0.25 g/L	8 hours	> 90 % Growth inhibition Size inhibition	(Zhang et al., 2007)
	E. coli	3.4 mM	24 hours	100 % Growth inhibition	(Reddy et al., 2007)
	S. aureus	1mM		100 % Growth inhibition	
	E. coli	10-5000 ppm	6 hours	98 % CFU Growth inhibition	(Adams et al., 2006)
	B. subtilis			5 % CFU Growth inhibition	
	E. coli	/	24 hours	IC_{50}: 15-43 mg/L	(Li et al., 2011)
	E. coli	/	24 hours	EC_{50}: 107.8-124.1 mg/L	(Baek and An, 2011)
	B. subtilis			EC_{50}: 73.7-99.9 mg/L	

	S. aureus			EC_{50}: > 125 mg/L	
	E. coli	50 mg/L	4 hours	85-100% Growth inhibition	(Ng et al., 2013)
	E. coli		30 minutes	LC_{50}: 0.13 mg/L	(Dasari et al., 2013)
	Marinobacter sp	2 mM	24 hours	80 % Growth inhibition	(Sinha et al., 2011)
	E. coli	150 mg/L	2 hours	82 % Growth inhibition	(Hu et al., 2009)
	E. coli	80 µg/mL	1 hour	27 % CFU Growth inhibition	(A. Kumar et al., 2011)
	E. coli	20 mg/L	2 hours	100 % CFU Growth inhibition	(Jiang et al., 2009)
	B. subtilis				
	P. fluorescens				
	S. aureus	10 mM	24 hours	> 50% Growth inhibition	(Jones et al., 2008, p. 200)
		MIC= 80-1200 µg/mL		For small to large NP	
	S. epidermidis	5 mM		> 95 % Growth inhibition	
	S. pyogenes				
	B. subtilis				
	E. coli	1 % solution	24 hours	Growth inhibition	(Espitia et al., 2013)
	S. aureus	10 % solution			
	S. choleraesuis				
	E. coli	7 mM	24 hours	2 order of magnitude of CFU	(Nair et al., 2009)
	S. aureus			Growth inhibition	
SiO_2	*E. coli*	10-5000 ppm	6 hours	60 % CFU Growth inhibition	(Adams et al., 2006)
	B. subtilis			15 % CFU Growth inhibition	
	E. coli	20 mg/L	2 hours	60 % CFU Growth inhibition	(Jiang et al., 2009)
	B. subtilis			40 % CFU Growth inhibition	
	P. fluorescens			75 % CFU Growth inhibition	
Al_2O_3	*E. coli*	600 mg/L	2 hours	60 % Growth inhibition	(Hu et al., 2009)
	E. coli	20 mg/L	2 hours	35 % CFU Growth inhibition	(Jiang et al., 2009)
	B. subtilis			60 % CFU Growth inhibition	
	P. fluorescens			75 % CFU Growth inhibition	
	E. coli	100 mg/L	24 hours	70 % Growth inhibition	(Simon-Deckers et al., 2009)
	S. aureus	10 mM	24 hours	50 % Growth inhibition	(Jones et al., 2008, p. 20)
La_2O_3	*E. coli*	600 mg/L	2 hours	68 % Growth inhibition	(Hu et al., 2009)
TiO_2	*E. coli*	0.1 mg/L	1 hour	1 order of magnitude of CFU	(van Grieken et al., 2010)
				Growth inhibition	
			6hours	6 order of magnitude of CFU	

				Growth inhibition	
		0.002 mg/L	2 hours	1 order of magnitude of CFU Growth inhibition	(Zhukova et al., 2012)
		0.02 mg/L	<1 hour	4 order of magnitude of CFU Growth inhibition	
	E. coli	10-5000 ppm	6 hours	25 % CFU Growth inhibition	(Adams et al., 2006)
	B. subtilis			30 % CFU Growth inhibition	
	Anabaena variabilis	1 mg/L	6 days	Total inhibition of nitrogen fixing activity	(Cherchi and Gu, 2010)
				EC_{50} 96h: 0.42-0.62 mg/L	
	V. fischeri	>20 g/L	96 hours	No Effect	(Heinlaan et al., 2008)
	V. fischeri	≥10 mg/L	96 hours	No Effect	(Zhu et al., 2011)
	S. oneidensis	100 mg/L	/	60% Growh rate inhibition	(Maurer-Jones et al., 2013, p. -)
	E. coli	10 ppm	1 hour	Bactericidal effect	(Planchon et al., 2013)
	E. coli	/	30 minutes	LC_{50}: 583 mg/L	(Dasari et al., 2013)
	E. coli	1200 mg/L	2 hours	56 % Growth inhibition	(Hu et al., 2009)
	E. coli	500 mg/L	24 hours	85 % Growth inhibition	(Simon-Deckers et al., 2009)
	E. coli	80 µg/mL	1 hour	19 % CFU Growth inhibition	(A. Kumar et al., 2011)
Fe_3O_4	*E. coli*	0.7 g/L	1 hour	Bactericidal effect	(Auffan et al., 2008)
FeO	*E. coli*	0.07 g/L	1 hour	70 % Growth inhibition	(Auffan et al., 2008)
FeOx	*E. coli*	24 mg/L	20 minutes	99 % Growth inhibition	(Schwegmann et al., 2010)
Fe_2O_3	*E. coli*	1200 mg/L	2 hours	93.5 % Growth inhibition	(Hu et al., 2009)
NZVI	*K. oxytoca*	10 mg/L	0.2-24 hours	No effect	(Sacca et al. 2013)
	E. coli	10 mg/L	24 hours	100 % Growth inhibition	(Diao and Yao, 2009)
	P. fluorescens	11 mg/L		100 % Growth inhibition	
SnO_2	*E. coli*	1200 mg/L	2 hours	60 % Growth inhibition	(Hu et al., 2009)
Sb_2O_3	*E. coli*	/	24 hours	EC_{50}: 236.2-298.6 mg/L	(Baek and An, 2011)
	B. subtilis			EC_{50}: 118.3-177.0 mg/L	
	P. fluorescens			EC_{50}: 246.8-425.5 mg/L	
CeO_2	Ordinary heterotrophic organisms		1-4 hours	EC_{50}: 0.18-0.28 mg/L	(Garcia et al., 2012)

	Ammonia-oxidizing bacteria		1-4 hours	EC_{50}: 0.21-0.05 mg/L	
	Mesophilic and Thermophilic anaerobic consortium		2 weeks	90 % Inihibition biomass EC_{50}: 0.16-0.26 mg/L	
	E. coli	>200mg/L	3 hours	99 % Growth inhibition	(Thill et al., 2006)
	Anabaena CPB4337		24 hours	EC_{50}: 0.27-7.5 mg/L, size dependant	(Rodea-Palomares et al., 2011)
	E. coli *B. subtilis*	50-150 mg/L	8 hours	Growth rate inhibition	(Pelletier et al., 2010)
Co_3O_4	*E. coli*		30 minutes	LC_{50}: 55.56 mg/L	(Dasari et al., 2013)
NO	*P. aeruginosa*	10mg/mL	16 hours	100% Growth inhibition	(Friedman et al., 2011)
	E. coli	20mg/mL	24 hours	"	
	K. pneumoniae	10mg/mL	24 hours	"	
	S. pyogenes	0.625mg/mL	16 hours	"	
	E. faecalis	2.5mg/mL	8 hours	90% Growth inhibition	

4. **Nanoparticles and bacteria: approach, interactions and effects**

A. Nanoparticles-nanoparticles and nanoparticles-bacteria interactions

The main manufactured nanoparticles presenting a commercial interest are Fe, Al, Ag, Au and Ni but also Al_2O_3, TiO_2, CuO, ZnO, ZrO_2 and SiO2 (Theodore and Kunz, 2005), ZnSe, CdSe, CdS (quantum dots)... The engineered nanoparticles express novel physicochemical properties compared to their bulk counterparts who make them of great interest (Hartung and Sabbioni, 2011). Nanoparticles have a size below hundred nanometer, such as natural colloids (particle sizes between 1 nm and 1 μm), who have been intensively studied in the past decades, the behavior of nanoparticles is assumed to be comparable to those of natural colloids (Delay and Frimmel, 2012; Jones, 2002; Lyklema, 2005). The suspensions of nanoparticles are expected to be unstable under relevant environmental conditions (Baalousha et al., 2008; Stone et al., 2010). In a given medium, the particles are subject to Brownian motion, such as microorganisms, that characterizes the whole random movements of particles. The Brownian motion move away the solid particles suspended in a fluid from their initial position to a random position. Within this fluid, the particles are subjected to forces arising from the presence of other particles. This movement induces a diffusion of particles that can diffuse into the suspension without interference from the other particles but only in a very dilute medium. However, in the environment or in ecotoxicity evaluation tests, the medium is highly charged leading to collisions between the particles (Stone et al., 2010; Tourbin, 2006). It was shown that for submicrons particles, the Brownian motion force is several orders of magnitude lower than colloidal forces (Chein and Liao, 2005). When looking at the nanoparticles interactions (with nanoparticles or microorganisms), interactions between particles are dominated by weak dipolar Van der Waals (VdW) forces, stronger polar electrostatic interactions and covalent interactions (Brar et al., 2010). When particles approach closely enough from each other, the repulsive forces tend to be predominant. Nevertheless, if the both particles have enough energy and are closely enough to overpass this barrier, the weak attractive VdW forces become the predominant forces, leading the particles to aggregates each other (Stone et al., 2010; Tsantilis and Pratsinis, 2004). The agglomeration of nanoparticles involved agglomeration of single particles into clusters and then agglomeration of these clusters into larger agglomerates. These agglomerates are not solid due to spaces between smallest aggregates and individual particles (Sterling Jr. et al., 2005). The particle interactions and aggregation depends on the viscosity of the fluids, the particle concentration, the ionic strength, the shape of the nanoparticles, the

hydrophobicity/hydrophilycity ratio, the mixing method, the characteristic of the electrolytes in the medium (Brar et al., 2010; French et al., 2009; R. D. Handy et al., 2008; Limbach et al., 2005; Pagnout et al., 2012; Sharma, 2009). For charged particles, such as nanoparticles in fluids, the stability of colloidal suspension is described by the Derjaguin–Landau– Verwey–Overbeek (DLVO) theory (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). This theory describes the theoretical interaction of attraction or repulsion between two closely charged particles assumed to be spherical and solid. At the origin, this theory was used to describe the interaction between a surface and a colloidal particle (Marshall et al., 1971) and is therefore applicable to bacterial microorganisms, because it has been demonstrated that their surfaces could be reduced to charged mineral surfaces into equations used for calculating the DLVO theory (Poortinga et al., 2002). In fact, it's the summation of the VdW forces and the electrical double layer (EDL) interactions (sum of the charges of the different molecules presents at the surface of the molecule of interest). The EDL thickness is highly dependent of the ionic strength of the media and an increasing ionic strength will led to a compressive EDL, leading to weaker interactions. Nevertheless, this theory was widely used to explain nanoparticles aggregation and interactions with biological surfaces. For the ecotoxicologist, DLVO theory gives a qualitative estimation of dispersion that can occurred in laboratory conditions despite the important limitations and weaknesses of this theory (no respect in NPs size, shape, surface coating) (Handy et al., 2012). To obtain more accurate results, the Extended Derjaguin-Landau-Verwey-Overbeek (XDLVO) theory for biological systems was modeled using an acid-base approach (He et al., 2008; Schwegmann et al., 2013; Snoswell et al., 2005; Zhang et al., 2011). Nevertheless, the acid-base interactions are not always operatives due to the distance that is maintained between the particles and the bacterial cells originates from the structural and chemical heterogeneities of the surfaces (Bos and Busscher, 1999). A model was proposed where the ions and water molecules can freely penetrate in a porous surface layer with fixed charges (Ohshima, 1994). It was then improved to take into account the complex structural organization of the bacterial cell surface (lipopolysaccharides, pili, fimbriae, peptidoglycane...). This model is the diffuse soft particle electrokinetic model (DSPE) where the bacterial electrophoretic mobility (generally used to determine the bacterial cell surface potential) is originated from the weighted average of potentials over the surface charge layer, and from the volume density of membrane-fixed charges (Duval and Ohshima, 2006). The majority of these models are based on electrokinetic measurements (zeta potential, electrophoresis mobility) and highlight the fact that depending on the surface potentials of the particles and bacterial cell, EDL thickness and VDW force strength, the particle were

deposited onto the wall, just suspended above or are aggregates and no physical interactions occurs (Chein and Liao, 2005; Zebardast et al., 2012; Zhang et al., 2008).

B. Membrane interaction and toxicity

The composition of bacterial cell walls determines parameters for adhesion of nanoparticles at their surfaces. The bacterial cell membrane is semipermeable and the bacterial outer membrane drive the cell adhesion process through their highly charged structure and their functional groups (carboxyl, hydroxyl, phosphate and amide) (Jiang et al., 2010; Omoike and Chorover, 2004; Parikh and Chorover, 2006). It is a physical barrier protecting microorganisms and supplying a stable cellular environment which regulates the movement of different types of molecules such as amino acids, the access to environmental information, the intercellular interactions (such as cellular attachment and gene transfer) and where energy transduction occurs (Jonak et al., 2004; Lyon et al., 2007; Zhao et al., 2013). Lipopolysaccharides (LPS), lipoteichoic acid (LTA), proteins, and phospholipids are the major biomolecules of the bacterial cells that can possibly interact with the nanoparticles.

Cell wall of Gram positive bacteria is a single layer that is 20-80 nm thick, composed of a cytoplasmic membrane surmounted by several layers of peptidoglycan (linear polymer of alternating subunits of N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM)). Perpendicularly insert into peptidoglycan sheets, lipoteichoic acids are anchored in the cytoplasmic membrane and their extremity is pointing outside the wall, in the medium. The peptidoglycan may contain teichoic acids constituting a felting leading to the formation of a "meshlike exoskeleton" (Bauman, 2004; Talaro, 2008). LTA basic structure consists of a soluble polymer that is tethered to a membrane anchor and faces the outer leaflet of the plasma membrane. The polymer is formed of alternating units of a polyhydroxy alkane, comprising glycerol, ribitol and phosphoric acid, joined to form phosphodiester units. Modifications of the repeating units are further modified; provide chemical diversity between various Gram positive bacterial species (Schneewind and Missiakas, 2014).

In Gram-negative bacteria, the cell wall is composed of a cytoplasmic membrane surmounted by a single layer of peptidoglycan (2-3 nm thick) with above another membrane structure called the outer membrane. The peptidoglycan of Gram-negative bacteria does not contain teichoic acids or LTA. The outer membrane of Gram-negative bacteria consists of a phospholipid bilayer in which all or parts of the phospholipids of the outermost layer were replaced by a unique component, the lipopolysaccharide (or endotoxin). The outer membrane is held together by divalent cations, such as Mg^{2+} and Ca^{2+}, by interactions between

phosphates on LPS molecules and by hydrophobic interactions between LPS and proteins (Bauman, 2004; Talaro, 2008).

In most physiological conditions and in environment, the bacterial cell surfaces are negatively charged. In aqueous media, the both LPS and LTA presents their hydrophobic sides embedded in the membrane whereas the hydrophilic side is turned to the outside of the cell. The LTA is composed of a long chain of anionic phosphodiester with neutral branches (glucosamine) and cationic branches composed of D-alanine. The LPS are composed of an oligosaccharide core with a hydrophobic A region embeds in the outer membrane and a hydrophilic O-antigen region turn to the outside of the cell. It has been estimated that the LPS cover the 3/4 of *E. coli* surface with an amount of approximately 3.5 million molecules and the remaining 1/4 correspond to membrane proteins (Li et al., 2010). The LPS of *E. coli* were shown to realize hydrogen bonds with TiO_2 (Jucker et al., 1997) and *E. coli* and *P. aeruginosa* strains with the longest LPS were shown to adhere in a higher way to TiO_2 thin film (20-500nm) than strain with truncated LPS (Li and Logan, 2004). The predominant role of the LPS in the interaction between bacterial cells surface and nanoparticles have also been evidenced in studies focusing on Au-NPs, Ag-NPs and CeO_2-NPs (Deplanche and Macaskie, 2008; Sondi and Salopek-Sondi, 2004; Thill et al., 2006). It is assumed that when there is no dissolution of nanoparticles and so no ionic adverse effects, the cytotoxicity of the nanoparticles against bacteria is mainly correlated to the structural damages of the bacterial cell surfaces. When looking at the bacterial surface biomolecules and the bacterial cells, it was found that Al_2O_3-NPs, TiO_2-NPs, and ZnO-NPs led to spectral changes of these biomolecules after NPs exposure under laboratory ambient condition when analyzed by Fourier transform infrared (FTIR) spectroscopy (Jiang et al., 2010). In this study, the lipopolysaccharides (G-) and lipoteichoic acids (G+) were able to realized hydrogen bonds and ligand exchange with the nanoparticles. Moreover, phosphodiester bond of L-α-phosphatidylethanolamine was broken, forming phosphate monoesters leading to disordered alkyl chain and disrupting bacterial membrane conducting to cell leakage. These modifications highlight the correlation between changes in proteins and bacterial cell phospholipids and NPs cytotoxicity. It was shown that the NP exposure induce changes in protein structure of bacterial surface biomolecules (β-sheets/α-helices ratio) indicating that the toxicity of Al_2O_3, TiO_2, and ZnO nanoparticles also seemed to be related with the function of the bacterial cell wall. More precisely, the ZnO-NPs were the most toxic of the three nanoparticles, possibly due to their solubility. By bonding to the cytoplasmic bacterial membrane and possibly leading to disrupt is integrity, NPs may interrupt the fundamental role

of electron transport phosphorylation and energy transduction process (Lyon et al., 2007). It is also important to know if interactions between bacterial cells surfaces and nanoparticles may lead to oxidation/reduction reactions. Indeed, microorganisms have potential to influence the catalytic characteristic of certain NPs by altering their oxidation state leading to their aggregation or sedimentation (Brar et al., 2010). In the case of CeO_2-NPs, which are strong oxidant, it has been shown that they can polymerize and decarboxylate small organic molecules (Cervini-Silva et al., 2005) and their adsorption on the bacterial surface of *E. coli* cells led to direct oxidizing mechanisms and expression of cytotoxicity (Lyon et al., 2007).

Physical impact of nanoparticles on bacterial cells could lead to the disruption of the membrane. Indeed, after exposition of *E. coli* cells to CuO-NPs concentration of 2 and 10 mg/L, both cellular wall and membrane were damage with the presence of deep holes (around 100 nm) on the surface of bacterial cells (Zhao et al., 2013). Moreover, it was shown that silica nanoparticles with a size below 22 nm could pierce a lipid bilayer which is the case of bacterial membrane (Roiter et al., 2008). ZnO-NPs and resulting Zn^{2+} ions penetration in the cell envelope led to bacterial membrane disorganization which is also a mechanism of bacterial growth inhibition (Brayner et al., 2006; Huang et al., 2008; Li et al., 2008). Exposing *E. coli* cells to 10 µg/ml of Ag-NPs completely inhibit the growth of cells. The leakage of sugars and proteins was observed and inactivation of the respiratory chain dehydrogenases occurs, suggesting that the permeability of the bacterial membranes was altered (Li et al., 2010). This also leds to the disorganization of membrane components and even dissolution and dispersion of the membrane vesicles. When exposed to 50 µg/ml of Ag-NPs, TEM and SEM images showed pits and gaps in bacterial cells, some membranes were fragmentary, indicating that the bacterial cells were severely damaged. It was also showed that by attaching to the bacterial cell surface membrane, Ag-NPs cause membrane permeability modifications, dissipation of the ATP pool and proton motive force, and finally cell death which confirmed what was previously highlight (Choi et al., 2008; Lok et al., 2006; Morones et al., 2005; Sondi and Salopek-Sondi, 2004). In a proteomic approach focusing on *E. coli* cells, short exposures of Ag-NPs induced a decrease in heat shock proteins and envelope proteins expression. This results in a reduction of the proton motive force leading to membrane potential shutdown and loss of cell viability (Lok et al., 2006). By binding to the *E. coli* bacterial cell surface, Ag-NPs were demonstrated to highly induce the *sodA* promoter highlighting the possibility of oxidative stress damage through ROS generation, especially the superoxide radicals (Hwang et al., 2008). Choi et al. demonstrated that exposing nitrifying bacteria to 1 mg/L of Ag-NPs end in various oxidation and dissolution of these nanoparticles (Choi et al., 2008). The Ag-

NPs were previously showed to be susceptible to oxygen oxidation, and the product of this process appeared to be more toxic than the initial nanoparticles (Lok et al., 2007), leading to think that bacteria may enhanced the nanoparticle toxicity against themselves. Moreover, when oxidative nanoparticles were in contact with bacteria the lipid peroxydation may happen. It consists in the oxidation of the double bounds present on fatty acids tails in phospholips membrane. It could conduct to osmotic stress, decreased in nutrient uptake and membrane permeability impact (Cabiscol et al., 2000; Lyon et al., 2007). It could lead to longer adverse effects against proteins and other molecules by generating free radicals that could be highly problematic when reacting with DNA (see below). The outer membrane of gram-negative bacteria contains porin forming channels with size generally assumed to be less than 4 nm, that allows the influx and efflux of hydrophilic low-molecular weight molecules and ions (Handy et al., 2012; Radzig et al., 2013). Even if it's theoretically possible that small nanoparticles uses these porins to overpass the bacterial wall, they are generally too small to allow the passive diffusion of NPs. Moreover, the porin channels are often charged due to their composition of amino acids that will also prevent the passive diffusion of NPs depending on their surfaces charges (Handy et al., 2012). The exposition of *E. coli* cells with OmpF and/or OmpC mutations to Ag-NPs evidenced that these mutants were 4 to 8 times more resistant to Ag-NPs, indicating that Ag-NPs toxicity may involve ionic silver. Indeed, Omp porins of *E. coli* cells had a diameter of around 1.0 nm, which allows the passage of ionic silver through these pores but not the Ag-NPs who, in this study, had a size of 8.3 nm. In *Vibrio cholera,* OmpU and OmpT porins proteins respectively have a size of about 0.55 nm and 0.43 nm, (Duret and Delcour, 2010) reinforcing the idea that only ionic compound may pass through porins proteins.

Regarding the toxicity mechanism for Ag-NPs, it was highlight that the partial oxidation of Ag-NPs was required to exhibit bactericidal properties, indicating that the release of silver ion were absolutely needed for the expression of nanoparticles toxicity. This work suggests that a synergistic mode of action from both nanoparticles and ions occur in the expression of toxicity against bacteria (Lok et al., 2007).

C. Ionic cytotoxicity

Dissolution of nanoparticles is a critical step for their toxicity evaluation. Au, TiO_2, and CeO_2 nanoparticles are highly insoluble under almost all environmental conditions and will strongly resist dissolution (Cornelis et al., 2011; Judy and Bertsch, 2014). At the opposite, Ag, ZnO, Al_2O_3, and Cu/CuO nanoparticles present a relative solubility and may partially dissolve in

many environments (Liu and Hurt, 2010; Unrine et al., 2010). Dissolution of nanoparticles deeply correlates to the solubility and the redox potential of the particles in a specific solution (Auffan et al., 2009; Pourbaix, 1974). The acute ecotoxicity of metals has been described by the biotic ligand model (BLM) (Di Toro et al., 2001) which is a chemical equilibrium-based model (Paquin et al., 2002). This model takes into account the sites of action of toxicity corresponding to biotic ligand. The purpose of this model is to describe a compound toxicity by measuring the interaction and accumulation between a free metal ion and the biotic ligand in the presence of other ion and organic matter (Di Toro et al., 2001; Erickson, 2013; Paquin et al., 2002). However, this model contains assumptions which may not always be justified in real situations but are necessary for modeling (Fabrega et al., 2011). Nevertheless, the dissolution of nanoparticles in complex media is such dependent on many parameters (pH, ionic strength, light...) and the sites of action of the nanoparticles are often not exactly known explaining why, to our knowledge, this model is not used to evaluate the toxicity of nanoparticles.

In the case of ZnO-NPs, nanoparticles were able to penetrate the cell envelope (Brayner et al., 2006; Li et al., 2008) but it was supposed to come from dissolution and recristalization process. Nevertheless, the role of Zn^{2+} ion is not clear (Franklin et al., 2007; Sawai, 2003). By binding to the microorganism cell membranes, Zn^{2+} was demonstrated to prolong the lag phase of the microbial growth cycle (Atmaca et al., 1998). For Ag-NPs, the release of Ag ions was shown to be contact and time dependent in the case of *E. coli* cells (Eduok et al., 2013) and silver granules can be detected at the surface of cell membranes (Fabrega, 2009). The release of ions may led to the presence of different oxidation state of Ag ions such as monovalent Ag^{+} (the most common one), Ag^{2+} or Ag^{3+} (Fabrega, 2009). The toxic effect of silver ions against bacterial cells is under investigations for more than 60 years (Franke et al., 2001; Li et al., 2010). In *E. coli* cells, Ag^{+} ions inhibit the uptake and exchange of phosphate, leading to accumulation of other essential molecules that are the mannitol, succinate proline and glutamine (Li et al., 2010; Schreurs and Rosenberg, 1982). In *Vibrio cholera*, Ag^{+} led to the shutdown of the proton motive force leading to cell death (Dibrov et al., 2002). By complexing to electron donor molecules composed with nitrogen, oxygen or sulfur, such as carboxylates, thiols, amines, phosphates..., and leading to their displacement from their usual binding site, the silver ions can inactivate enzymes and may also inhibit the of oxidation energetic substrates (glucose, succinate, fumarate, glycerol...) (Ahearn et al., 1995; Cioffi et al., 2004; Feng et al., 2000; Kumar and Münstedt, 2005; Li et al., 2010; Morones et al., 2005). It was shown that in *E. coli*, with thiol groups of cysteine, Ag^{+} replace the hydrogen bonds by

a –S–Ag interaction, decreasing the enzymatic potential and leading to cell death (J. Y. Kim et al., 2008; K.-J. Kim et al., 2008). Moreover, the Ag ions may inhibit the respiratory enzymes leading to the reactive oxygen species formation. Moreover the silver ions were capable to bind to the DNA and to increase the previous adverse effects (Bragg and Rainnie, 1974; Carlson et al., 2008; Feng et al., 2000; Holt and Bard, 2005; Lansdown and Williams, 2007; Slawson et al., 1992; Trevors, 1987). Nevertheless, the composition of the surrounding media can drastically shut down the possible toxicity of Ag ions. Indeed, by strongly binding to Ag^+, chloride ions lead to the precipitation of a AgCl complex decreasing Ag bioavailability (Fabrega, 2009). It was showed that Cl^- ions, when they are present in high concentration in the solution, may also bring back from bacterial cells membrane fixation the Ag ions and formed a newly bioavailable complex of $AgCl_2$ (Silver, 2003). In the literature, it is assumed that the mechanism of Ag-NPs toxicity and Ag ions were practically the same, but the antimicrobials activity of Ag-NPs was much more efficient than for Ag ions (in the nM range *vs.* µM range) (Fabrega, 2009). However, in a study comparing the effects of Ag-NPs and $AgNO_3$, the authors found that the presence of many common ligands in the solution such as Cl^- , S_2^- , PO_4^{3-}, and cysteine were able to reduce the toxicity of silver nitrate without affecting those of Ag-NPs. This result suggests that toxicity was not only correlated with the presence of free Ag ions (Xiu et al., 2011). Indeed, when the toxicity was provoked by ionic silver, it was shown that Ag0 cluster formation may lead to a decrease of about 80% of Ag^+ concentration (Morones et al., 2005). Experiments with ZnO-NPs and zinc acetate solution after exposition of *Cupriavidus necator* show different pattern in the toxicity mechanisms. Indeed, after exposure, the protein expression profiles were different according to the zinc types. The ZnO-NPs exposition lead to increasing production of membrane bound proteins, whereas zinc acetate exposure increased the production of metabolism associated proteins. With zinc acetate, growth rate increased for concentration of zinc between 25nM and 1mM. This result suggests that the mechanisms of toxicity for both treatments are particle-specific (Neal et al., 2012). Furthermore, in the case of ZnO-NPs, it was shown that the Zn^{2+} concentrations after ZnO-NPs dissolution stays very low due to the formation of zinc carbonate which is insoluble mineral (Reed et al., 2012). With the presence of phosphate and sulfur in the wastewater, the concentration of Zn^{2+} was deeply reduced due to the formation of zinc phosphate mineral (Judy and Bertsch, 2014). The same type of toxicity modulation was observed for nanoparticles and ions released from nanoparticles. If some nanoparticles are able to dissolve in environment, insoluble nanoparticles may exert specific effects unrelated

to release of metal ions (Judy and Bertsch, 2014; Meyer et al., 2010; Shoults-Wilson et al., 2011; Yin et al., 2011).

D. Modulation of toxicity

All types of nanoparticles may aggregates under environmental conditions dramatically reducing their bioavailability, mobility, and toxicity (Gao et al., 2009). In the literature, it was intensively demonstrated that exposure media composition, pH, ionic strength, hydrophobicity, natural organic matter (NOM)/dissolved organic matter (DOM) concentration, and surface chemistry affect the electrostatic repulsion and the steric repulsion between individualized nanoparticles (Judy and Bertsch, 2014; Moreau et al., 2007). These parameters deeply impact the coagulation and the aggregation of nanoparticles leading to their "inactivation" (Auffan et al., 2009; Choi et al., 2008; Lyon et al., 2007; Sudheer Khan et al., 2011). Moreover, the presence in the solution of high concentrations of divalent cations may drastically increase the aggregation process (Espinasse et al., 2007; Pagnout et al., 2012; Zhang et al., 2009). In environment these parameters lead to the formation of heteroaggregates, corresponding to a mix of nanoparticles and environmental particles (Espinasse et al., 2007; Lowry et al., 2012). The adsorption of NOM to nanoparticles is pH-dependent, and related to negative surface charge (Yang et al., 2009). NOM will adsorb to active sites on the nanoparticle surface, reducing the overall surface charge of the particle, decreasing the possible interactions with the microorganisms (Hyung et al., 2007; Neal, 2008) and even promoting their dispersion (Terashima and Nagao, 2007). At the opposite, at higher concentrations, NOM will form a highly negative charge coating enhancing steric hindrances (Illés and Tombácz, 2006) promoting the dispersion of the nanoparticles (Diegoli et al., 2008; Judy and Bertsch, 2014). In liquid medium, the Ag-NP antimicrobial effect decrease in comparison of what was observed on agar plates, due to the coagulation of nanoparticles (Sondi and Salopek-Sondi, 2004). It was demonstrated that sulfidation of Ag-NPs could altered the surface charge of Ag-NPs leading to dramatically reduce dissolution rate of Ag-NPs (Levard et al., 2011). The sulfidation process of nanoparticles was demonstrated to be highly influenced by pH, organic matter and dissolved oxygen (Judy and Bertsch, 2014). In a study where synthetic ZnS-NPs were used with amino acids of sulfate reducing bacteria from a biofilm, the EPS synthesis and excretion were leading to a rapid nanoparticles aggregation (Moreau et al., 2007). When exposing cells to 10 mg/L of CuO-NPs, the growth inhibition rate was 45.4 %. The toxicity was assumed to be caused by the particles because no toxicity was measured when the cells were exposed to Cu^{2+} ions. Disorganized cell walls and

disrupted cellular morphology were observed on NPs treated bacteria. What was surprising was the location of the physical damages, located on the ends of the cells, suggesting that this part of the cells were more sensitive to CuO-NPs. Moreover, in this study, they adds fulvic acids in their experiments to understand the impact of the dissolved organic matter. They found that when fulvic acids were present, the CuO-NPs were interacting with them, reducing the direct contact between bacterial cells and nanoparticles, decreasing the toxicity express by these nanoparticles (Chen et al., 2011; Li et al., 2008; Zhao et al., 2013). In another study, it was shown that when CeO_2-NPs are covered with organic molecules, the oxidative action of these nanoparticles was still occurring but no toxicity were measured (Lyon et al., 2007).

E. Oxidative mechanisms

After adsorption of nanoparticles on bacteria, in addition to physical interactions, photocatalytic reactions may occur when nanoparticles have semiconductor properties and when light absorption occurs. The nanoparticles presenting semiconducting properties are varied, the most used being TiO_2, ZnO, Fe_2O_3, CdS and ZnS. When these nanoparticles are illuminated by photons whose energy is greater or equal to the valence band, they are absorbed and a photogenerated electron hole is created. At the same time, an electron transfer from the valence band to the conduction band occurs. Depending on the redox potential of the surface adsorption of nanoparticles, an electron transfer or the photogenerated hole (h +) occurs. In the case of TiO_2, the reactions arising from such adsorption generates either the direct oxidation of the organic matter present in the vicinity of the nanoparticles or generate the creation of reactive oxygen species (ROS) such as superoxide ions (O2° -) , the highly reactive hydroxyl radical (OH°), H_2O° and hydrogen peroxide (H_2O_2) (Benabbou et al., 2007; Brayner et al., 2006; Brunet et al., 2009; Kikuchi et al., 1997; Seven et al., 2004). It is important to note that specific surface area of nanoparticles enhance production of ROS (Singh et al., 2009). These ROS are highly reactive molecules that are able to completely disturb homeostasis of bacterial intracellular environment by adversely reacting with cellular macromolecules (DNA, proteins and lipids) (Pelletier et al., 2010). Transition metal nanoparticles and ions released by some of these types of nanoparticles (such as cadmium, chromium, cobalt, copper, iron, nickel, titanium and zinc) may induce the conversion of cellular oxygen metabolic products to hydroxyl radicals (Singh et al., 2009). Currently, during a photocatalytic exposition of bacteria to UV-A low intensity light, it appears that the main target of photogenerated ROS is the bacterial membrane (Cheng et al., 2007; Ibáñez et al., 2003). Hydroxyl radicals are supposed to be the main elements who acts in the expression of

the bactericidal action (Singh et al., 2009), however, these reactive species can diffuse only very little in the medium (below 1µm) and their lifetime does not exceed nanoseconds (Carre, 2013; Cho et al., 2005, 2004a; Foster et al., 2011; Grassian, 2005; Ireland et al., 1993; Markowska-Szczupak et al., 2011; Văcăroiu et al., 2009). Moreover, they are only produced in contact with water in the case of TiO_2-NPs (Linsebigler et al., 1995). However, it seems to be sufficient to generate adverse effects (Dhandapani et al., 2012; Rajagopal et al., 2006). As the hydroxyl radicals cannot diffuse into the medium and cannot cross permeable physical barriers due to their surface charge (Kikuchi et al., 1997). Hydrogen peroxide, because of its lack of electric charge, may be the reactive species capable of diffusing in the medium and to generate and induce cell death (Foster et al., 2011). This suggests that the simultaneous action of several ROS is the cause of bactericidal effects that can be observed in these studies (Carre, 2013).

The rutile crystal form allows TiO_2-NPs to be activated by UV-A from the beginning of the visible light spectrum (λ<413 nm) which leads to the generation of photocatalysis reactions. These photocatalysis reactions can occur with other semiconductor nanoparticles, however, the lack of stability of these particles lead to much shorter phenomena and therefore less impacting reactions affecting bacterial microorganisms in the long term. Thus, CdS and ZnS-NPs are unstable in water and prone to sulphate oxidation and ZnO-NPs are less stable than TiO_2-NPs in liquid phase. It has been shown that the low intensity UV-A radiation can induce DNA damage, lipid oxidation and protein alterations (Bosshard et al., 2010; Carre, 2013; Santos et al., 2013; Tyrrell, 1973). It is therefore necessary to take into account part of photochemical damage generation during the use of potential photocatalytic nanoparticles in the analysis of the effects of nanoparticles on bacteria.

When using P25 TiO_2-NPs NPs at a concentration ranging from 0.1 à 2.5 g/L and with 10 minutes of irradiation under UV-A, a 3-log reduction in the viability of *E. coli* cells was obtained clearly indicating that solar energy is sufficient to induce photocatalytic reactions leading to the inactivation of bacteria (Benabbou et al., 2007). This inactivation after low UV radiation was also highlighted on enterobacteria, *E. cloacae.* After 40 minutes of exposure to a concentration of 0.1 g/L of P25 TiO_2-NPs, a 4-log reduction in cell number was observed (Ibáñez et al., 2003). It was also demonstrated that the coupled systems Cu-TiO_2-NPs exposed to low light intensity, comparable to the day light energy intensity, could lead to a decrease of more than one log in *E. coli* copper resistant strain after 4 hours of exposure (K Sunada et al., 2003). In addition to these photocatalytic effects, adsorption of nanoparticles (ZnO, TiO_2,

Ag...) on microorganisms or microorganisms inclusion within nanoparticle aggregates induce changes in membrane permeability, even in the absence of light, leading to an increase in cell death (Adams et al., 2006; Gogniat et al., 2006; Li et al., 2008; Maness et al., 1999). These ROS will first oxidize the outer membrane by forming micropores (for gram-negative bacteria).

In addition to the effects observed with natural low-energy light, the production of ROS in the dark is supposed to happen. However, this assumption is questionable. Indeed, it has been shown that toxic effects of TiO_2-NPs under dark condition were due to hydroxyl radical (OH°) formation and carbon-centered radicals generation occurred after the cleavage of the C–H bond in a model molecule (Fenoglio et al., 2009). The production of reactive oxygen species under dark conditions was also questioned with the work realized on *S. typhimurium* cells after exposure to P25-TiO_2-NPs (Jomini et al., 2012). Regarding the rutile ultrafine TiO_2-NPs, they express the same reactivity in the dark than those observed under illumination (Fenoglio et al., 2009; Gurr et al., 2005; Long et al., 2006; Sayes et al., 2006; Theogaraj et al., 2007). Another study focusing on the whole-cell-array stress genes expression in *E. coli* highlight that under dark condition, TiO_2-NPs induced oxidative stress, cell membrane damages and DNA adverse effects (Gou et al., 2010).

F. Intracellular effects

It has been shown that after bacterial exposure to nanoparticle, NPs can potentially cross the bacterial cell wall and ended in the cytoplasm (Brayner et al., 2006; Huang et al., 2000; A. Kumar et al., 2011; Sondi and Salopek-Sondi, 2004). After penetration, the mechanism of lipid peroxydation was initiated leading to adverse effects on intracellular components of bacterial organisms (Caballero et al., 2009; Carre, 2013; Rincón and Pulgarin, 2003; K. Sunada et al., 2003). In a study where *Pseudomonas aeruginosa* was exposed to silver nanoparticles with a size over 80 nm, the Ag-NPs were able to cross the both inner and outer membrane (Xu et al., 2004). Internalization and reductive deposition of Pd-NPs in the periplasmic space of *Shewanella oneidensis* have also been found (Windt et al., 2006). Despite these works, there is very few evidence of nanoparticles penetration in bacterial cells (Neal, 2008) and this possibility is still under severe debate and seems to be highly unlikely (Lyon et al., 2007). This suggests that the intracellular effects that can be observe are cause by indirect mechanisms when there is no disruption of the bacterial membrane. Nevertheless Handy and colleagues in 2012 also noticed that even if prokaryotic cells were unable to realize endocytosis, the work of Lonhienne et al., showed that a species of Planctomycete was

able to realize the same internalization process (Lonhienne et al., 2010). If this mechanism is also realizable by the microorganisms, this could led to the interactions of intracellular components with nanoparticles and engendered adverse effects for the cytoplasmic biomolecules (Nel et al., 2009). It has been shown that Ag ions were capable to bind to the thiol groups of enzyme, especially those involved in the ATP production which can negatively impact the bacterial metabolism (Li et al., 2010). The Ag-NPs were shown to penetrate *E. coli* bacterial cells and persist in the intracellular compartments releasing Ag^+ ions which were supposed to be one of the possible toxicity mechanisms for Ag-NPs (Xiu et al., 2011). This silver ion generation may conduct to ROS generation and ROS protein oxidation (Handy et al., 2012). In the case of NZVI, an exposure of 3 hours of the *K. oxytoca* strain resulted in the overproduction of tryptophanase encoded by the *tnaA* gene (Sacca et al., 2013). This enzyme who converts tryptophan to indole, was identified as an activator molecule for the *tnaA* gene (Gerth et al., 1993), ammonia, pyruvate, and have been associated with an oxidative stress response. In this study, the over expression of tryptophanase led to a protective action against the potential adverse effects of the NZVI (Sacca et al., 2013). Regarding the effects of CuO-NPs, it was evidenced that exposition to 10 mg/L significantly increased the K^+ ion efflux indicating that the membrane bacterial cells were compromised (Zhao et al., 2013). In the proteomic study of Loak et al., in 2006, loss of the proton motive force after exposure to Ag-NPs also induced massive loss of intracellular potassium. Moreover, the cellular ATP levels were also negatively affected leading to the loss of cell viability. Thus, it was supposed that cytoplasmic material may also leaked in the extracellular medium due to the disruption of bacterial cells membranes.

When Ag-NPs enter the bacterial cells, they form a low molecular weight region inside the bacteria leading to the formation of a conglomerate of bacteria in order to protect their DNA from the Ag-NPs (Li et al., 2010). When no intracellular nanoparticles were found and a genotoxicity was obtained, this led to think that another indirect genotoxic mechanism occurred, probably involving ionic silver. It corresponds to what we observed in a previous work, where no nanoparticles was observed inside *S. typhimurium* cells, but genotoxicity was detected (Jomini et al., 2012). To highlight these intracellular effects, the detection of nanoparticle genotoxic adverse effect is one of the methods that can be implemented. The Ames test is one of the few tests of bacterial genotoxicity but also one of the easiest way to assess the genotoxic potential of nanoparticle. To measure both potential ROS generation and genotoxic effects, the strain of *S. typhimurium* TA102 was specially developed for the detection of oxidative mutagens (Levin et al., 1982). When the Ames test is used in a

conventional manner (on agar), this test always produces negative results with P25-TiO_2-NPs, ZnO-NPs and ultrafine TiO_2-NPs (Dufour et al., 2006; Nakagawa et al., 1997; Pan et al., 2010; Warheit et al., 2007) except the work of Maenosono and collaborators who obtained very slightly positive results with the TA100 strain of *S. typhimurium* when studying FePt-NPs capped with $(CH_3)_4NH_4OH$ (Maenosono et al., 2007). In our previous work, we used the Ames fluctuation method with a preexposition step to detect TiO_2-NPs genotoxicity under dark condition. We observed that all the three TiO_2-NPs tested were genotoxic to the TA102 strain without any penetration in the bacterial cells, reinforcing the idea of an indirect mechanism of genotoxicity induction by ROS or physical overcrowding (Jomini et al., 2012). Iron nanoparticles could be quickly oxidized into iron ions (Kumar, 2006; Lo et al., 2006; Zhang and Elliott, 2006), leading to OH°-induced purine and pyrimidine alterations (Zastawny et al., 1995). Moreover, Fe-NPs could led to excessive level of iron ions within the bacterial cells conducing to the over realization of the Fenton reaction who produce highly reactive oxygen species and results in localized DNA damages (Cabiscol et al., 2000; Lyon et al., 2007; Rai et al., 2001; Singh et al., 2009). It was demonstrated by X-ray absorption near edge structure spectroscopy that Ag-NPs were able to realized Ag-N and/or Ag-O bond, suggesting that Ag-NPs could interact with proteins or DNA (Bovenkamp et al., 2013). The effects of ROS production possibly occurring after Ag-NPs exposition were investigated by studying the influence of mutations in genes of the base excision repair (BER) system (Radzig et al., 2013). The most common lesion after oxidative damage in bacterial cell was production of 8-oxoguanine (8-oxoG) who led to the generation of oxidative mutagenesis (Farr and Kogoma, 1991). When realizing a mutant who carries mutations inactivating the *mutM*, *mutS*, *mutT* genes, *E. coli* resistances to Ag-NPs was decreased by a factor 3. When the *mutY* gene was inactivate, the *E. coli* resistance to Ag-NP was decreased by a factor of 10. When the mutation inactivates the *nth* and *xthA* genes a decrease of about 2.5 fold for MIC value was observed after Ag-NPs exposure. The *mutY* gene code for the DNA glycosylase short-patch base excision repair pathway in order to remove adenines from mismatches. The *mutS* gene initiates the long-patch mismatch repair on daughter DNA strands after binding to the mismatches. The *mutT* gene removes 8-oxodGTP after hydrolysis of the nucleotide pool in order to prevent his incorporation into DNA by polymerases. *Nth* and *xthA* genes encode for the endonuclease III and exonuclease III and *nth* gene participates in the removal of 8-oxoG from 8-oxoG/G mispairs. The same oxidative effects on DNA were observed with $AgNO_3$ suggesting that the same pathways and mechanisms were involved in silver toxicity against bacteria (Radzig et al., 2013). The adverse effects of Ag-NPs were also observed after *E. coli*

DNA extraction and gel electrophoresis which highlight the fragmentation of the DNA strand revealed by the presence of a tail in the gel and according to these authors. This report was the first description of such DNA fragmentation caused by nanoparticles (Tamboli and Lee, 2013). It was also recently shown that daylight exposure of *E. coli* DH5α cells associated with the presence of Ag/ZnO-NPs heterostructure (Ag island doped-ZnO heterostructure) may led to generation of ROS and could damage plasmid DNA (Zhang et al., 2014). By impacting bacterial cells and their ability to grow by several mechanisms, nanoparticles may eventually impact bacterial communities' structure and dynamics.

5. Nanoparticles effects on bacterial communities

The increasing use and release of nanoparticles in environment create a great concern about their ecotoxicological effects. The effects of nanoparticles on bacterial cells and populations described in the previous chapter suggest that the impact of nanoparticles will affect microbial communities. Thus, exposition to manufactured nanoparticle/nanomaterial may conduce to shifts in the microbial composition of natural samples from environmental compartment (Handy et al., 2012). As discussed in the first part of this work, molecular fingerprinting techniques are essential for the evaluation of these bacterial composition modifications.

A. Impact of nanoparticle/nanomaterial in WWTP

The increasing use of nanoparticle/nanomaterial based-applications may lead to relevant amount of nanoparticles release in the environment, mainly in the aquatic compartment. Nanoparticles rejected into natural water were first reported for TiO_2 nanoparticles after emission from paints applied on building facades (Kaegi et al., 2008), and the same authors found also a released from paints containing Ag nanoparticles (Kaegi et al., 2010). The nanoparticles may also be rejected by abrasion process or during textile washing (Gottschalk et al., 2009; Guiot et al., 2009). Wastewater containing NPs can be directly released in the environment but they could also be collected and redirected to a WWTP. Indeed, the WWTP are the major receptor for effluents containing NP from accidental leaks (Boxall et al., 2007), manufacturers (Gottschalk et al., 2013; Kiser et al., 2010) and home-using applications (Weir et al., 2012).

When reaching the WWTP, nanoparticles end up in contact of the activated sludge. The activated sludge contains a wide range of different organic surface chemistries, such as Gram-positive, Gram-negative, filamentous bacteria (Kiser et al., 2010) and exopolymers.

Moreover, due to the several stage of anaerobic digestion involved in wastewater purification (solubilization, hydrolysis, acidification and methanisation) these microorganisms were particularly important. When in the WWTP process, nanoparticles were associated with the solid fraction of the activated sludge. It's the case of SiO_2-NPs which were sorbed to wastewater biomass and covered the majority of it surface (Kiser et al., 2012, 2010) and this observation was also done with TiO_2-NP (Kiser et al., 2012; Luppi and de Cortalezzi, 2013), CeO_2-NP (Limbach et al., 2008), Ag-NP (Choi et al., 2008). Regarding the impact of Ag NPs in WWTP on activated sludge biomass, it has been shown that nitrifying bacterial assemblages were modified. Indeed, it was found that at 1 mg/L of Ag in the nitrifying suspension, the nitrification inhibition by Ag-NPs, was 86.73% and from all the Ag species tested, Ag NPs presented the highest inhibition rate on nitrifying bacterial communities (Choi et al., 2008). The same observation was done with 1 mg/L of total Ag (coming from Ag-NP) when using a respirometric assays, 41.4% of nitrification inhibitions was observed, reinforcing the idea that Ag-NPs were particularly toxic to nitrifying bacteria (Liang et al., 2010). This study also showed that after a shock load of nanosilver (20 mg/L), it will take at least 25 days to completely clean out the system. However, the nitrification inhibition will continue for a period superior to a month, leading to an increase in ammonia/nitrite emissions but will not impact the heterotrophic community responsible for the organic matter removal. The T-RFLP microbial community structure analysis indicated that the *Nitrobacter* species were totally removed after the shock load and the ammonium-oxidizing bacteria and nitrite-oxidizing bacteria decreased after nanoparticles exposure. Moreover, a 90 % size dependent inhibition of natural nitrifying community was observed (Choi and Hu, 2008; Choi et al., 2008). Thus, the bactericidal effect of silver nanoparticles on bacterial communities performing nitrification within the WWTP may cause an adverse effect on the entire process of water disinfection.

When regarding the effects of TiO_2-NPs on biological removal of nitrogen, phosphorus and impact on bacterial community in activated sludge, it was found that 1 and 50 mg/L of TiO_2-NPs had no acute effects after short term exposure (1 day). After long term exposure, corresponding to 70 days, concentration of 50 mg/L of TiO_2-NPs significantly decreased the total nitrogen (TN) removal efficiency from 80.3% to 24.4%. For the phosphorus removal, no effect was observed in both situations. When performing bacterial community analysis by DGGE profiles, the exposition to a concentration of 50 mg/L of TiO_2-NPs significantly reduced the diversity of the microbial community in the activated sludge. To confirm these community modifications, FISH analysis was performed. It highlighted the decrease after

long term exposure of nitrifying bacteria, especially ammonia-oxidizing bacteria explaining why the process of ammonia oxidation was impacted. By looking at the toxicity mechanism, these authors found that the long-term exposure to a concentration of 50 mg/L of TiO_2-NPs inhibited the activities of ammonia monooxygenase and nitrite oxidoreductase which was consistent to what was previously observed (Zheng et al., 2011).

Regarding the aerobic heterotrophic microbial community of the activated sludge, respiratory inhibition tests were realized (Luppi and de Cortalezzi, 2013) with P25-TiO_2-NPs and Ag-NPs, and they both showed respiratory inhibition at concentrations comprised between 10 mg/l and 100 mg/l. TiO_2-NPs used under dark condition also led to respiratory inhibition. When paying attention to the anaerobic community, TiO_2-NPs, Al_2O_3-NPs and SiO_2-NPs in doses up to 150 milligram per gram of total suspended solids (mg/g-TSS) express no inhibitory effect (Mu et al., 2011). At the opposite, ZnO-NPs express a dose-response inhibitory effect against this bacterial community. The ZnO-NPs also inhibited the hydrolysis, acidification and methane generation sludge steps, and led to acetate kinase, protease and coenzyme F420 inhibition. The inhibitory effect on methane generation was assumed to be mainly due to $Zn^{2+,}$ which has been demonstrated by the literature (Zayed and Winter, 2000) but the other observed effect were assumed to came from ZnO-NPs.

By impacting the entire process of wastewater purification realized in the WWTP, nanoparticles can reduce the quality of water resources while leading to a significant increase in the pollution of surface water generated by discharges of untreated water. As the association and adsorption of nanoparticles on the organic fraction of activated sludge was reported to be the principal mechanism for nanoparticle removal in WWTPs (Ganesh et al., 2010; Kiser et al., 2010, 2009; Limbach et al., 2008), dependent on the elimination of used sewage sludge.

Despite the removal of activated sludge on a regular periodic period, depending on the nanoparticles, an important part of the initial amount entering the WWTP remains in the discharged water. It was observed in the case of TiO_2-NPs, that only 42% of the incoming nanoparticles were partially removed at the end of the purification process (Kiser et al., 2009). Considering the removal efficiency of wastewater treatment plants when regarding the nanoparticles or other type of pollutants, it is possible to think that municipal WWTPs are important sources of environmental contaminant release, allowing potential loophole into surface waters, soils, and air (Gottschalk et al., 2009; Kiser et al., 2010; Liang et al., 2010). This pathway consists in biosolids, liquid effluent, and potentially aerosol generation (Handy et al., 2012; Limbach et al., 2008; Mueller and Nowack, 2008). Thus, it is important to note

that NP could be relased by intentionnal overflow discharge in WWTP during heavy rain episode like in the case of TiO_2-NPs with discharged variation of 2 to 10% of the total inflow (Mueller and Nowack, 2008). The set of physico-chemical processes taking place within a WWTP could lead to cellular degradation and organic matter matrices present in rich amount in the process. These substances can react with the nanoparticles surfaces and significantly modify their fate in different environmental compartments (Kiser et al., 2012; Limbach et al., 2008). The transition of liquid effluents containing nanoparticles through WWTPs will be a significant parameter in the final spatial distribution of nanoparticles in the environment (Kiser et al., 2010).

B. Impact of nanoparticle/nanomaterial in natural waters

The increasing manufactured products available for nanoparticle/nanomaterial based-applications may lead to relevant amount of nanoparticles release in the environment mainly in the aquatic compartment. Thus, apart from accidental releases, the nanoparticle/nanomaterial released into the natural water was first reported for TiO_2 nanoparticles after emission from paints applied on building facades (600µg/L of Ti) (Kaegi et al., 2008), and the same authors found also a release from paints containing Ag nanoparticles (145µg/L of Ag and even more than 30% of the Ag-NPs after one year) (Kaegi et al., 2010). The nanoparticle/nanomaterial may also be released by abrasion process or during textile washing (Gottschalk et al., 2009; Guiot et al., 2009). To our knowledge there are very few studies regarding the impact of nanoparticles on bacterial community from natural waters.

Despite evidence of toxicity against pure culture of bacterial cells even at relatively high concentration, < 100 mg/L (Auffan et al., 2008; Diao and Yao, 2009; Lee et al., 2008), the adjunction of ZVI-NP led to an increased number of both viable and total bacterial cells abundances which was an evidence of an healthy and active bacterial community. The rank-abundance plots realized after DGGE analysis of an indigenous river water bacterial community revealed that the diversity and structure of this community were not impacted by the ZVI-NP (Barnes et al., 2010).

When focusing on Ag-NPs effects on natural bacterial community in natural waters, it was shown that an exposition to carboxy-functionalized AgNPs at 0.01 to 1 mg-Ag/L impact the bacterial community structure, diversity and significantly reduced the bacterial metabolic activity (alkaline phosphatase) without recuperation within 5 days. The DGGE analysis showed that the exposed bacterial communities were 54 to 56% different from the control.

Moreover, these changes were very fast for the high dose (0.1 and 1.0 mg-Ag/L) and tended to be less severe for the low exposure concentrations (0.01 and 0.02 mg-Ag/L). The T-RFLP analysis shows 4 types of responses: the intolerant, the recovering, the tolerant and the stimulated bacteria. This study was the first to present bacterial community tolerance and even stimulation after Ag-NPs treatment. The low treatment dose also allowed recovery activities as equivalent as the control after 5 days. The hypothesis for bacterial community resistance and even stimulation was the presence of bacterial cells containing metal gene resistance or could be due to the higher sensitivity of the most represented bacterial phylotypes (Das et al., 2012).

Still with regards to Ag-NPs, a microcosm study also showed a significant nanoparticles dose and shape-effect relationship on bacterial community structure and bacterial abundance (Doiron et al., 2012). Thus, a decrease in the number of OTUs was observed after exposition to 5 µg/L of Ag-NPs (41 OTU vs 44 in the control) and 50 µg/L (28 OTU vs 44 in the control). As previously observed the modification in species richness occurs quickly and signs of recovery after 48 hours are notable even at the highest concentration.

In our previous work (Jomini et al., in preparation), bacterial community modification was also highlight. P25-TiO_2-NPs were used to expose bacterial community from natural water. After 15 days of exposure under dark condition to avoid photocatalytic reaction, abundance, community structure and diversity were measured. It was observed that when exposed to increasing concentrations of P25 TiO_2-NPs (from 1-100 mg/L), the total amount of planktonic bacterial cells increased and a sharp decreased occurred for the biofilm when exposed to the highest concentration. DGGE analyses were performed to assess the effect of P25 TiO_2-NPs on diversity and structure of the community. The NMDS ordinations of PCR-DGGE fingerprint profiles highlight an important difference in samples exposed to 100 mg/L of P25 TiO_2-NPs compared to the control. For the biofilm community, modifications occur for each concentration of P25 TiO_2-NPs used. The diversity assessment by clone library analysis show a decreased in the number of sequences when the samples are exposed to P25 TiO_2-NPs in agreement with the index of Shannon (H'). Thus, on the RDP comparison, an important decreased in the *Proteobacteria* is observed, most especially with the *Betaproteobacteria* (41 in the control *vs*. 26 OTUs) despite the increasing number of sequences for the *Oxalobacteraceae*. A large increased occurred in the *Bacteroidetes* (24 in the control *vs*. 43 OTUs) due to the *Cytophagaceae* and, especially, the gender *Emticicia* after exposition to 100 mg/L of P25 TiO_2-NPs. At the opposite, the biofilm expressed a different evolution with increasing diversity under 100 mg/L of TiO_2-NPs exposition (31 in the control *vs*. 38 OTUs),

decreased occurring in the Betaproteobacteria mainly due to the *Burkholderiales* especially the *Leptothrix* and from the *Comamonadaceae*.

It is important to know that 99% of the total amount of bacteria live attached as surfaces biofilm (Thiéry et al., 2012). Bacterial communities present in natural aquatic biofilms are a potential target of the harmful effects of nanoparticles. Indeed, when studying a Singapore Harbour site where biofilms were grown up and after a 24 hours exposition to increasing concentrations of Ag-NPs (0-2000 µg/L), a significant decreased in biofilm biomass and volume was observed (Fabrega et al., 2011). The cluster and phylogenetic analysis made after performing T-RFLP showed a significant impact on marine biofilm community structure without impacting the major bacterial groups. Relative abundance of these major groups were decreased, such as biofilm development and species succession suggesting that Ag-NPs may have potential long-term adverse effects on community structure and function. Regarding the gold nanorods contamination of natural river water column in a mesocosm study, the biofilm, who represent only 0.5% of the total biomass, recovered 60% of the amount of Au-NPs (Ferry et al., 2009). This observation suggests that biofilm could be the major receptor for nanoparticles in the environment. It was also demonstrated that gold nanoparticles could induce morphological change in biofilm, destabilize *L. pneumophila* biofilms and promote their dispersion (Raftery et al., 2013; Stojak et al., 2011). Yeo and Nam observed that Ti accumulation in biofilm can reach a concentration of more than 3.5 µg/L during microcosm experiments after an exposure of 17 days to 1.8 mg/L of TiO_2-NP. By having adverse effect on biofilm structure, diversity, succession and by accumulating within biofilms, nanoparticles may impact the whole ecosystem. Indeed, bacterial biofilm communities play a key role in the production and degradation of organic matter, the degradation of many environmental pollutants, and the cycling of nitrogen, sulfur, and many metals (Davey and O'toole, 2000). In biofilms, bacteria were known to modulate gene expression in order to change biofilm morphology to avoid grazing (Matz et al., 2004, 2005). By impacting the bacterial composition of the biofilm, nanoparticles may completely change the biofilm ability to modulate gene expression and led to a much larger grazing by higher organisms (Raftery et al., 2013) which could ultimately alter the different biofilm function and adversely affect higher trophic level development and stability (Schmitt et al., 1995; Sheldon and Walker, 1997; Stojak et al., 2011). Moreover, biofilms are at the basis of food web for many organisms and if accumulation occurs into it, it can offer a magnification route for higher level organisms. Indeed, biofilm are the major food source for heterotrophic flagellates and ciliates in most aquatic systems (Huws et al., 2005), they are grazed by various organisms

(Scholz and Boon, 1993), as for example detritivores (Ferry et al., 2009). It is consider that biofilms consumption by protozoa is the major source of bacterial mortality in freshwater environments and a key parameter for protozoa development (Iriberri et al., 1995; Sanderson et al., 1989). Because microorganisms help to regulate and maintain ecosystem health and function (Janvier et al., 2007; Kaye et al., 2005), impacting the microbial community will conduct to affect the entire ecosystem (Kanerva et al., 2008). Moreover, when the dietary resource of a food web is impacted by a xenobiotic, the development and growth of aquatic invertebrates are negatively influence (Hall and Meyer, 1998; Lowell et al., 1995).

The migration process from biofilm to higher organisms was recently highlighted by Yeo and Nam, who showed that migration of TiO_2-NP from biofilms to mudsnail can reach 7% within 17 days of exposition (Yeo and Nam, 2013). According some authors, the morphological changes in biofilms after exposition to a xenobiotic mainly affect protozoa (Burns and Walker, 2000; Mojtahid et al., 2011). Impacting the biofilm may also engender perturbation and a lower food quality for shrimp. It has been shown that biofilm microorganisms provide essential nutrients as complementary food source for shrimp culture (sterols, amino acids, vitamins and fatty acids) and reduction of microbial diversity may totally change the specific contributions in food quality of the different bacterial taxa (Thompson et al., 2002). By presenting adverse effects on bacterial free cells and on biofilms, nanoparticles will impact higher organism levels that need the bacterial functions. Thus, in natural waters system, invertebrate shredders decompose plant litter and play a key role in detritus food web by transferring energy to higher trophic levels (Graça and Canhoto, 2006). They prefer to fed on litter colonized by aquatic bacterial cells and fungi, which increased the food quality and palatability to shredders (Pradhan et al., 2012; Suberkropp et al., 1983). When exposing the shredder *Allogamus ligonifer* to pre-contaminated leaves with concentrations of CuO-NPs 75 mg/L, a significant reduction of growth rate and leaf consumption was detected (Pradhan et al., 2012). These authors showed in a previous work that CuO-NPs had negative effects on bacterial cells and on the leaf litter colonization process (Pradhan et al., 2012) leading to a severe reduction in decomposition by bacterial population. To our knowledge, the first study highlighting the biomagnification of nanomaterial from bacterial cells to protozoa was made by Werlin and colleagues (Werlin et al., 2011). In this study, CdSe quantum dots that have accumulated in *Pseudomonas aeruginosa* bacteria have been transferred and biomagnified in the *Tetrahymena thermophila* protozoa who feed on them. They noticed that cadmium concentrations were approximately five times higher in the protozoa predator than in bacteria. By inhibiting protozoan food vacuoles digestion, bacteria treated with CdSe quantum dots

were differentially toxic to the *T. thermophila*. Important amount of intact quantum dots remains in the protozoa cells that didn't lyses leading to potential route for biomagnification of the quantum dots. This work was then completed by the study of TiO_2-NPs impact on *Tetrahymena thermophila* and accumulation that can occurred when protozoa were fed with direct "coated" nanoparticles-bacteria or through trophic transfer (Mielke et al., 2013). When nanoparticle exposure was realized by contaminated food with "coated" Pseudomonas aeruginosa, *T. thermophila* population showed lower growth rate than when fed on bacterial cells without nanoparticles. TiO_2 was internalized into food vacuoles whatever the feeding mode. This study reinforces the idea that nanoparticles could be transferred into higher trophic levels through food webs and that the food web could be impacted in a negative way.

The work of Bundschuh and colleague also showed that when the amphipods *Gammarus fossarum* was exposed to TiO_2-NPs under ambient-UV irradiation, the adverse effect did not came from leaf-associated nanoparticles but came from direct exposure of organisms. What was interesting was the non impact on feed selection of the TiO_2-NPs leaf-association, which was in contrast to what had been seen previously (Bundschuh et al., 2011). As also observed for marine amphipods with copper nanoparticles and UV irradiation, the photocatalytic effect seems to had the worst adverse effect (Bundschuh et al., 2011; Liess et al., 2001). These photocatalytic effects were also reported with *Cerodaphnia dubia* where the median lethal concentrations (LC_{50}) of TiO_2-NPs were found to be 8 µg/mL in case of light exposure and 32 µg/mL under dark exposure, and 74.3 µg/mL after 72 hours with Al_2O_3-NPs (Pakrashi et al., 2013). For the *Daphnia maagna*, higher toxicity response were noticed, with an LC_{50} of 2µg/mL for ZnO-NPs, 79 µg/mL for CuO-NPs and 1µg/mL for Ag-NPs. It was shown that in the case of Al_2O_3-NPs there were evidences of internalization of the particles after TEM images highlighting a clear visualization of disruptions along the digestive tract leading to a disturbed metabolism conducting to death (Pakrashi et al., 2013). Impacting these organisms may engender adverse effect for even higher organisms. Fish are predators for organisms like daphnids, and the effects of nanoparticles on fish have been reviewed in several scientific papers (Farré et al., 2009; R. D. Handy et al., 2008; Kahru and Dubourguier, 2010; Klaine et al., 2008; Menard et al., 2011). In the case of TiO_2-NPs, very high LC_{50} values are available, such as the 48-h LC_{50} for *P. promelas* with concentration up to 500 mg/L (Hall et al., 2009), the 96-h LC_{50} for *O. mykiss* with value up to 100 mg/L (Warheit et al., 2007). The nanoparticles will be able to cause adverse effects along the food chain but also for the offspring of organisms. It was shown that some NPs, such as silica, silver, and gold, were able to overpass the embryonic barriers and deeply penetrate in zebrafish embryos even at early

life stages (Browning et al., 2009; Fent et al., 2010; Lee et al., 2007). This penetration contributes to generate adverse developmental effects (Bacchetta et al., 2012). They also demonstrated that TiO_2-NPs can led to malformations generation in the *Xenopus laevis* development due to the large amount present in the digestive tract causing mechanical injuries. Thus, the impact of nanoparticles on bacterial organisms in natural waters may have severe implication for healthy functioning of the whole ecosystem.

C. Impact of nanoparticle/nanomaterial in soil

When the nanoparticles/nanomaterials are in WWTP, they are associate with the sludge. After the end of the purification process, the sludge can be dried and then used in landfill applications (Benn and Westerhoff, 2008). Thus, in the United States, 60% of the average 5.6 million tons of biosolids produced in WWTP is then land applied (Colman et al., 2013). Moreover, nanoparticles such as nanoscale zerovalent iron (nZVI) may be used in soil environmental remediation due to their increased reactivity and enhanced mobility for treatment of organochlorine pesticide and polychlorinated biphenyl contaminated environments (Zhang, 2003). According to Ge et al., 2011, there are two types of mechanisms that can cause nanoparticle adverse effect to soil. The first one is the direct toxicity leading to cells toxicity which had been made by scientists working on nanoparticles toxicity against pure culture (Ge et al., 2011). The second one is based on the indirect adverse effects of the nanoparticles, including impact on food web. The adverse food web effects are the same than in waters, as impacting the prey-predator relation between bacteria and protozoa (Mielke et al., 2013; Werlin et al., 2011). Direct effect was observed for Ag NPs who led to mechanical injury on an arctic soil microbial community (A. Kumar et al., 2011). These authors exposed an arctic soil to Ag-NPs over a 6 months period and observed that there was a decrease in the number of 16S rRNA gene sequences of the major bacterial orders suggesting a decreased of bacterial diversity. The same type of structural impact was observed with zero-valent iron nanoparticles (ZVIN) on soil microbial community (Fajardo et al., 2012). The decreased in bacterial diversity caused by the nanoparticles was also highlighted with gold nanorods who reduced soil microbial community structural diversity (Nogueira et al., 2012). Changes in the structural diversity may have implications on microbial processes. Modifications in diversity were also evidenced by exposing in microcosm a grassland soil to various concentrations of TiO_2-NPs and ZnO-NPs over 60 days. The T-RFLP analysis showed that bacterial community diversity and structure were impacted without modifying the soil characteristic. Exposure time explained 26.6% and exposure dose explained 24.7% of the community variations for

both nanoparticles (Ge et al., 2011). After 15 days, community dissimilarity increased in a linear way to increasing TiO_2 and ZnO-NPs and after 60 days, the response was exponential to increasing nanoparticles concentrations. They also observed that the drying process occurring in the soil could amplified the effects of the nanoparticles by concentrating them around bacterial cells. With the same nanoparticles, it was shown that soil protease, peroxidase and catalase activities were significantly inhibited (Du et al., 2011). The addition of copper (Cu-NPs) and ZnO-NPs were also shown to decrease the Shannon diversity index of soil and to drastically decreased the substrate utilization by the bacterial communities (22 in the control to 2 in the sample exposed to Cu-NPs and from 22 to 11 for the sample exposed to ZnO-NPs) (Collins et al., 2012). Moreover, the NZVI conducted to a significant decreased in the size of the bacterial community, a decline in the abundance of sensitive phylotypes and an increase in the abundance of tolerant and competitive phylotypes of bacteria (Tilston et al., 2013).

Coleman et al., in 2013, have shown that when biosolids were applied on soils, the soils were the major receptor for the sink Ag-NPs, with a recovery of nearly 99% (Colman et al., 2013). They also demonstrated that the nanoparticles associated with each other, because within 15 minutes, the Slurry+Ag-NPs were intimately associated with the TiO_2-NPs. The microbial activity changed with an increase in the flux of N_2O and a lower extracellular enzyme activity. The N_2O flux was 350% higher in the treated soil than in the control, which is a very dramatically problem for the human population because N_2O is a very important greenhouse gas and is also the dominant stratospheric ozone depleting substance (Colman et al., 2013). In addition, bacterial community structure analysis realized by DGGE profiles showed differences between iron oxide magnetic nanoparticles treated and untreated samples and reveal concentration-dependent effects of γ-Fe2O3 nanoparticles on bacterial community. Moreover, the effect of γ-Fe2O3 was greater than those of Fe3O4 on the bacterial community structure. Invertase and urease activities in γ-Fe2O3 (0.84 and 1.26 mg/g) treated samples were significantly increased and no impact on dehydrogenase and phosphatase activity were noticed (He et al., 2011). According to Kim et al., 2013, the measurement of enzyme activities can potentially be used to assess the impact of NPs on the soil. They reported that the enzyme activity of dehydrogenases can be used as an indicator of the oxidase capacity of soil microorganisms, and was highlighted to be very sensitive to CuO-NPs and ZnO-NPs.
When exposed to silver NPs, there was a rapid decline in microbial diversity (drastically decline in OTUs richness, from 142 to 101 OTUs in exposed sample than in control just after 1 day) but a recovery occurred within 50 days. This study suggest than even if Ag-NPs

transformation occurred they still had an impact on plants and microbes (Colman et al., 2013). In arctic soil experiments, Ag-NPs were demonstrated to be specifically toxic to Rhizobiales, nitrogen-fixing bacteria presenting a major role for plants (A. Kumar et al., 2011). This was also observed by Frenk et al., in 2013, by using a sandy-loam Bet-Dagan soil with 1% CuO-NPs (Frenk et al., 2013). This suggests that impacted bacterial communities living in soil may have adverse effects for other organisms depending on the soil compartment. Thus, exposing *Eisenia fetida* earthworm to neutron-activated nanoparticles for 7 days conduct to nanoparticles accumulation (Oughton et al., 2008). Worms exposed to 10 mg/L of TiO2-NPs byproducts also showed bioaccumulation and a significant decreased of phagocytosis (Bigorgne et al., 2011).

By willingly applying biosolids who eventually contains nanoparticles on soil, plants exposure may happen. When TiO_2 nanoparticles were present in the soil solution surrounding the primary root of *Zea mays* seeds, the leaf growth and the transpiration were rapidly inhibited. The external nanoparticles presence cause reduced water availability and a physical inhibition of the apoplastic flow through nanosized root cell wall pores. Thus TiO2-NPs treatment also reduced the hydraulic conductivity of cell wall and decreased the particle exclusion diameter of root cell wall pores from 6.6 to 3.0 nm (Asli and Neumann, 2009). It has been shown that translocation of TiO_2-NPs into plant shoots can be achieved. Titanium with a size of 5 nm have been found in leaves of *A. thaliana* (Kurepa et al., 2010), and titanium dioxide nanoparticles, over 100 nm, have been found able to be uptake and translocated into *Nicotiana tabacum* leaves (Ghosh et al., 2010). It also have been demonstrated that Ti originating from Ti-NPs accumulate in the plants (Jacob et al., 2013) but interaction with phosphorus can occurred. When especially looking at the TiO2-NPs, phosphate may adsorb to the surface of the particles and inhibited these effects or can be a competitor for receptor of the plants (Jacob et al., 2013). Concerning the uptake of titania and the interaction with phosphorus, it was observed that eutrophic growth conditions may decrease the Ti uptake and, at the opposite, oligotrophic conditions may conduct plants to be more vulnerable to nanoparticles (Jacob et al., 2013). For other plant organisms, metal oxide NPs were found to be toxic, even at very low concentrations. In the case of *C. sativus*, the IC_{50} values of CuO-NPs and ZnO-NPs were respectively 376 mg/L and 629 mg/L.

Thereby, nanoparticles can be found in the different environmental compartments and cause adverse effects for various trophic levels. Biomagnifications of nanoparticles along the trophic chains can lead to a spread of deleterious effects and can, therefore, lead to loss of

function performed by each biological compartment, from bacteria to higher organisms, engendering damages for ecosystems and for their good functioning.

Conclusion

Applications and products containing nanoparticles continue to expand rapidly, with actually over 1880 products commercially available and, according to estimation, a financial value for 2014 estimated at 2.6 trillion dollars (www.nanotechproject.org, 2014). The expanded commercialization and use of these nanoparticles-based products will conduct to increasing release in the environment engendering interrogation from the scientist community on the risk for the environment and the human health. Despite the intensive evaluation of the potential adverse effect of these nanoparticles, key data are lacking to fully understand the potential risk for the environment and, in the context of this review, for the bacteria.

With limiting nanoparticles characterization, variations in experimental methods (exposure time, medium, analytical techniques limitations, short-time response ...) and endpoints of interest, bacterial response to nanoparticles exposure are subjected to variations leading to antagonist results among research team. Depending on the chosen nanoparticles, toxicity against bacterial free living cells appears to require very low concentration during exposition but when cells are associated into biofilms, resistance increased. Moreover, in complex media under laboratory condition, aggregation and complexation of nanoparticles occurs, highly reducing interactions between bacteria and nanoparticles and decreasing their toxicity. Many studies have reported data examining the toxicity of nanoparticles to a variety of bacterial organisms without considering physico-chemical modifications that will undergo in the aquatic compartment. Thus, most of the data available have limited environmental relevance due to the realization of none environmentally relevant exposure scenarios. This led us to think, that under environmental condition, nanoparticles may exert less potential adverse effect. Thereby, there is an urgent need to assess more precisely the potential adverse effect by launching realistic ecological risks assessment experiments. Aggregation and complexation process may conduct nanoparticles to sediment in a faster way, presenting a higher risk for bacteria associated in biofilm. As we noticed that biofilm may be the first living compartment for nanoparticles accumulation, we conclude that evaluation of the potential adverse effect of nanoparticles against biofilm should be a priority.

Molecular techniques such as genomics, transcriptomics and proteomics are emerging techniques dealing with early response of bacterial cells to xenobiotic exposure and may be very useful tools to assess the adverse effects engender by the nanoparticles and potentially not detected by other techniques. Moreover, these techniques can be used to fill in the missing

information in order to characterize and to better assess the potential adverse effects of nanoparticles on bacteria.

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A l'origine de ces travaux de thèse, les résultats de toxicité des TiO_2-NPs vis-à-vis des microorganismes décrits dans la littérature étaient beaucoup plus sujet à questionnement que maintenant. En effet, très peu d'études s'intéressaient aux effets des TiO_2-NPs à l'obscurité afin de s'affranchir des effets photocatalytiques qui eux, sont bien connus. Les études visant à étudier ces nanoparticules sans illuminations étaient contradictoires. Ainsi, Huang et collaborateurs et Jiang et collaborateurs ne détectaient aucune toxicité des TiO_2-NPs à l'encontre de la souche bactérienne *E. coli* (Huang *et al.*, 2000 ; Jiang *et al.*, 2009). A l'opposé de ces observations, Hu et collaborateurs obtenaient une dose létale avec 50% d'effet en utilisant une concentration de 1104,8 mg/L en nanoparticules alors que Simon-Deckers et collaborateurs observaient une DL_{50} de 100 mg/L (Hu *et al.*, 2009 ; Simon-Deckers *et al.*, 2009). Il a ensuite été admis que ces contradictions découlaient principalement des variations pouvant exister entre les protocoles expérimentaux utilisés par chaque équipe, que ce soit au niveau des propriétés physico-chimiques des nanoparticules (taille, charge de surface, pourcentage anatase/rutile), de leurs préparations (mise en suspension par sonication ou non, filtration, centrifugation), de la préparation cellulaire (milieu de croissance, phase de croissance, lavages éventuels), ou encore des conditions d'exposition (temps, milieu d'exposition, concentration en sel, en nanoparticules, nombre de cellules, agitation ou non) et du type de mesure de toxicité (BackLight, courbes de croissance, CFU). Cette thématique de recherche étant une thématique émergente, un certain nombre de calibrations et un effort de caractérisations plus important devaient être fournis. Dans cette optique, caractériser au mieux les nanoparticules utilisées en déterminant la charge de surface en fonction du pH, des ions présents, le point isoélectrique, l'état d'agrégation, la morphologie des agrégats, l'effet de la sonication, a été une priorité dans notre approche. En connaissant ces données, il a alors été possible de déterminer quels paramètres physico-chimiques étaient importants dans l'expression de la toxicité des nanoparticules vis-à-vis des bactéries. Par la suite, nous avons tenté d'appliquer nos découvertes à différents niveaux d'évaluation de l'impact du nano TiO_2 sur les bactéries. Cette première partie montre à quel point les avancées scientifiques dans l'étude des nanoparticules sont rapides et à quel point les réponses fournies sont précieuses pour comprendre les phénomènes de toxicité éventuels.

Chapitre II : Rôle des interactions de phases dans la toxicité des nanoparticules de dioxyde de titane sur les bactéries

I. Rôle des interactions électrostatiques dans la toxicité des nanoparticules de dioxyde de titane vis-à-vis d'*Escherichia coli.*

Le titane est un métal très abondant sur terre et dont les oxydes métalliques sont très utilisés, aussi bien dans des procédés industriels que dans l'agroalimentaire. Ces formes dites « bulk » tendent à être remplacées par leurs formes nanoparticulaires avec pour objectif une augmentation de l'efficacité des rendements de réactions et opérations au sein desquels interviennent ces oxydes. De fait, les TiO_2-NPs sont de plus en plus produites, utilisées et se retrouvent à terme dans l'environnement, en particulier dans le compartiment aquatique, réceptacle de toutes les pollutions terrestres. L'augmentation de ces rejets a conduit la communauté scientifique à s'interroger fortement sur l'impact éventuel des nanoparticules ou dérivés de nanomatériaux sur l'environnement.

En tant qu'acteurs majeurs des cycles biogéochimiques, les microorganismes procaryotes, et en raison de leur présence dans tous les milieux, il est raisonnable de les considérer en tant que cible d'un éventuel impact de TiO_2-NP. L'objectif de ces travaux est d'évaluer la toxicité de ces nanoparticules en fonction des interactions électrostatiques qui peuvent s'opérer entre bactéries et nanoparticules. Cette étude a mis en évidence que lorsque le milieu d'exposition des microorganismes aux nanoparticules présentait une faible force ionique, la viabilité cellulaire de la souche bactérienne *Escherichia coli* était beaucoup plus affectée à pH 5,5 qu'à pH 7,0 ou 9,5. A pH 5,5, les nanoparticules sont chargées positivement et interagissent fortement avec les bactéries qui, elles, sont chargées négativement. Cela conduit également à une forte accumulation et adsorption des nanoparticules à la surface des microorganismes. Ce même phénomène est observé dans une moindre mesure à pH 7,0 du fait de la charge neutre des nanoparticules de titane. A l'inverse, lorsque le pH est de 9,5, les deux éléments sont chargés négativement ce qui conduit à des phénomènes de répulsion. Cette étude a également mis en évidence la modulation des effets toxiques des nanoparticules lorsque différents électrolytes sont utilisés. Ainsi, l'utilisation de NaCl, $CaCl_2$, Na_2SO_4 conduit à une réduction graduelle des effets toxique à pH 5,5 alors que cette toxicité est augmentée à pH 9,5. Ces variations et modulations sont fortement corrélées aux modulations des charges électrostatiques des microorganismes et des nanoparticules.

Ces travaux ont permis d'acquérir des connaissances fondamentales et des pistes de réflexion sur les interactions modulant les effets toxiques des nanoparticules de titane sur la souche modèle *Escherichia coli* dans un contexte d'évaluation du risque environnemental.

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Role of electrostatic interactions in the toxicity of titanium dioxide nanoparticles toward *Escherichia coli*

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ABSTRACT

The increasing production and use of titanium dioxide nanoparticles (NP-TiO_2) has led to concerns about their possible impact on the environment. Bacteria play crucial roles in ecosystem processes and may be subject to the toxicity of these nanoparticles. In this study, we showed that at low ionic strength, the cell viability of *Escherichia coli* was more severely affected at pH 5.5 than at pH 7.0 and pH 9.5. At pH 5.5, nanoparticles (positively charged) strongly interacted with the bacterial cells (negatively charged) and accumulated on their surfaces. This phenomenon was observed in a much lower degree at pH 7.0 (NP-TiO_2 neutrally charged and cells negatively charged) and pH 9.5 (both NP-TiO_2 and cells negatively charged). It was also shown that the addition of electrolytes (NaCl, $CaCl_2$, Na_2SO_4) resulted in a gradual reduction of the NP-TiO_2 toxicity at pH 5.5 and an increase in this toxicity at pH 9.5, which was closely related to the reduction of the NP-TiO_2 and bacterial cell electrostatic charges.
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1. Introduction

Titanium dioxide is a naturally occurring mineral that primarily exists in three crystalline forms, known as rutile, anatase, and brookite. TiO_2 has been used commercially since the early 1900s. Because of its whiteness, brightness and opacity, TiO_2 is currently the most widely used white pigment in the world, with a total annual production of 4 million tons [1]. It is used in many consumer products including paints, plastics, inks, papers, coatings, ceramics, and textiles. In addition, it has been approved by the U.S. Food and Drug Administration (FDA) for use in food, drugs, cosmetics, and food contact materials.

In the past two decades, TiO_2 has also received considerable attention owing to its potential for use in heterogeneous photocatalysis. When illuminated under UV light, TiO_2 decomposes organic compounds by participating in a series of oxidation reactions. Specifically, it generates electrons and superoxide ions ($O_2^{\bullet -}$) in the conduction band and positive holes and hydroxyl radicals ($^{\bullet}OH$) in the valence band. Owing to its high reactivity, chemical stability, low cost and non-toxicity, TiO_2 has become the most widely used semiconductor for numerous technological processes, leading to the development of a wide range of sterilization, sanitation, and remediation applications in water, air and even in the solid phase.

The massive production and use of titanium dioxide inevitably leads to its release into the environment and raises the question of its toxicity. To date, the majority of TiO_2 used industrially is not nanoscale and considered chemically inert and safe for human health and ecosystems. However, with the development of nanotechnology, TiO_2 nanoparticles have been produced more extensively and found wider applications owing to their unique physicochemical properties when compared to the bulk. The share of TiO_2 nanoparticles (NP-TiO_2) in the total U.S. TiO_2 production was estimated to be 2.5% in 2009 and 10% by 2015, and it is estimated that the industry will be completely converted by 2025 [2]. The booming demand for NP-TiO_2 has spurred significant public alarm regarding its possible impact on the environment. Indeed, nanoparticles exhibit distinct properties when compared to their bulk counterparts, which can make them more reactive and therefore more likely to produce adverse effects.

Industrial products and wastes tend to end up in waterways despite safeguards. Consequently, as nanotechnology industries start to come on line with larger scale production, it is inevitable that nanoscale products and by-products will enter the aquatic

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environment and affect organisms living in it [3,4]. More than one million microorganisms per milliliter inhabit the aquatic environment and they are vital for efficient functioning of any ecosystem. These organisms constitute the primary agents of early transformation of organic matter and regeneration of nutrients, and also serve as food for higher trophic levels. Accordingly, any factor that can affect the microbial community structures are of great concern. Surprisingly, relatively few studies have dealt with the direct effects of NP-TiO_2 on bacteria and most of the literature to date has focused on its effects under UV light within the framework of photocatalytic applications [5,6]. The toxicological effects of TiO_2 nanoparticles toward bacteria under dark condition are still the subject of debate. For example, on the laboratory model strain *Escherichia coli*, some authors found that the NP-TiO_2 were not toxic when compared to other metal oxide nanoparticles [7,8], while others found them to be toxic, but at EC_{50} values ranging from 100 mg/l to 1104.8 mg/l [9,10]. These contradictory results may be explained in part by multiple causal factors, including the intrinsic characteristics of TiO_2 nanoparticles (size, shape, surface area, physical and chemical properties, dispersion state) and *E. coli* cells (physiological state, surface properties) used in these studies, exposure conditions for toxicity assessment (pH, ionic strength, temperature, duration, nanoparticle and cell concentrations) and even the method used to assess the toxicity (UFC, growth inhibition, viability assays).

Nanoparticles can cause toxicity toward bacteria via several mechanisms that can be related to their sorption onto the cell wall (direct toxicity), such as interruption of transmembrane electron transfer, modification of the membrane potential, physical damage resulting in leakage of the cell contents, or, depending on the nature of the nanoparticle, the ability to generate secondary products such as reactive oxygen species or dissolved toxic ions (indirect toxicity) [11]. In the case of scarcely water-soluble nanoparticles, such as TiO_2 nanoparticles, interfacial electrostatic interactions and physico-chemical parameters of the medium that can affect them (pH, ionic strength, electrolyte composition, temperature), may play important roles in determining their toxicity. The paramount role of electrostatic interaction was previously shown in the initial mechanism by which antimicrobial cationic peptides target the negatively charged lipid membranes of bacteria [12,13]. In such cases, the relationship between electrostatic interaction and antimicrobial activity has been established by the strong correlations between the surface charge of the peptide and the bacteria, the membrane binding activity, and the biocidal effect. Increasing the pH of the medium or the ionic strength has been shown to reduce the activity of most antimicrobial peptides and partially contribute to the selectivity of the antimicrobial peptides by weakening the electrostatic interactions required for the initial interaction.

In the present study, we investigated the role that electrostatic interactions play in the toxicological effects of TiO_2 nanoparticles toward *E. coli*. Relationships were established between the physicochemical parameters (pH, ionic strength and electrolyte composition), loss of bacterial viability, charge surface of both NP-TiO_2 and bacteria, and NP-TiO_2 adhesion onto the cell wall.

2. Materials and methods

2.1. Preparation of the NP-TiO_2 stock suspension

TiO_2 nanopowder Aeroxide P25 was provided by Evonik Degussa GmbH (Frankfurt, Germany). These nanoparticles are widely used as photocatalysts and have been well-investigated. Aeroxide P25 is an 80:20 anatase:rutile mixture with a primary particle size of about 25 nm and specific surface area of $50 \pm 15\ m^2/g$. The suspension was prepared by dispersing 100 mg of NP-TiO_2 in 10 ml sterile ultrapure water (milli-Q water, 18.2 MΩ cm). The resultant suspension was then probe-sonicated (Sonics Vibra-cell 750 W, Sonics & Materials, frequency 20 kHz, 3 mm micro tip, amplitude 40%) for 30 min at 4 °C to homogenize and break apart the larger agglomerates.

2.2. NP-TiO_2 surface charges and aggregation

The NP-TiO_2 stock suspension (100 μl) was suspended in 10 ml of sterile ultrapure water (milli-Q water, 18.2 MΩ cm) supplemented with various concentrations of NaCl, $CaCl_2$, or Na_2SO_4. When necessary, the natural pH (pH 5.5) was modified by adding HCl or NaOH. Electrophoretic mobility and hydrodynamic diameters were measured on a Zetasizer NanoZS (Malvern Instruments, UK).

2.3. Preparation of the bacterial cell suspension

E. coli MG1655 was used as a model bacterium. Cells were inoculated in 100 ml of a Luria-Bertani broth and then incubated at 37 °C and 150 rpm until exponential phase of growth was attained ($OD_{600\,nm}$: 0.5). The cells were subsequently harvested by centrifugation at $8000 \times g$ for 10 min, after which they were washed twice with sterile milli-Q water. The bacterial suspension obtained was adjusted to an $OD_{600\,nm}$ of 4.0 (10^9 cells/ml) and used immediately for assays.

2.4. Bacterial surface charges and aggregation

A bacterial cell suspension (500 μl) was suspended in 50 ml of sterile milli-Q water supplemented with various concentrations of NaCl, $CaCl_2$, or Na_2SO_4. When necessary, the natural pH (pH 5.5) was modified by adding HCl or NaOH. Bacterial electrophoretic mobility measurements were conducted using a Zetaphoremeter IV (CAD Instrumentations, Les Essarts le Roi, France) in a quartz suprasil cell at 24 °C from the reflection of a laser beam by bacteria tracked with a charge-coupled device camera. Image analysis software was used to process recorded images in real time to calculate the electrophoretic mobilities from the displacement (migration motion) of the bacteria subjected to a constant direct-current electric field (800 V/m). Different cycles were recorded to yield 100 measurements of the bacterial mobility.

Bacterial aggregation was assessed by measuring the change in light transmission ($OD_{600\,nm}$) through the bacterial suspension. The extent of aggregation was determined based on the difference between the absorbance at time zero and after 24 h.

2.5. Toxicity assessment

Experiments were conducted in 100 ml flasks containing 50 ml of sterile milli-Q water (natural pH 5.5 or NaOH-modified pH 9.5) supplemented with various concentrations of NaCl, $CaCl_2$, or Na_2SO_4. Briefly, 500 μl of the *E. coli* bacterial suspension and 500 μl of the NP-TiO2 stock suspension (or mQ water for the control) were prepared as previously described and then added to the electrolyte solutions to obtain final concentrations of 10^7 cells/ml and 100 mg/l of TiO_2 nanoparticles. The flasks were then incubated at room temperature on a dark rotary shaker (150 rpm). After 20 h of exposure, the toxicity of NP-TiO_2 toward *E. coli* was investigated using the Drop Count Method, growth curve inhibition, and the BacLight bacterial viability kit.

For the Drop Count Method, nine drops (20 μl per drop) were used for each dilution, transferred onto solid Luria Bertani (LB) agar medium and incubated at 37 °C for 24 h. The percentage of viable cells was determined by comparing the number of colony forming units (CFU) produced by the exposed and unexposed samples.

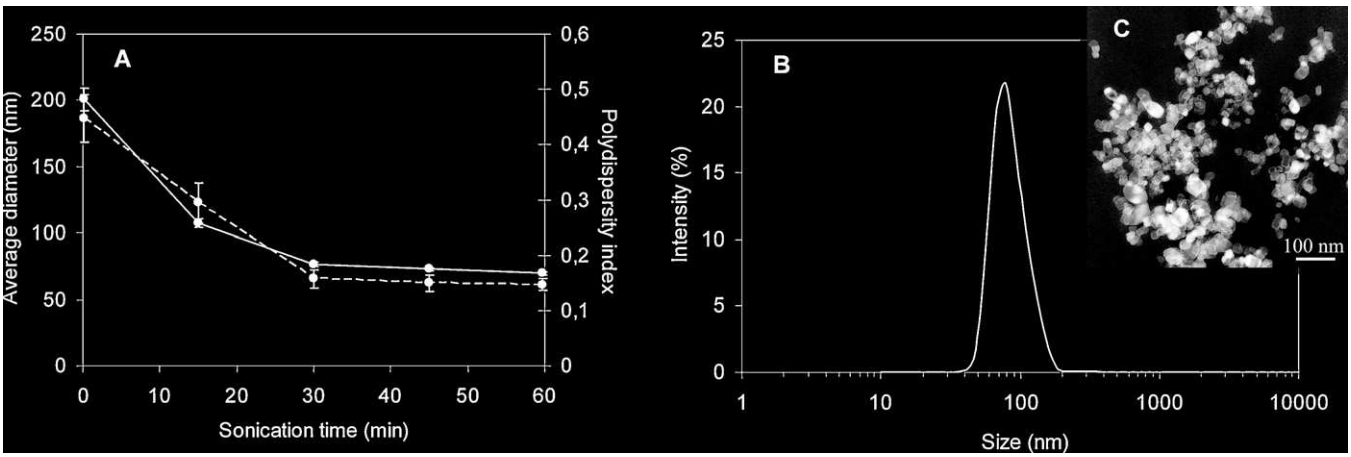


Fig. 1. Physical characterization of the NP-TiO_2 stock suspension. (A) Hydrodynamic diameter and polydispersity index of the NP-TiO2 after various sonication times in mQ water. (B) Particle size distribution (B) and transmission electron microscopy image (C) of the NP-TiO_2 after 30 min of sonication in mQ water.

For the bacterial growth inhibition in liquid medium, 500 μl of exposed or unexposed cells were transferred to 250 ml Erlenmeyer flasks containing 50 ml of LB medium. The flasks were maintained at 37 °C on a rotary shaker (150 rpm) and growth was monitored by measuring the $OD_{600\,nm}$ every hour. The percentage of viable cells was determined by comparing the absorbances obtained from exposed and unexposed samples in the exponential phase of growth.

The Live/Dead BacLight bacterial viability kit (Molecular Probes, Eugene, OR.) was used according to the manufacturer's instructions. This kit employs SYTO 9 and propidium iodide to differentiate cells with intact (live organisms – stained in green) and compromised (dead organisms – stained in red) membranes. BacLight-stained samples were filtered onto 0.2-μm-pore-size black polycarbonate filters and cells were viewed using an epifluorescence microscope (Olympus model BX41) with the appropriate filter cube. The percentage of viable cells was determined by comparing the number of red and green cells obtained with the exposed and unexposed samples.

For these three methods, each set of experiments was repeated at least three times to ensure the data reproducibility. Each time, the percentage of viable cells was determined by comparing samples at the same pH or electrolyte concentration.

2.6. Scanning electron microscopy

Bacterial cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) overnight at 4 °C. The cells were then filtered through 0.2 μm polycarbonate Nucleopore filters, after which they were washed with 0.2 M cacodylate buffer (pH 7.2) three times for 5 min each. The cells were subsequently dehydrated through a graded series of ethanol solutions (30%, 50%, 70%, 90% and 100%) for 5 min and then desiccated by successive bathing in hexamethyldisilazane-ethanol (HMDS diluted at 75%, 50% and 25%) for 5 min and one bath in pure HMDS for 10 min. Next, the filters were then placed under vacuum until the HMDS completely evaporation. Finally, the cells were observed with a HITACHI S-4800 field emission scanning electron microscope at the Service Commun de Microscopies Electroniques et de Microanalyses (SCMEM, Nancy, France).

2.7. Statistical analysis

All results presented are based on at least three repetitions. Statistical evaluation was conducted using the STATISTICA software. One-way Kruskal–Wallis ANOVA was used, followed by Mann–Whitney *u*-test. A $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Size characterization of the NP-TiO_2 stock suspension

Aeroxide P25 powder was suspended in milli-Q water and probe-sonicated as described in Section 2. When sonication was applied, DLS measurements revealed that the average hydrodynamic diameter of the dispersion, as the polydispersity index, decreased significantly, and became relatively constant after 30 min (Fig. 1A). The hydrodynamic diameter obtained (30–220 nm, average size: 78 nm) was larger than the primary particle sizes obtained by TEM (20–80 nm, average size: 23.8 nm) (Fig. 1B) and given by the manufacturer (average size: 24 nm). These findings suggest that the NP-TiO_2 stock suspension is largely composed of small aggregates that cannot be readily broken up. Similar observations were made by authors using Aeroxide P25 (Degussa) as a model [8,10,14,15]. Thus prepared, the stock suspension is stable and no change in the size distribution was observed over one month.

3.2. Effect of pH on nanoparticle toxicity

The toxicological effects of NP-TiO_2 toward *E. coli* were investigated under three pH conditions (pH 5.5, 7.0 and 9.5) by the following conventional methods: direct plating on LB medium (Colony Forming Unit/ml), analysis of growth curve inhibition after exposure, and a fluorescence-based assay using the Live/Dead BacLight Kit. As shown in Fig. 2A, similar results were obtained using all three of these methods. Furthermore, NP-TiO_2 was found to be toxic toward *E. coli* at acidic pH (viable cells: 0.1–1%), less toxic at pH 7.0 (viable cells: 60–70%) and not toxic at basic pH (viable cells: 97–100%) after 20 h of exposure.

To assess our initial hypothesis that electrostatic interactions play an essential role in determining the toxicity of nanoparticles toward bacteria, electrophoretic mobility measurements were conducted on both NP-TiO_2 and *E. coli* cells at various pH values (Fig. 2B). The isoelectric point (IEP) of the TiO_2 Aeroxide P25 was found to be located around pH 7, which is consistent with previously reported results [10,16]. At pH values below 5.5 and above 9.5, nanoparticles exhibit net positive and negative charges, respectively, resulting in good stability of the suspension owing to high electrostatic repulsions between nanoparticles (Fig. 2C).

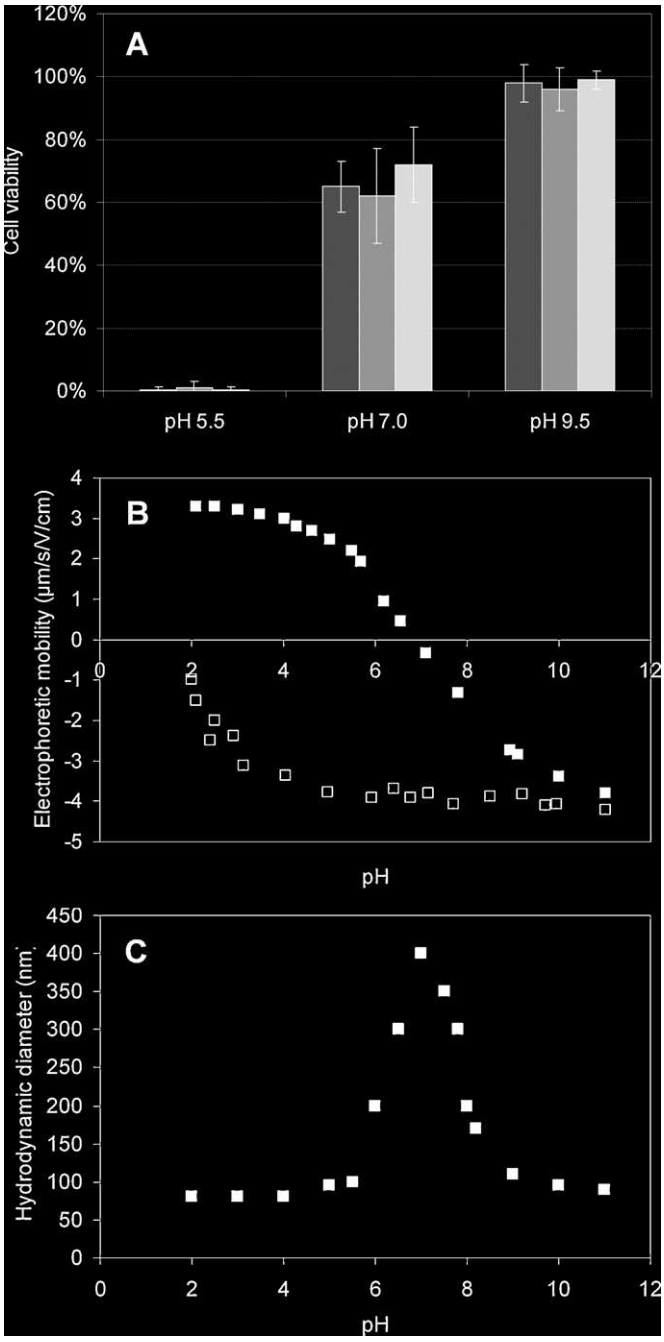


Fig. 2. *Escherichia coli* cell viability after 20 h of exposure to NP-TiO_2 at pH 5.5, 7.0 and 9.5 (A). Cell viability was assessed using the Drop Count Method (clear gray columns), growth curve inhibition (dark gray columns) and Live/Dead Baclight assay (black columns) as described in Section 2. The results were expressed as a percentage by comparing NP-TiO_2 exposed and unexposed cells subjected to the same exposure conditions. (B) The electrophoretic mobility of both NP-TiO_2 (black squares) and *Escherichia coli* cells (open squares) at various pH values in mQ water supplemented with 0.1 mM NaCl. (C) The hydrodynamic diameter of NP-TiO_2 after 5 min in mQ water supplemented with 0.1 mM NaCl.

Conversely, as expected from the DLVO colloidal stability theory [17,18], around the IEP, attractive van der Waals forces dominate over the repulsive electrostatic forces, causing rapid nanoparticle aggregation. In less than five minutes, the average size of the aggregates reached ~400 nm. For the *E. coli* MG1655 cells, the IEP was found around pH 2. Like most of bacteria, mobility does not change much with pH in the range of 4–10 (~−3.8 μm/s/V/cm) due to the constancy of the degree of ionization of the surface groups (carboxylic and phosphate) on the cell wall [19–21].

These measurements revealed a close correlation between NP-TiO_2 toxicity and the charge of the nanoparticle surface, which determined the nature and strength of their electrostatic interaction with anionic components of the bacterial cell wall. At pH 5.5, where NP-TiO_2 showed toxicity toward *E. coli*, fully dispersed positively charged nanoparticles (+2.2 μm/s/V/cm) were attracted by the bacterial surface. Indeed, these particles stick and accumulate on the membrane as observed on the SEM micrograph (Fig. 3A). Conversely, at pH 9.5, where no NP-TiO_2 toxicity was observed, nanoparticles are negatively charged (−3.2 μm/s/V/cm) and therefore repelled by the bacterial surface. SEM observations revealed that under this pH condition, NP-TiO_2 cannot adsorb to the bacterial cell wall (Fig. 3B). At pH equal to the nanoparticle IEP, where low, but significant NP-TiO_2 toxicity was observed, nanoparticles strongly aggregate and due to their overall neutral charge slightly interact with the bacterial surface components likely through other interfacial forces (e.g. attractive van der Waals forces) (Fig. 3C). These results are in good agreement with those obtained by several researchers investigating scarcely water-soluble nanoparticles, suggesting that contact between nanoparticles and bacteria is necessary for manifestation of the biocidal activity [22–24]. This contact is strongly dependent on the interfacial electrostatic interactions between nanoparticles and bacterial cells (NP-cell interactions), and between nanoparticles (NP-NP interactions), which, in turn, are directly related to the pH of the medium.

3.3. Effects of electrolytes on nanoparticle toxicity

The effects of various electrolytes (NaCl, $CaCl_2$, Na_2SO_4) on the NP-TiO_2 toxicity toward *E. coli* were investigated at pH 5.5 and pH 9.5 by direct plating on LB medium and growth curve inhibition, as described in Section 2. Incoherent data were obtained with the Baclight kit, as a result of a priori interference between the fluorochomes and the electrolytes at high ionic strength; therefore, these data are not shown. The results presented in Fig. 4 revealed that both toxicity assessment methods showed the same tendency. At pH 5.5, increases in ionic strength induced a decrease in the NP-TiO_2 toxicity (Fig. 4A–C). The cell viability, which was close to 0.1–1% after 20 h of exposure to nanoparticles without electrolytes, reached 20–30% in 100 mM NaCl and 90–100% in 50 mM $CaCl_2$ or Na_2SO_4. Conversely, an increase the ionic strength at pH 9.5 induced an increase of the NP-TiO_2 toxicity (Fig. 4D–F). The cell viability, which was initially close to 90-100% in the absence of electrolytes, was only 20–30% in 100 mM NaCl or 50 mM Na_2SO_4, and was 65–85% in 50 mM $CaCl_2$. Regardless of the fact that ionic strength influences the nanoparticle toxicity, these results demonstrated that the magnitude of the effect is strongly dependent on the electrolyte nature.

This clear relationship between the electrolyte concentration and composition, and the toxicity of NP-TiO_2 toward *E. coli*, strongly supports a direct influence of surface electrostatics on the nanoparticle/nanoparticle and nanoparticle/bacteria interactions. Therefore electrophoretic mobility measurements (Fig. 5) and aggregation/coagulation experiments (Fig. 6) were conducted on both nanoparticles and bacteria, at pH 5.5 and 9.5.

3.3.1. Effects on the nanoparticle–nanoparticle interactions

For charged particles such as NP-TiO_2, the stability of the colloidal suspension is described by the DLVO theory [17,18]. The electrostatic forces (which are related to the particle charge and give rise to the electrophoretic mobility) are repulsive for same-charged particles, and opposed to the attractive van der Waals forces. The pH, electrolyte concentration, and ion valence, determine the value of the electrostatic forces, and hence the

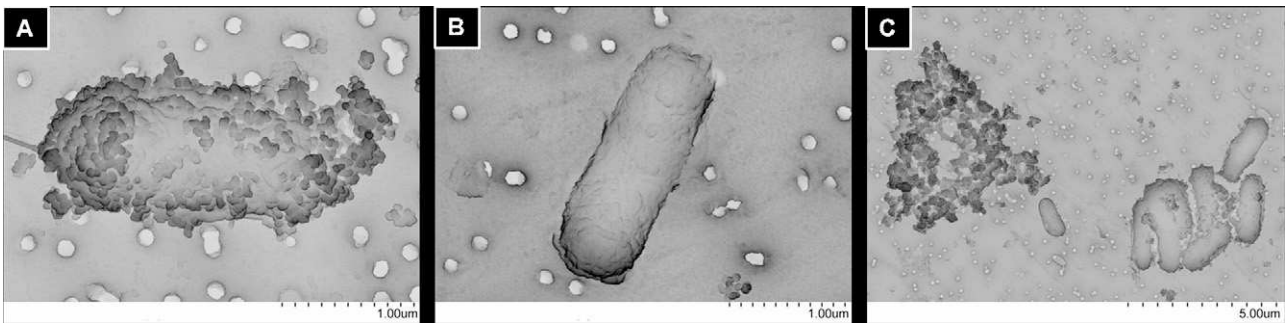


Fig. 3. Scanning electron microscopy image of *Escherichia coli* after 20 h of exposure to 100 mg/l NP-TiO_2 in water at pH 5.5 (A) and 9.5 (B) and 7.0 (C); followed by filtration on polycarbonate filters (0.22 μm, Millipore) and dehydration. Images show bacteria covered by TiO_2-NP at pH 5.5 (distribution of Ti confirmed by EDS spectra) but not at pH 9.5. They also show the strong aggregation of NP-TiO_2 at pH 7.0, which correspond to the nanoparticle IEP.

attractive-repulsive force balance. If electrostatic repulsion dominates, the particles repel each other and the suspension is dispersed. Reversely, weak electrostatic repulsion allows the attractive van der Waals forces to dominate and the particles experience mutual attraction and coagulate. At pH 5.5, the NP-TiO_2 are positively charged (Fig. 2B). Increasing the NaCl concentration results in screening of the positive surface charges by the Cl^- counterions and in decreasing of the electrostatic potential (Fig. 5A). The divalent anion SO_4^{2-} is much more effective than the monovalent Cl^- for screening positive charges of the nanoparticles (Fig. 5A vs. C). Thus, the NP-TiO_2 electrophoretic mobility decreases to zero with only 5 mM Na_2SO_4 (+0.17 μm/s/V/cm) when compared to a concentration of 100 mM with NaCl (+0.23 μm/s/V/cm), and it is ultimately reversed (NP overcharging) at 50 mM Na_2SO_4 (−1.01 μm/s/V/cm). As a result, nanoparticles aggregate into clusters that quickly exceed the micron size at these electrolyte concentrations (Fig. 6A vs. C). The results also showed that the valence of the counterions has a significant effect on the surface potential of the nanoparticles. Thus, charge screening by Cl^- is much weaker when adding 50 mM $CaCl_2$ (+1.71 μm/s/V/cm) to the medium instead of 100 mM NaCl (+0.23 μm/s/V/cm) (Fig. 5A vs. B). A further explanation for this phenomenon is the formation of stable $CaCl^+$ complexes [25] that decrease the number of free Cl^- ions in the medium that are able to interact with nanoparticles.

At pH 9.5, where the NP-TiO_2 is negatively charged (Fig. 2B), the behavior is symmetrical according to pH 5.5. Divalent cations Ca^{2+} are more effective at screening the negative charges of the nanoparticles than Na^+ at the same Cl^- counterion concentration (Fig. 5D vs. E). It is also interesting to note that no significant difference has been shown when using SO_4^{2-} instead of Cl^- with Na^+ as a counterion (Fig. 5D vs. F). Thus, the electrophoretic mobility of NP-TiO_2 is close to zero when there is only 5 mM $CaCl_2$ (−0.09 μm/s/V/cm), but is still highly negative in 100 mM NaCl (−1.86 μm/s/V/cm) or 50 mM Na_2SO_4 (−2.07 μm/s/V/cm). As previously observed with Na_2SO_4 at pH 5.5, increasing the $CaCl_2$ concentration to 50 mM results in nanoparticle overcharging (+1.18 μm/s/V/cm) (Fig. 5E). Fig. 6 indicates that a significant decrease in the NP-TiO_2 surface potential induces their rapid aggregation, which is as expected from the theory (Fig. 6D–F).

3.3.2. *Effects on the nanoparticle–cell interactions*

The electrophoretic mobility of *E. coli* is also affected by the pH, ionic strength and valence of the ions (Fig. 5). However, as demonstrated by Duval and Gaboriaud [26] in their review paper,

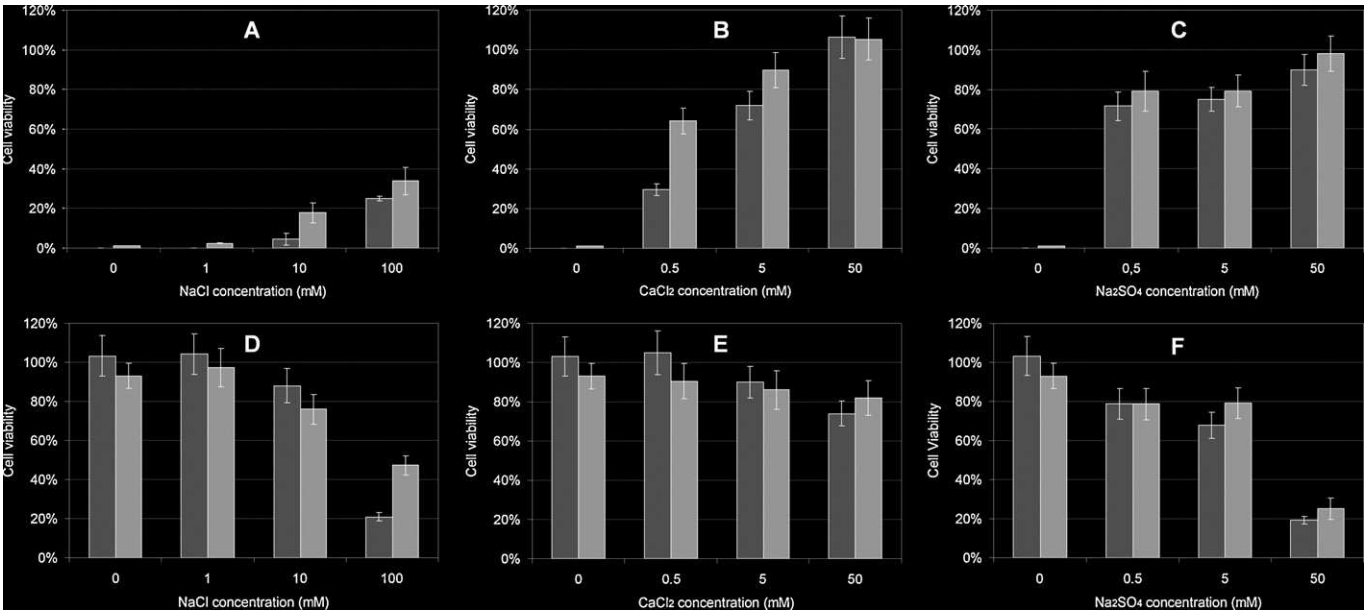


Fig. 4. *Escherichia coli* cell viability after 20 h of exposure to 100 mg/l NP-TiO_2 as a function of (A) NaCl, (B) $CaCl_2$, (C) Na_2SO_4 at pH 5.5 and (D) NaCl, (E) $CaCl_2$, (F) Na_2SO_4 at pH 9.5. Cell viability was assessed using the Drop Count Method (clear gray columns) and the growth curve inhibition (dark gray columns) as described in Section 2. Results are expressed as a percentage based on comparison of the NP-TiO_2 exposed and unexposed cells subjected to the same conditions.

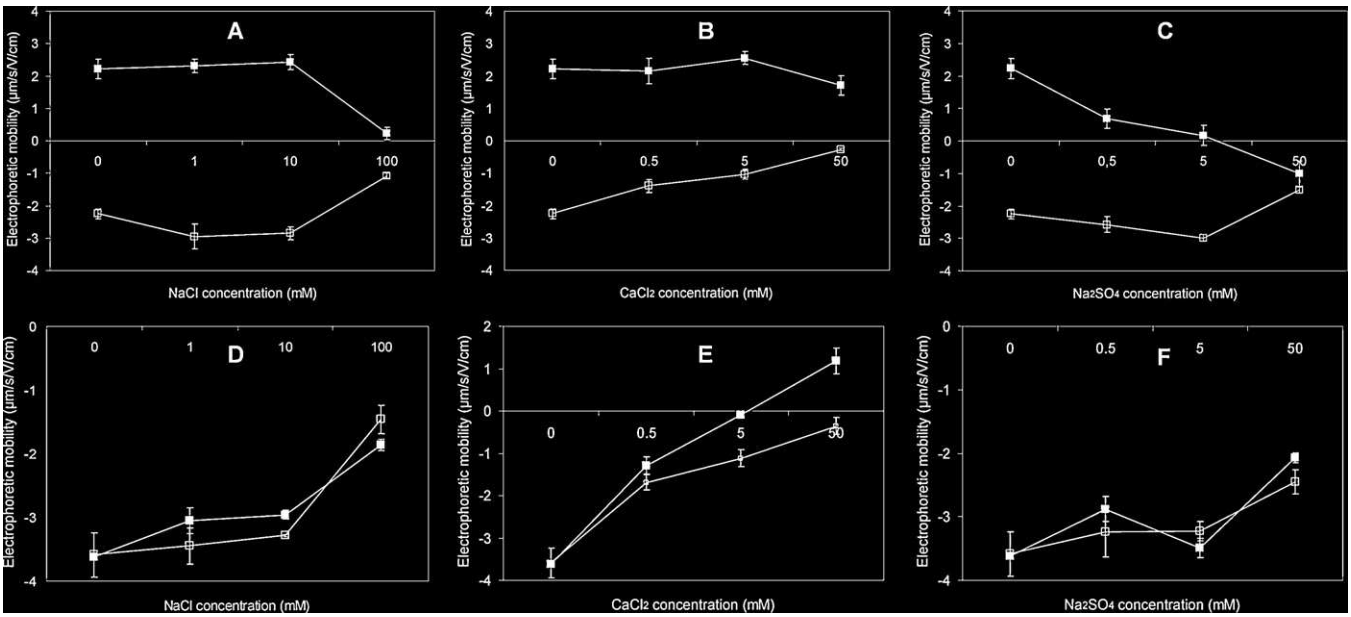


Fig. 5. The electrophoretic mobility of both NP-TiO_2 (black squares) and *Escherichia coli* cells (open squares) as a function of (A) NaCl, (B) $CaCl_2$, (C) Na_2SO_4 at pH 5.5 and (D) NaCl, (E) $CaCl_2$, (F) Na_2SO_4 at pH 9.5.

the electrophoretic mobility of microbial particles cannot be rigorously explained by the approaches classically followed for hard and impermeable particles. Indeed, the outmost bacterial surface is composed of complex structural organizations such as pili, fimbriae, fibrils, polymeric fringes, lipopolysaccharides, peptidoglycan, that can extend hundreds of nanometers toward the surrounding solution. The diffuse soft particle electrokinetic formalism (DSPE) developed by Duval and Ohshima [27] describes the electrophoretic mobility in terms of volume charge density, hydrodynamic properties and diffuse character of the permeable charged soft surface layer at the periphery of the particle. Indeed, in terms of the so-called soft layer model, electrophoretic mobility is basically described as originating from the weighted average of potentials over the surface charge layer, and from the volume density of membrane-fixed charges [28]. At high ionic strength, where the potentials are low due to the charge screening effect of the electrolytes, the first term decreases so that the mobility is determined mainly by the second term, meaning that a bacterial cell can exhibit a non-zero mobility.

At pH 5.5, a significant decrease in the *E. coli* mobility was observed for 0.5 mM $CaCl_2$ (−1.39 µm/s/V/cm) compared to a concentration of 100 mM NaCl (−1.08 µm/s/V/cm) (Fig. 5A vs. B). Similar observations were observed at pH 9.5 with the addition of 0.5 mM $CaCl_2$ (−1.69 µm/s/V/cm) or 100 mM NaCl (−1.46 µm/s/V/cm) to the medium (Fig. 5D vs. E). It is interesting to note that, at high $CaCl_2$ concentrations, the electrophoretic mobility asymptotically reached a nonzero plateau value at both pH values (∼−0.3 µm/s/V/cm), which was characteristic of the presence

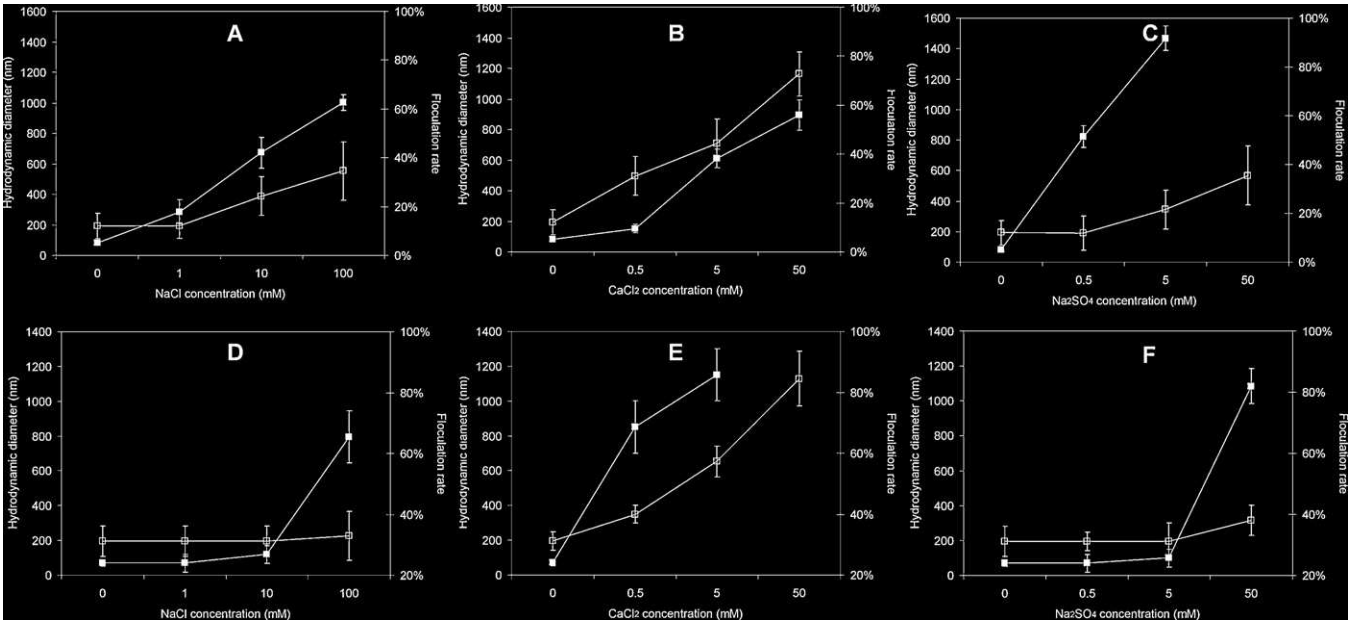


Fig. 6. Hydrodynamic diameter of NP-TiO_2 (black squares) and flocculation rate of *Escherichia coli* cells (open squares) after 5 min and 20 h in mQ water supplemented with (A) NaCl, (B) $CaCl_2$, (C) Na_2SO_4 at pH 5.5 and (D) NaCl, (E) $CaCl_2$, (F) Na_2SO_4 at pH 9.5.

of a soft, hydrodynamically permeable layer around the bacterial cells [29]. As observed for hard (impermeable) particles, adding electrolytes to the medium induces lower electrostatic repulsion between bacterial cells, leading to their aggregation and flocculation (Fig. 6). As expected, Ca^{2+} is more effective than Na^{+} at screening the negative charges of bacteria. Indeed, after 24 h, 75% of the cells were flocculated with 50 mM $CaCl_2$ at pH 5.5 (Fig. 6B), while only 35% were flocculated with 100 mM NaCl (Fig. 6A) or 50 mM Na_2SO_4 (Fig. 6C). At pH 9.5, 85% of cells were flocculated with 50 mM $CaCl_2$ (Fig. 6E) against 34% with 100 mM NaCl (Fig. 6D) and 39% were flocculated with 50 mM Na_2SO_4 (Fig. 6F).

The results presented above show that both the ionic strength and nature of the electrolytes may affect the interfacial electrostatic forces acting between charged particles, e.g. reduction of the attractive electrostatic interactions between oppositely charged surfaces and reduction of the repulsive electrostatic interactions between similarly charged surfaces. Based on the hypothesis that the toxicity of nanoparticles toward bacteria strongly depends on interactions between each other, this phenomenon can broadly explain variations in the magnitude of the NP-TiO_2 toxicity observed with the addition of electrolytes to the medium. In a medium with a $pH < IEP_{NP}$, adding electrolytes induces a synergistic effect on the NP-TiO_2 toxicity by reducing the NP-cell interactions and promoting the nanoparticle aggregation. It was previously shown that the degree of nanoparticle aggregation might play a major role in determining their toxicity [30,31]. Conversely, adding electrolytes to a medium at $pH > IEP_{NP}$ induces antagonistic toxicological effects by reducing the electrostatic repulsion between NP-TiO_2 and bacteria while promoting nanoparticle aggregation. To a lesser extent, the bacterial aggregation/flocculation observed at high ionic strength may also affect the toxicity by protecting the cell surface against the damage caused by the NP-TiO_2.

4. Conclusion

The results presented herein demonstrate (i) the toxicity of TiO_2 nanoparticles on the laboratory model strain *E. coli* in the absence of light, (ii) the paramount role that interfacial electrostatic interactions (NP-cell vs. NP-NP vs. cell-cell interactions) play in determining the extent of this toxicity, and (iii) the importance of physico-chemical parameters such as pH, ionic strength, and electrolyte composition in controlling these interactions. All these data provide fundamental knowledge essential to a better assessment of in vitro nanoparticle toxicity. They also give important clues in understanding the behavior of nanoparticles in aqueous media and their possible interactions with bacteria in the context of the environmental risk assessment.

Acknowledgments

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Les travaux réalisés précédemment ont mis en évidence l'importance des interactions de surfaces dans l'apparition d'un effet biocide des nanoparticules de dioxyde de titane à l'encontre des bactéries Gram négative telle que *Escherichia coli*. Cependant, les interactions électrostatiques ne peuvent à elles seules être le moteur de la toxicité, la membrane externe bactérienne doit également jouer un rôle prépondérant dans la modulation de ces interactions. Ces premiers résultats nous amènent donc à analyser à une échelle locale la nature des déterminants biophysiques des interphases bactériennes, à savoir la présence, la nature et les propriétés de structures de type LPS et/ou fimbriaes. La balance hydrophobe/hydrophile généré par ses structures ainsi que leurs élasticité mécanique vont également avoir un impact sur les phases d'approche des nanoparticules vers ou depuis les interphases bactériennes. Ces paramètres vont également moduler les phénomènes de déposition et/ou de pénétration des nanoparticules sur ou dans les structures périphériques bactériennes et leur toxicité éventuelle. Afin d'étudier la topologie et les propriétés physiques (nanomécaniques et électrohydrodynamiques) des interphases bactériennes sur leurs capacités à adsorber et accumuler les NP-TiO_2 et à modifier leur sensibilité/résistance par rapport à une exposition aux NP-TiO_2, nous nous sommes intéressés à des mutants d'*Escherichia coli* présentant des délétions génique au niveau de l'opéron *rfa* qui conduit à la génération de mutants bactériens présentant des fimbriaes ou des LPS tronqués. En effet, en jouant sur la longueur des LPS ainsi que sur les différents groupements chimiques exposés dans le milieu, la réponse des organismes bactériens à une exposition à des nanoparticules pourrait être modulée.

II. Rôle de l'interphase bactérienne dans l'expression de la toxicité des nanoparticules de dioxyde de titane.

Cette étude a mis en évidence que l'exposition à une concentration de 100 mg/L de P25-TiO_2-NP, engendrait pour l'ensemble des souches bactériennes (sauvage et mutantes) présentent une diminution de 1 log de leur population à l'exception de la souche JW3606 qui subi une diminution de 3 log. Cette souche présente une mutation au niveau du gène *rfaG* conduisant à une diminution de la taille des LPS, une diminution de leur niveau de phosphorylation mais aussi à la disparition du flagelle. L'analyse en microscopie à force atomique montre une diminution de l'élasticité de la membrane bactérienne par rapport à la souche témoin BW25113. L'analyse de la mobilité électrophorétique des souches bactériennes a ensuite été réalisée. Celle-ci montre que la souche témoin présente la charge de surface la moins électronégative alors que la souche JW3606 est la plus chargée négativement. Les nanoparticules de titane utilisées présentent quant à elles une charge de surface positive. Les forces d'interactions électrostatiques entre la souche JW3606 et les nanoparticules sont donc favorisées dans ces conditions ce qui peut expliquer la toxicité plus importante observée avec cette souche bactérienne. Cependant, les résultats de toxicité laissent à penser que la structure des LPS n'est pas le seul paramètre influençant la toxicité des P25-TiO_2-NPs c'est pourquoi l'analyse de l'ensemble des protéines de la membrane externe des différents mutants a été réalisée. Cette analyse à mis en évidence une accumulation de la flagelline de 50 à 500 fois supérieure au témoin BW25113 et à la souche JW3596. De plus, les protéines impliquées dans les porines sont également impactés par cette mutation (OmpR diminué d'un facteur 14, OmpF et OmpW diminué de 50%). La souche JW3606 étant la seule souche bactérienne présentant une forte sensibilité aux nanoparticules, il semble que la sensibilité au P25-TiO_2-NP soit fortement dépendante de la mutation de type *rfaG* qui supprime le flagelle et à l'accumulation de FliC, au niveau de la membrane bactérienne. Ainsi, il semblerait que la toxicité des P25-TiO2-NPs pour les organismes bactériens soit dépendante de la structure de la membrane externe bactérienne (longueur, présence/absence des LPS), de sa composition protéique mais également des protéines sous jacentes et de l'accumulation éventuelles de certaines d'entre elles.

Rôle de l'interphase bactérienne dans l'expression de la toxicité des nanoparticules de dioxyde de titane

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Résumé

L'exposition à une concentration de 100 mg/L de P25-TiO_2-NP conduit à une diminution de 1 log de leur population pour les souches bactériennes sauvage et mutantes à l'exception de la souche JW3606 qui subi une diminution de 3 log. Cette souche présente une mutation au niveau du gène *rfaG* (diminution de la taille des LPS, de leur niveau de phosphorylation, disparition du flagelle). L'analyse en AFM montre une diminution de l'élasticité de la membrane bactérienne par rapport au témoin BW25113. L'analyse de la mobilité électrophorétique montre que la souche témoin présente la charge de surface la moins électronégative alors que la souche JW3606 est la plus chargée négativement. Les P25-TiO_2-NPs sont quant à elles positive. Les forces d'interactions électrostatiques sont donc favorisées dans ces conditions ce qui peut expliquer la toxicité plus importante observée avec cette souche bactérienne. L'analyse de l'ensemble des protéines de la membrane externe des différents mutants à mis en évidence une accumulation de la flagelline de 50 à 500 fois supérieure au témoin BW25113 et à la souche JW3596 pour la souche JW3606. De plus, les protéines impliquées dans les porines sont également impactés par cette mutation (OmpR diminué d'un facteur 14, OmpF et OmpW diminué de 50%). La souche JW3606 étant la seule souche bactérienne présentant une forte sensibilité aux nanoparticules, la sensibilité au P25-TiO_2-NP semble fortement dépendante de la mutation de type *rfaG* qui supprime le flagelle et conduit à l'accumulation de FliC au niveau de la membrane bactérienne. Ainsi, il semblerait que la toxicité des P25-TiO2-NPs pour les organismes bactériens soit dépendante de la structure de la membrane externe bactérienne (longueur, présence/absence des LPS), de sa composition protéique mais également des protéines sous jacentes et de l'accumulation éventuelles de certaines d'entre elles.

Introduction

Les avancées technologiques ont permis la mise au point de nanoparticules utilisées dans un nombre croissant de processus industriels et de produits de consommation courante (Gavankar et al., 2012). La production et l'utilisation de ces nanoparticules vont conduire à leur dispersion et leur propagation dans l'environnement, ce qui peut présenter un risque pour l'ensemble des processus ecosystémiques et la maintenance d'un bon état écologique (Chen and Mao, 2007; Holden et al., 2012). Le dioxyde de titane (TiO_2-NPs) est une des nanoparticules les plus utilisées puisqu'il entre dans la composition de ciment, de verre aux propriétés autonettoyantes, dans des peintures, ainsi que comme agents désinfectants dans des systèmes de dépollution des eaux, les écrans solaires (Chen and Mao, 2007; Liu and Zhao, 2007; Theron et al., 2008; Tratnyek and Johnson, 2006). Après leur dispersion dans l'environnement, les nanoparticules de TiO_2 peuvent entraîner une modification de la structure et de la composition des communautés bactérienne à la fois dans les sols (Ge et al., 2011) et les eaux douces naturelles (Jomini et al., soumis). De plus, l'exposition des microorganismes à ces nanoparticules peux conduire à la modification des processus de fixation biologique de l'azote, de l'oxydation du méthane mais également modifier la minéralisation du carbone organique (Ge et al., 2012). Nos précédents travaux sur la souche modèle *Escherichia coli* ont mis en évidence une relation directe entre l'adsorption de nanoparticules de TiO_2 à la surface des cellules bactériennes et la toxicité. Cette adsorption est conditionnée, entre autre, par les interactions électrostatiques entre bactéries et nanoparticules, lesquelles interactions sont gouvernées par les propriétés physico-chimiques des bactéries et nanoparticules (en particulier leurs charges respectives) ainsi que la composition du milieu électrolyte dispersant (nature des ions, force ionique, pH) (Pagnout et al., 2012). Ces résultats nous amènent donc à analyser à une échelle locale (nanométrique) la nature des déterminants biophysiques des interphases bactériennes (présence/nature/propriétés de structures type LPS et/ou fimbriae, balance hydrophobe/hydrophile, élasticité mécanique des structures membranaires) régulant les phases d'approche des nanoparticules vers/depuis les interphases bactériennes, la déposition/pénétration de celles-ci sur/dans les structures périphériques bactériennes et leur toxicité éventuelle.

La membrane externe d'*Escherichia coli* est une barrière semiperméable organisée en bicouche lipidique présentant une distribution asymétrique des phospholipides et des lipopolysaccharides (LPS) qui la composent. Les LPS représentent les 3/4 de la surface d'*E. coli* (3,5 millions de molécules) et sont presque exclusivement ancré dans le feuillet externe de la bicouche lipidique alors que les phospholipides sont confinés au feuillet interne ce qui

assure leur protection (Li et al., 2010; Parker et al., 1992). Les LPS sont composés de trois régions principales: le lipid A qui sert d'ancrage hydrophobe dans la bicouche lipidique, le core des LPS composé d'une chaîne d'oligosaccharide phosphorylés de 10 à 15 sucres servant d'ancrage pour l'antigène O, constitué d'une répétition d'unités oligomériques formant un polysaccharide à structure variable (Chang et al., 2010; Yethon et al., 2000). Il a été mis en évidence que les LPS d'*E. coli* pouvaient établir des interactions hydrogènes avec le TiO_2 (Jucker et al., 1997), et que l'adhésion de souches d'*E. coli* et de *P. aeruginosa* était plus importante pour les souches présentant les LPS les plus long sur des films de TiO_2 (20-500 nm) (Li and Logan, 2004). Des études réalisées avec d'autres nanoparticules telle que celle d'Au, d'Ag et de CeO_2 ont mis en évidence le rôle déterminant de ces structures dans les interactions entre bactéries et nanoparticules (Deplanche and Macaskie, 2008; Sondi and Salopek-Sondi, 2004; Thill et al., 2006). Afin d'examiner plus précisément le rôle de la membrane externe bactérienne dans les interactions avec le TiO_2-NPs et la toxicité pouvant en résulter, nous allons examiner la toxicité du P25 sur différents mutants d'*Escherichia coli* présentant des délétions géniques au niveau de l'opéron *rfa.* Cet opéron, décrit pour la première fois par Subbaiah and Stocker en 1974, contient la plupart des gènes impliqués dans la synthèse de glycosyltransférases responsables de l'assemblage des LPS mais aussi dans la synthèse des sucres constitutifs des LPS et dans le transfert des unités formant l'antigène O sur le core des LPS (Kadam et al., 1985; Karp et al., 2007; Subbaiah and Stocker, 1964; Yethon et al., 2000). Les mutations au sein de cet opéron conduisent à l'expression d'un phénotype bactérien de type "deep-rough", caractérisé par une pléiotropie conduisant à des modifications de la composition et de la structure de la membrane externe (Schnaitman and Klena, 1993). Cette instabilité conduit à une sensibilité accrue aux composés hydrophobes mais également à une diminution de l'expression des protéines membranaires pouvant conduire à un relargage d'enzymes periplasmic dans le milieu de culture (Raetz and Whitfield, 2002). Ces mutants présentent également une diminution de la sensibilité des LPS aux interactions spécifiques avec des bactériophages (Silverman and Benson, 1987). De plus, la composition en phospholipides de la membrane externe est augmentée et leur répartition au sein de la bicouche lipidique s'en trouve modifiée (Mäkelä and Stocker, 1984; Parker et al., 1992). Il a également été rapporté que les mutants présentant un phénotype de type « deep rough » ne présentaient plus de flagelle et de pili (Ames et al., 1974; Parker et al., 1992). L'objectif de ces travaux est de déterminer quel est le rôle de l'organisation de la surface bactérienne dans l'expression de la toxicité des TiO_2-NPs à l'encontre chez *E. coli*. Pour ce faire, la souche bactérienne sauvage d'*E. coli* K-12 BW25113 et ses mutants isogéniques ont

été étudiés pour déterminer le rôle des LPS et l'impact de leurs tailles sur les interactions entre TiO_2-NPs et bactéries. La toxicité des TiO_2-NPs a été évaluée sur la souche sauvage et les mutants et, afin de comprendre les différences de toxicité pouvant être observées, la mobilité électrophorétique de chacun a été mesurée. De plus, la visualisation de la surface bactérienne et sa caractérisation a été réalisée grâce à l'utilisation d'imageries en microscopie à force atomique. Dans le but de caractériser au mieux les mutants les plus sensibles aux TiO_2-NPs et de comprendre le rôle des interactions de surface lors de l'exposition aux nanoparticules, une analyse comparative de l'ensemble des protéines de la membrane externe bactérienne des différentes souches a également été effectuée par une approche de protéomique quantitative off gel.

Materials and Methods

Bacterial strains

The wild type strain used in this study is *E. coli* BW25113, obtained from the Keio collection (1) of the Coli Genetic Stock Centre (Yale University). From this strain, isogenic mutants with specific deletion in the *rfa* operon were derived. These strains corresponds to the *E. coli* JW3606-1 with a *rfaG* (Kn^r) deletion, JW3601-1 with a *rfaJ* (Kn^r) deletion, JW3605-1 with a *rfaP* (Kn^r) deletion, JW3603-2 with a *rfaB* (Kn^r) deletion, JW4277-1 with a *fimA* (Kn^r) deletion, JW4281-1 with a *fimF* (Kn^r) deletion, JW4282-2 with a *fimG* (Kn^r) deletion, JW4283-3 with a *fimH* (Kn^r) deletion and JW3596-1 with a *rfaC* (Kn^r) deletion. The effect of each mutation regarding the LPS can be seen on figure 1.

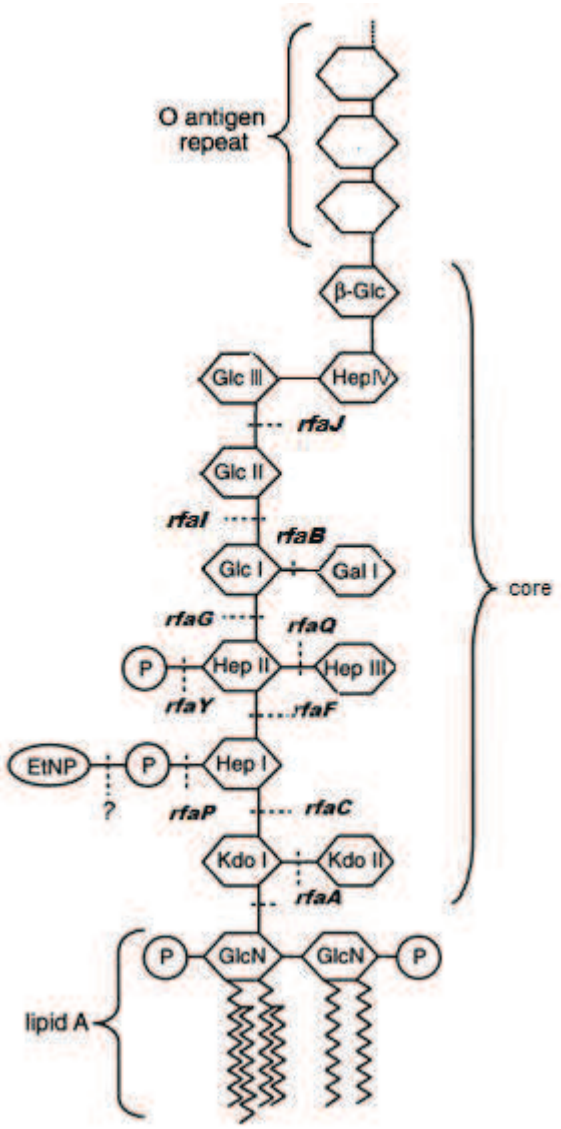


Figure 1: Lipopolysaccharide structure of E. coli BW25113 and each rfa mutation impact in the core structure. Abbreviations are as follows: GlcN, D-glucosamine; Kdo, 3-deoxy-D-manno-octulosonic acid; Hep, L-glycero-D-manno-heptose; P, phosphate; EtNP, 2-aminoethyl phosphate; Glc, D-glucose; and Gal, D-galactose. This figure is modified from (Chang et al., 2010) and from (Klein et al., 2014).

Bacterial cultures

All bacterial strains were grown in M9 salts medium (900mL of ultra pure water, 100 mL of M9 10X, 1 mL MgSO4 1M, 1 mL CaCl2 0.1M, 1 mL, Thiamine 10mg/mL, 10 mL Glucose 20%, 2 mL, Proline 10mg/mL, 2.5 mL, Uridine 10mg/mL) supplemented with kanamycin to a final concentration of 30µg/mL at 37 °C except for the control wild type strain., *E. coli* BW25113 and the isogenic *rfa* mutants were first streaked from frozen stock on LB agar

medium supplemented with kanamycin (LB broth containing 1.5% (w/v) agar) and incubated at 37°C over night. Isolated colonies were inoculated in 200 ml of a M9 salts medium broth supplemented with kanamycin and then incubated at 37°C until exponential phase of growth was reach (OD_{600} nm: 0.5). The cells were subsequently harvested by centrifugation at 5000 × g for 8 min, after which they were washed twice with sterile KNO_3 10mM. The bacterial suspension obtained was adjusted to an OD_{600} nm of 4.0 (10^9 cells/ml) and used immediately for assays or freeze dried for ITRAC analysis.

Used nanoparticles

TiO_2 nanopowder Aeroxide P25 was provided by Evonik Degussa GmbH (Frankfurt, Germany). Analyses showed that it was a mixture of anatase and rutile forms (~ 84 % anatase and 16 % rutile) with an average primary particle size of 23 ± 4.9 nm. Dynamic light scattering (DLS) measurements revealed that the average hydrodynamic diameter of the nanoparticle stock suspension ranged between 60 and 80 nm, this size corresponding to an agglomerated state. The determined isoelectric point was approximately of pH 6.8. For more information on the used nanoparticles, see our previous work (Pagnout et al., 2012).

Toxicity assessment

Experiments were conducted in 100 ml flasks containing 20 ml of sterile milli-Q water (natural pH 5.5) supplemented with a concentration of 10 mM KNO_3. Briefly, 200 µl of each *E. coli* mutant bacterial suspension and 200 µl of the NP-TiO_2 stock suspension (or mQ water for the control) were prepared as previously described (Pagnout et al., 2012) and then added to the electrolyte solutions to obtain a final concentrations of 10^7 cells/ml and 100 mg/l of TiO_2 nanoparticles. The flasks were then incubated at room temperature on a dark rotary shaker at 150 rpm. After 20 h of exposure, the toxicity of NP-TiO_2 toward each mutant of *E. coli* was investigated using the Drop Count Method.

For the Drop Count Method, nine drops (20 µl per drop) were used, transferred onto solid Luria Bertani (LB) agar medium and incubated at 37 ◦C for 24 h. The percentage of viable cells was determined by comparing the number of colony forming units (CFU) produced by the exposed and unexposed samples. All experiments were prepared in triplicate.

AFM measurements

Glass slides were freshly prepared upon immersion in 0.2% PEI solution for 30 minutes, extensively rinsed with Milli-Q water, dried with nitrogen and stored in a sterile Petri dish. One mL of bacterial culture (OD600 nm, 0.5–0.6) was directly deposited onto the PEI-coated glass slide for 30 minutes. Each bacterial sample was extensively rinsed with 50 ml of 1 mM KNO3 and then was immediately transferred into the AFM liquid cell with addition of 2 ml of KNO3 solution. Finally, we mention that bacteria immobilized onto the PEI-coated substrate were extensively rinsed with KNO3 in order to get rid of un-adhered free bacteria and possibly remaining residues from minimum growth medium. The Mode PeakForce QNM (Quantitative Nanomechanical Property Mapping) was used with a nominal spring constant of 0.32-0.35 N/m and a nominal resonance frequency of 1 KHz to measure the elasticity of the bacterial cells membrane.

Bacterial electrophoretic mobility

After bacterial preparation, the cells were diluted to reach a final OD_{600} of 0.4 and were then exposed to various concentrations of P25-TiO_2-NPs for 20 hours under agitation and in the dark. The bacterial cells solution was then resuspended to an OD_{600} of 0.04 in increasing concentration of KNO_3 salts to reach final concentration of 1-2.5-5-10-25-50-100-250-500mM and bacterial electrophoretic mobility was assessed. The electrophoretic mobility was also assessed after exposing bacterial cells to 0.1mg/L of P25-TiO_2-NPs in 10 mM KNO_3 for 20 hours and then resuspended in increasing concentration of KNO_3 as previously done. These measurements were conducted using a Zetaphoremeter IV (CAD Instrumentations, Les Essarts le Roi, France) in quartz Suprasil® cell (Heraeus Quarzglas GmbH & Co., Hanau, Germany) at 24 ◦C. It consisted in measuring the reflection of a laser beam by bacteria tracked with a charge-coupled device camera. Image analysis software was used to record images in real time used to calculate the electrophoretic mobilities from the displacement (migration motion) of bacteria subjected to a constant direct-current electric field (800 V/m). Different cycles were recorded to yield bacterial mobility measurements.

Isolation of cell membranes

Cell membranes were isolated according to the method described in Molloy et al. with slight modification. Briefly, pelleted cells were resuspended in 6 mL of 50 mM Tris/HCl pH 7.3 containing 0.7 mg of DNase I. Cells were broken in a French press twice at 0.965 kBar and centrifuge at 2500 g for 10 min. The supernatant containing the broken cells was diluted 10 times with ice cold 0.1M sodium carbonate (pH11) and stirred slowly at 4°C for 1 h.

Carbonate treated membrane were pelleted by ultracentrifugation at 115 000 g for 1 h in a Kontron TFT 50.38 rotor, washed in 50 mM Tris/HCl pH 7.3 and the final pellet was collected by ultracentrifugation at 115 000 g for 20 min. Cell membranes were dissolved in 300 µL of 500 mM TEAB (Tetra Ethyl Ammonium Bromide)/ 0.1% SDS and denatured 10 min at 95°C. Protein concentration was estimated by the method of Bradford with bovine gamma globulin as standard (BioRad) and protein quality was checked by protein separation on a SDS-PAGE 12% and staining with Coomassie blue R250 (Bradford, 1976). Protein concentration was adjusted to 3µg/µL with 500 mM TEAB/ 0.1% SDS/20% ACN (Acetonitrile).

ITRAQ labeling and MSMS proteins identification and relative quantification.

Isobaric peptide labeling

The volume corresponding to 90 µg of protein for each sample were reduced (30mM DTT, 30 min at 56°C), alkylated (60mM Chloroacetamide, 30 min at 25°C), digested (1µg trypsine, over night at 30°C). The pH was checked to assure a complete digestion. Then samples were dried in a vacuum concentrator (Eppendorf), resuspended in 35µl water and labeled with iTRAQ reagents according to the protocol of the iTRAQ Reagents kit (Applied Biosystems). Samples were labeled as follows with a different isobaric tag. In the experiment WTA, WTB, 1596A, 1596B, 1601A, 1601B, 1606A and 1606B samples were labeled with 113, 114, 115, 116, 117, 118, 119 and 121 respectively. Labeled samples were then combined.

IEF Off-gel fractionation

iTRAQ reagent excess and detergent were eliminated by using an SCX column (Strong Cation exchange, ABSciex). Briefly: SCX column was prewashed with 2ml of cleaning buffer (25% ACN, 10mM KH2PO4, 1M KCl pH3) and equilibrated with 2mL of loading buffer (25% ACN, 10mM KH2PO4, pH3). Sample was resuspended by 1ml of loading buffer acidified with 20µl of KH2PO4 10% to obtain pH 3, percolated on column then washed with 2mL of loading buffer. Retained peptides were eluted with 500µl of elution buffer (25% ACN, 10mM KH2PO4, 350mM KCl, pH3). Eluted peptides were desalted by using a Sep-Pak C18 column (Waters). Briefly, C18 column was activated with 3ml of 90% ACN, 0.1% TFA (Trifluoroacetic acid Fluka) and equilibrated with 3mL of 0.1% TFA. Peptides were percolated on column, retained fraction was washed with 2mL of 0.1% TFA and eluted with 1ml of 70% ACN, 0.1% TFA then dried in a vacuum concentrator (eppendorf). Peptides were

prepared for an Off-gel isoelectrofocalisation on 13 cm strip pH 3-10 as indicated in Agilent 3100 Off-Gel fractionator kit-quick start guide. After focusing, each fraction was collected. To extract peptides trapped in strip gel, 200µl of 50% methanol 1% formic acid were added to each tank of the frame and incubated for 30min. Methanol-extracted peptides were pooled with their respective fraction then dried in a vacuum concentrator then resuspended in 100µl of 10% ACN and 0.1% TFA solution.

LC-MSMS

1µg of each fraction were then analyzed in LC-MS-MS. Analyzes were realized using an Ultimate 3000 Rapid Separation liquid chromatographic system coupled to a hybrid LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Briefly, peptides were loaded and washed on a C18 reverse phase precolumn (3 µm particle size,100 A pore size, 75 µm i.d., 2 cm length) The loading buffer contains 98% H_2O, 2% ACN and 0.1% TFA. Peptides were then separated on a C18 reverse phase resin (2 µm particle size,100 A pore size, 75 µm i.d.,15 cm length) with a 97 min "effective" gradient from 100% A (0.1% formic acid and 100% H_2O) to 10% B (80% ACN, 0.085% formic acid and 20% H_2O) in 33 min then to 40% B in 64 min. The Linear Trap Quadrupole Orbitrap mass spectrometer acquired data throughout the elution process and operated in a data dependent scheme with full MS scans acquired with the orbitrap, followed by up to 10 LTQ MS/MS CID spectra and 10 LTQ MS/MS HCD on the most abundant ions detected in the MS scan. Mass spectrometer settings were: full MS (AGC: 1*106, resolution: 6*104, m/z range 400-2000, maximum ion injection time: 500 ms); MS/MS (minimum signal threshold: 500, isolation width: 2Da, dynamic exclusion time setting: 60 s, Ion Trap MSn AGC Target: 5*103 and maximum injection time: 200 ms, FTMS MSn AGC Target: 5*104 and maximum injection time: 200 ms). The fragmentation was permitted of precursor with a charge state of 2 and 3.

Spectral Processing and Database Searching

The software used to generate .mgf files is Proteome discoverer 1.3. The threshold of Signal to Noise for extraction values is 3. The node Spectrum grouper was used to group HCD and CID scans. The mgf files were submitted to Protein Pilot version 4. The database used was Escherichia coli K12 extract from database Uniprot. FDR calculations were performed using a reverse database. Search effort was set to Rapid ID and instrument used was Orbi-FT MS (1-3 ppm), LTQ MSMS. Analyze was performed using Trypsin as enzyme and carbamidomethylation as fixed modification. For iTRAQ quantitation, bias correction and

background correction were applied to Proteinpilot research. A protein was considered to be significantly identified when 2 or more high confidence (>95%) unique peptides were assigned, the protein identification had to have a 5% local False Discovery Rate. The relative quantification was done using the BW25113 biological duplicates as references samples. Ratios of fold change and the associated p-value were calculated for each biological duplicates (mutant/BW25113) then the median value of the four ratios was obtained. For the biological analysis, only proteins with a median ratios above the threshold ratio and with 3 Pval <0.05 were considered significantly deregulated. The threshold ratio corresponds, for each comparison, at the first and the last 10% from the Gaussian distribution of the mutant/BW25113 protein ratio values. Up and down threshold ratio values are 4.74, 0.21; 4.81, 0.20 and 6.12, 0.16 for the strain 3606, 3601 and 3596 respectively.

Results and Discussion

Toxicity assessment

Après exposition de la souche sauvage BW25113 et de ses mutants isogénique à une concentration de 100 mg/L de P25-TiO_2-NPs, la toxicité a été déterminée par la méthode de croissance « en drop », les résultats obtenus sont représentés sur la figure 2. Toutes les souches présentent une sensibilité aux nanoparticules se traduisant par une diminution du nombre de CFU. Cependant, la souche JW3606 est la plus sensible aux nanoparticules, avec une diminution de 3 log du nombre de CFU. La souche JW3606 présente une délétion du gène *rfaG* conduisant à la suppression de l'activité de la glycosyltransférase I (Kadam et al., 1985). Ceci provoque une déficience en glucose des LPS et la perte de toutes les unités distales (Mäkelä and Stocker, 1981). Cette mutation conduit à une disparition complète des groupements phosphate sur Hep II et une substitution de 40% des phosphates sur HepI ce qui conduit à une réduction de 80 % de la phosphorylation totale des heptoses dans la chaîne des LPS et à une diminution de plus de la moitié de leurs longueurs (Yethon et al., 2000). Li and Logan (2004) ont montré que les organismes présentant les plus grand LPS présentent une adhésion plus importante avec un film fin de TiO_2 (20-500 nm) que les souches avec des LPS tronqués. Les LPS ont été démontrés comme étant capables de réaliser des liaisons hydrogène avec le TiO_2 chez *E. coli* (Jucker et al., 1997) confirmé par analyse spectroscopique par transformation de Fourier (FTIR) lors de l'exposition d'*E. coli* à Al_2O_3-NPs, TiO_2-NPs, et ZnO-NPs (Jiang et al., 2010). Ces modifications pourraient conduire à des interactions plus importantes entre la surface bactérienne et les nanoparticules pouvant expliquer cette toxicité.

Cependant, la taille des LPS ne semble pas être le paramètre le plus important puisque la souche JW3596 présentant la mutation au niveau du gène *rfaC* ne présente dans ses LPS que l'ancrage du lipide A et l'acide 3-deoxy-D-mannooctulosonic connu sous le nom de keto-deoxyoctulosonate (KDO) n'est pas la plus sensible. La composition de la partie des LPS exposés dans le milieu semble jouer un rôle majeur dans l'établissement des interactions entre la membrane externe et les P25-TiO_2-NPs. Ceci tend à confirmer les observations de la littérature puisqu'il a été mis en évidence que la perte de la majeure partie de la phosphorylation des heptoses sur la chaîne des LPS conduisait à une diminution de la stabilité de la membrane externe en permettant la création de pontage inter-LPS (Chang et al., 2010; Yethon et al., 1998). Il a été suggéré que ces modifications conduiraient à favoriser l'agrégation des organismes bactériens. Afin de vérifier cette possibilité, des observations en microscopie à force atomique (AFM) on été réalisées.

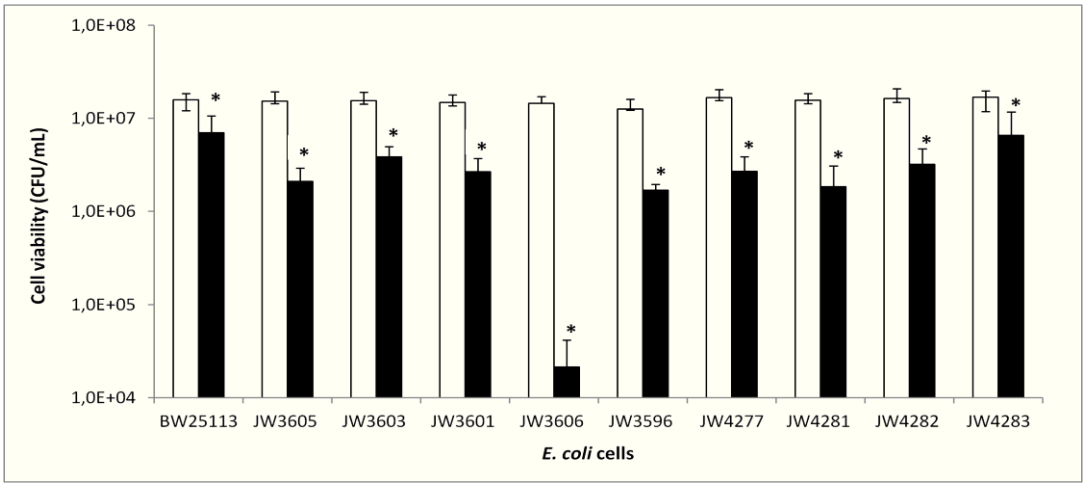


Figure 2: Toxicité du P25-TiO_2-NPs sur *Escherichia coli* BW25113 et ses mutants après exposition à une concentration en nanoparticule de 100 mg/L dans du KNO_3 10 mM pendant 20 heures. Les barres blanches représentent le nombre d'unité formant colonie sans exposition au P25-TiO_2-NPs et les barres noires correspondent au nombre de CFU après exposition au P25-TiO_2-NPs. Les différences statistiques sont déterminé par le test de Wilcoxon–Mann–Whitney avec une p-value <0.05 (*).

Cells morphology and mechanical properties

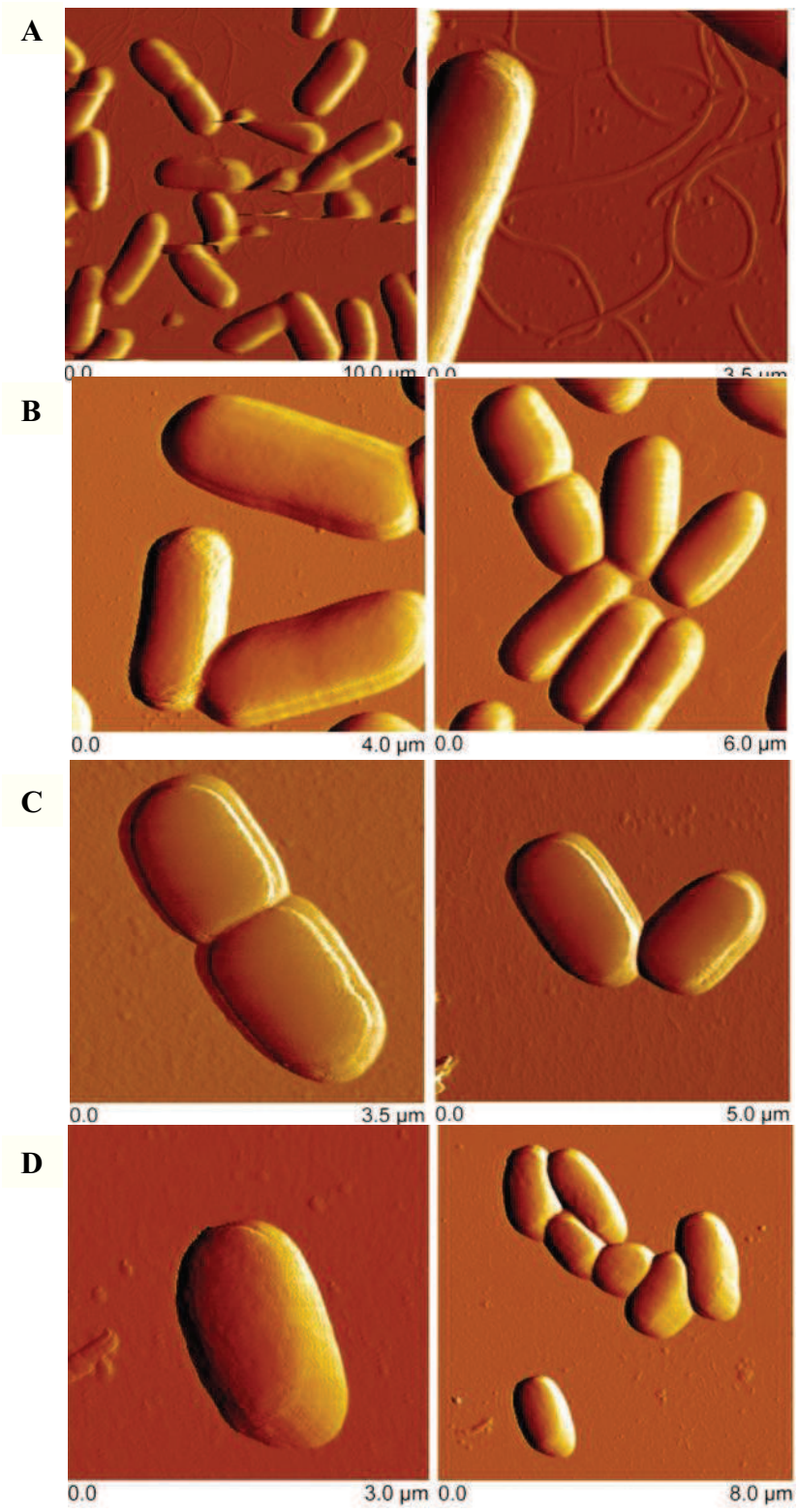


Figure 3: Observation au microscope à force atomique des souches d'*E. coli* BW25113 (A), JW3601 (B), JW3606 (C) et JW3596 (D) après croissance en M9 et suspension en KNO_3 1mM.

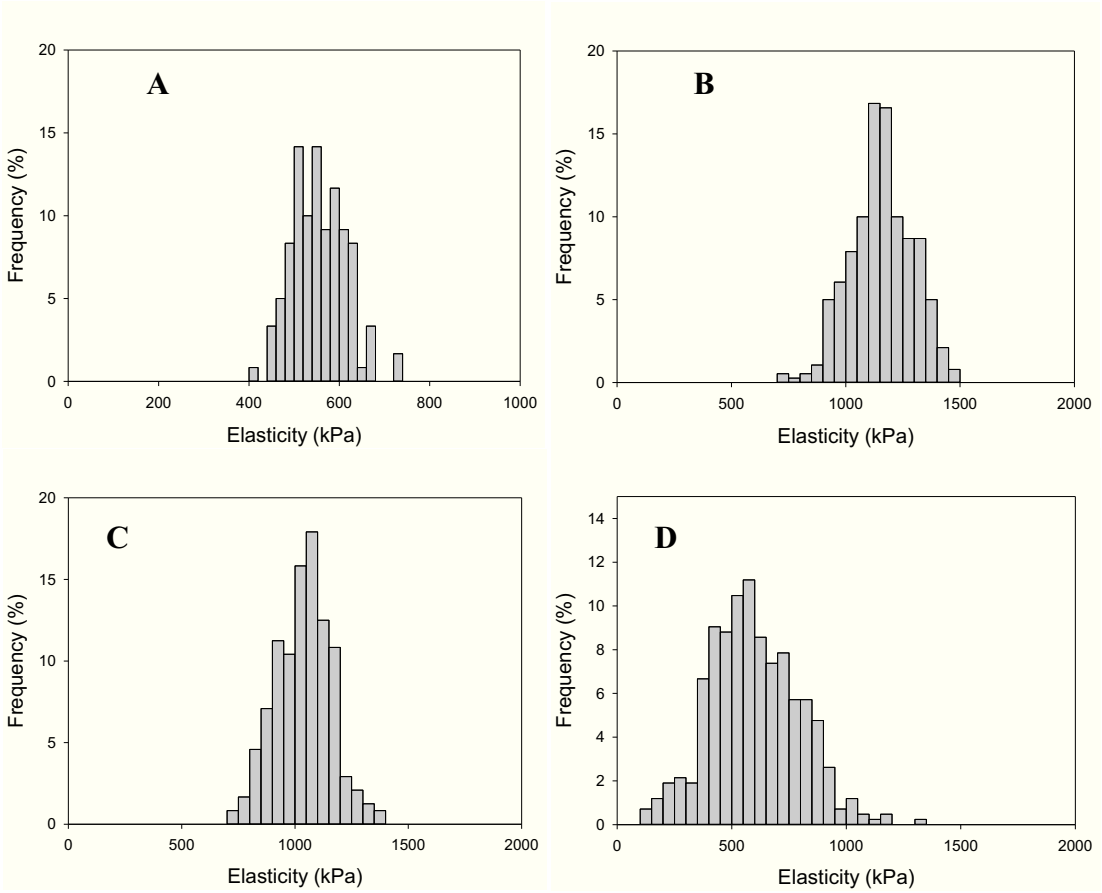


Figure 4: Mesure en AFM du module de Young des souches d'*E. coli* BW25113 (A), JW3601 (B), JW3606 (C) et JW3596 (D) après croissance en M9 et suspension en KNO_3 1mM.

Les mesures de toxicité réalisées précédemment ont mis en évidence des différences entre les mutants et la souche sauvage BW25113. Cependant, pour comprendre au mieux les variations observées, la suite des analyses a été restreinte à 4 souches bactériennes, à savoir la souche sauvage BW25113 ; une souche présentant une taille de LPS intermédiaire, JW3601 ; la souche la plus sensible, JW3606 et ; la souche présentant une membrane externe pratiquement nue, JW3596. Afin de mieux appréhender l'impact de la structure de la membrane externe de chaque souche bactérienne sur les interactions entre bactéries et nanoparticules, des observations en AFM et des mesures de propriétés nanomécaniques ont été réalisées. Conformément à ce qui est décrit dans la littérature, seule la souche sauvage BW25113 présente des flagelles (Fig 3A). En effet, une mutation au sein de l'opéron *rfa* conduit à la disparition de la synthèse des flagelles et des pilis (Ames et al., 1974; Parker et al., 1992). Afin de confirmer l'absence de ces appendices, un test de mobilité sur gélose a été réalisé et seule la souche sauvage s'est révélée mobile (Donnée non affichées). Au niveau de la morphologie, les cellules de mutants semblent très proches les unes des autres, mis à part la souche sauvage qui présente une forme plus allongée typique des souches d'*E. coli*. Nos observations microscopiques n'ont pas permis de mettre en évidence des phénomènes d'agrégation plus important pour la souche JW3606 en comparaison des autres mutants. Cependant, l'étude des propriétés nanomécaniques de la membrane externe de ces quatre souches met en évidence deux populations. En effet, la mesure en mode "peak force mapping" en contact intermittent réalisée avec une fréquence de vibration du levier de 1KHz permet la mesure du module de Young corrélé à l'élasticité de la membrane bactérienne (dépendante de sa composition). Dans notre étude, ces mesures mettent en évidence un niveau similaire de l'élasticité de la membrane externe des souches JW3601 et JW3606 avec des valeurs d'élasticité respective de 1200 et 1300 ± 150 KPa, alors que la souche témoin BW25113 et la souche JW3596 présentent des valeurs d'élasticité respectives de 500 et 650 ± 150 KPa (Figure 4). Ainsi, les deux souches bactériennes JW3601 et JW3606 présentent l'élasticité la plus faible puisque la force à appliquer pour déformer la membrane est la plus grande. Du fait de la plus haute toxicité détecté sur la souche mutante JW3606, cette diminution de l'élasticité pourrait être un des facteurs conduisant à une augmentation de la sensibilité aux contacts de surfaces et à la pression physique des nanoparticules sur la membrane externe. L'élasticité de la membrane externe pourrait jouer un rôle majeur dans la sensibilité des souches bactériennes aux nanoparticules. Malgré la grande précision de cette technique, les surstructures de la membrane externe de ces souches bactériennes ne peuvent être distinguées en microscopie AFM puisque la valeur d'indentation de la pointe permettant la mesure du module de Young

est de l'ordre de 20 nm. L'analyse en mode force-volume autoriserait l'utilisation de forces plus importantes ce qui permettrait d'atteindre la zone de turgescence des bactéries conduisant ainsi à une différenciation plus fine de leurs spécificités mécaniques.

Electrophoretic mobilities assessment

Les propriétés nanomécaniques de la membrane externe des souches bactériennes utilisées ne présentent pas de différence significative entre les différents mutants. Pour compléter leur caractérisation, la mesure de la charge de surface de chaque souche a été réalisée par détermination de leur mobilité électrophorétique. Dans l'environnement, la très grande majorité des organismes bactériens présentent une charge de surface négative du fait de la présence de différents groupes fonctionnels tels que des acides carboxyliques, des hydroxyles ou des phosphates (Blake et al., 1999; Kiwi and Nadtochenko, 2004; Rincón and Pulgarin, 2004). Ainsi, nos précédents travaux ont mis en évidence l'importance des interactions électrostatiques entre la surface bactérienne et les P25-TiO_2-NPs pour la détection d'une toxicité/génotoxicité (Jomini et al., 2012; Pagnout et al., 2012). Les propriétés nanomécaniques de la membrane externe bactérienne ne semblent pas être le seul paramètre impliqué dans la toxicité observée, le troncage des LPS par les différentes délétions doit également avoir un impact sur la charge de surface de la membrane externe (Figure 5). La charge de surface de la souche témoin BW25113 n'a pu être mesurée du fait de la présence de flagelle qui conduit les bactéries à se mouvoir dans la solution de KNO_3 malgré l'application du champ électrique qui doit être le seul paramètre influençant le mouvement des cellules. Pour les trois souches mutantes, on remarque l'apparition d'un plateau dans la charge de surface lorsque la mesure est effectuée pour une concentration en KNO_3 supérieure à 250 mM. Ceci s'explique par l'écrantage total des charges de la double couche électronique présente à la surface des bactéries (Figure 5 A). Pour des concentrations plus faibles en KNO_3, on remarque que la souche JW3606 est la plus électronégative sur toute la gamme de concentration. Pour une concentration en KNO_3 de 10mM, utilisé lors de la mesure de la toxicité, la souche JW3606 présente une valeur de mobilité électrophorétique de -4.8 µm/s/V/cm alors que la souche JW3601 et la souche JW3596 présente des valeurs respectives de -3.8 µm/s/V/cm et -3.6 µm/s/V/cm (Figure 5B). L'exposition du phosphate de l'Heptose II de la chaîne des LPS suite à la mutation *rfaG* conduit à une électronégativité plus importante pour la souche JW3606. Cependant, la perte de près de 80% de la phosphorylation des heptoses aurait suggéré que cette souche ne serait pas la plus électronégative. Néanmoins, cette électronégativité plus importante peut expliquer en partie la sensibilité accrue de la

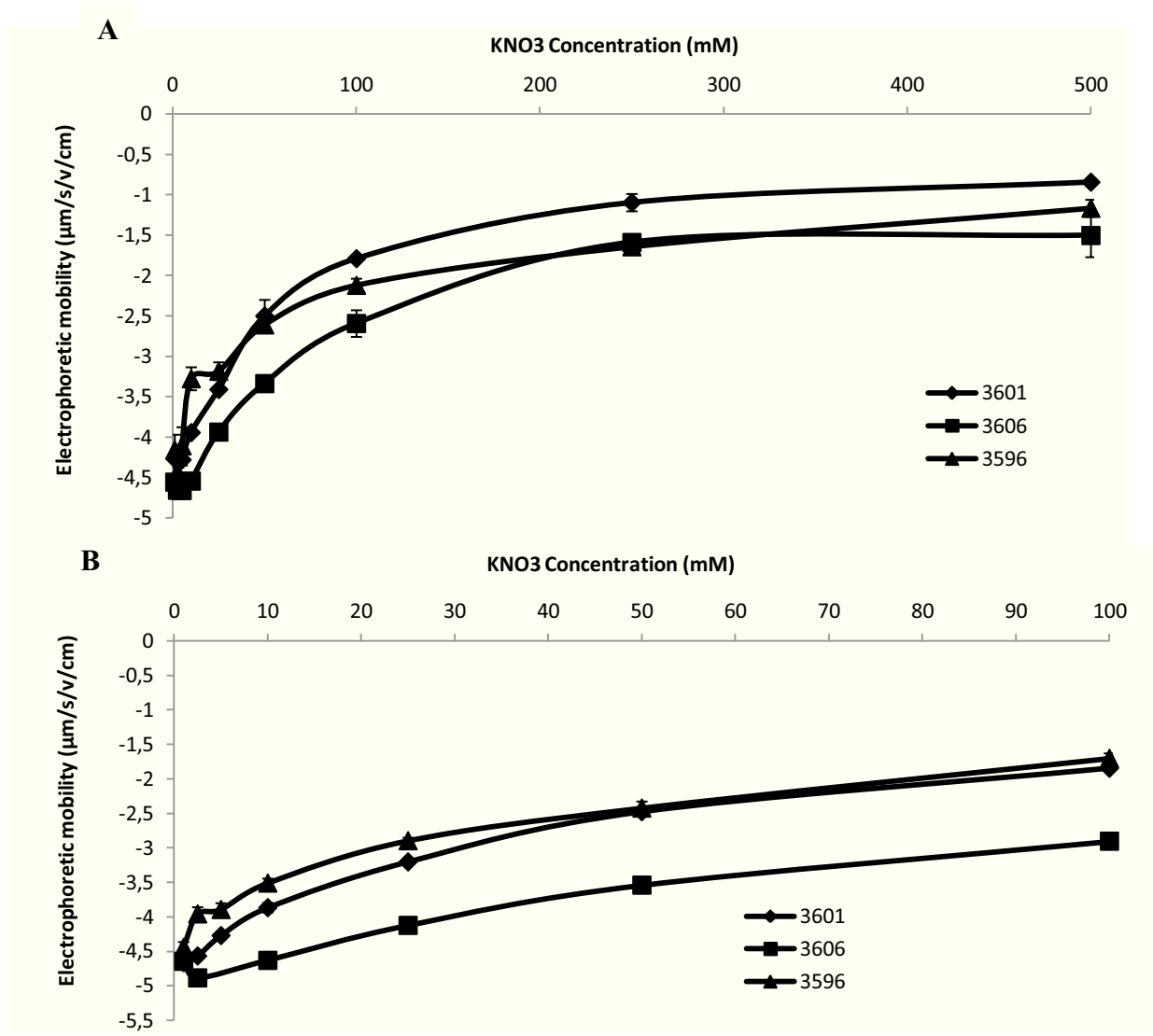


Figure 5: Mesure de mobilité électrophorétique des mutants coli JW3601 (losange noir), JW3606 (carré noir) et JW3596 (triangle noir) après suspension dans des solutions de molarité croissante en KNO3. Le graphique A présente le plateau atteint par la valeur de mobilité pour chaque souche bactérienne entre 1 et 100mM de KNO3. Le graphique B permet de différencier plus finement les mutants entre eux.

souche à l'exposition aux P25-TiO2-NPs. En effet, ces bactéries sont exposées aux nanoparticules dans un milieu salin faiblement chargé (KNO3 10mM) qui diminue les interactions interparticulaires en favorisant les répulsions électrostatiques et promeut les forces attractives de Van der Walls (Derjaguin and Landau, 1941; Pagnout et al., 2012; Verwey and Overbeek, 1948). La charge négative plus importante et la diminution de l'encombrement et de la stabilisation stérique de la surface des bactéries provoquées par la diminution de la longueur des LPS conduit à des interactions plus importantes entre cette souche JW3606 et les P25-TiO2-NPs ce qui pourrait expliquer que la toxicité mesurée sur cette souche soit plus importante.

Les résultats de toxicité laissent à penser que la structure des LPS de la membrane externe n'est pas le seul paramètre influençant la toxicité des P25-TiO2-NPs pour *E. coli* mais que la composition même de la membrane peut influencer la sensibilité des différentes souches. Ainsi, dans le but de mieux comprendre le mécanisme de toxicité, l'analyse des protéines de la membrane externe de ces quatre souches a été réalisée.

ITRAQ analysis:

Afin de pouvoir comparer le contenu en protéines de la membrane externe de chaque souche, une analyse de quantification relative des protéines par spectrométrie de masse à l'aide de la technique de marquage ITRAQ (Isobaric Tags for Absolute and Relative Quantification) a été réalisée. Les extraits protéiques ont été préparés par précipitation des protéines membranaires au carbonate de sodium (Molloy et al., 2000). L'observation du contenu protéique de ces échantillons sur gel SDS-PAGE 1D a montré qu'ils contiennent plus d'une centaine de protéines. Afin de réduire la complexité de ces extraits, un fractionnement peptidique a été réalisé par isoéléctrofocalisation (IEF off gel) après le marquage ITRAQ, puis les peptides contenus dans les différentes fractions ont été identifiés par spectrométrie de masse. L'analyse de quantification relative des protéines a été réalisée en utilisant la souche sauvage BW25113 comme référence. L'utilisation d'un marquage ITRAQ 8-plex a permis l'analyse en duplicats biologiques de chaque souche. Les critères de sélection des protéines ainsi que les calculs des ratios et des ratios seuils sont décrits dans la partie « matériels et méthodes ». L'analyse des résultats a permis de mettre en évidence 50 protéines qui présentaient une variation significative dans au moins une des conditions (table supl. 1) et plus précisément 25 protéines dérégulées significativement dans la souche 3606. Parmi les protéines dérégulées, certaines semblent particulièrement intéressantes et seront discutées ci-dessous.

Ainsi, la souche JW3606 présente une forte modification de son taux en flagelline. La concentration en flagelline est 55 fois plus importante au niveau de la membrane externe de la souche JW3606 par rapport à la souche sauvage BW25113 et 500 fois plus importante que la souche JW3596. En ce qui concerne la souche JW3601, son taux est équivalent à celui de la souche sauvage. Le système de régulation d'OmpR couplé à des histidines kinases est également diminué d'un facteur 14 dans la souche JW3606 par rapport à la souche sauvage, 12,5 fois par rapport à la souche JW3596 et 6 fois par rapport à la souche JW3601. Une diminution de 50 % du taux d'OmpF et OmpW est également observée pour chacune des souches mutantes. Les gènes de type *Omp* codent pour des protéines constituantes des porines qui sont fondamentales pour la régulation de la pression osmotique. OmpF est un indicateur de la présence de faible pression osmotique et OmpR est un régulateur transcriptionnel de l'osmorégulation des cellules bactériennes qui gère de ce fait la production de OmpF en cas de faible osmolarité ou de OmpC en cas de forte osmolarité (Rampersaud et al., 1994). Les protéines de type OmpW, découvertes récemment (Albrecht et al., 2006; Hong et al., 2006), sont quand à elles supposées être impliquées dans la protection de la cellule face à un stress environnemental et contre les mécanismes de phagocytose, face à des mécanismes d'oxydation, et jouent également un rôle dans le transport de petites molécules à travers la membrane externe des bactéries (Gil et al., 2007; Hong et al., 2006; Wu et al., 2013; Xu et al., 2005). La diminution de ces protéines de régulation de l'état osmotique et de résistance face aux conditions environnementales défavorables laisse à penser que ces mutants bactériens sont plus sensibles que la souche sauvage à l'exposition aux nanoparticules. Cependant, il semblerait qu'elles sont capables d'enclencher des mécanismes de protections adéquats, en ce qui concerne les souches JW3596 et JW3601 étant donné que seule la souche JW3606 est sensible aux P25-TiO_2-NPs. Il apparaît donc que cette sensibilité pourrait être fortement liée à la mutation de type *rfaG* et à l'accumulation de la flagelline, seule protéine vraiment exprimé très différentiellement dans la souche JW3606 en comparaison des autres souches. La flagelline est une protéine synthétisée à partir du gène *fliC*. Elle est la seule protéine qui compose le flagelle suite à l'assemblage hélicoïdal rigide de plus de 20 000 sous unités de FliC (Aldridge et al., 2006; Guzzo et al., 1991; Macnab, 2004) ne se réalisant qu'après la transcription d'un promoteur de type III sous l'action de protéines chaperonnes encodées par *fliS* qui se fixe à la partie α-hélicoïdal amphipathique C-terminale formant un hétéro-dimer qui évite sa polymérisation (Apel and Surette, 2008; Minamino, 2013). La disparition du flagelle est liée à la mutation *rfaG* (Parker et al., 1992), elle proviendrait de la non incorporation du glucose I dans le core des LPS rejoignant les observations de Komeda et

collaborateur qui ont mis en évidence l'absence de flagelle lors d'une mutation de *galU* (Komeda et al., 1977). Néanmoins, à l'inverse de cette équipe, nous observons une augmentation de la flagelline au sein de la préparation de protéines membranaires de la souche JW3606. La synthèse du flagelle étant supprimée par la mutation *rfaG*, l'accumulation de la flagelline ne devrait pas intervenir puisque une régulation négative est supposée intervenir sur la synthèse du flagelle et des pilis lors de cette mutation (Parker et al., 1992). Komeda et al., 1977, ont également observé une diminution des ARNm codant pour la flagelline ce qui rejoint ces observations. L'organisation de l'ancrage du flagelle (C et MS ring ancré dans la membrane cytoplasmique, P and L rings ancrés dans la membrane externe) laisserait à penser que la flagelline s'accumulerait préférentiellement dans le cytoplasme (Macnab, 2004). Cependant, une étude s'intéressant à 452 espèces bactériennes a mis en évidence que près de 60% des espèces présentaient de deux jusqu'à treize copies du gène *fliC* dans leurs génomes (Zhang et al., 2012). Ainsi, la mutation *rfaG* ne stopperait pas la synthèse de la flagelline mais empêcherait l'assemblage du flagelle, ce qui pourrait conduire à l'accumulation de celle-ci au niveau de la membrane externe, à proximité de son site d'utilisation. Les bactéries Gram négatives produisent à la surface de leur membrane externe des vésicules membranaires externes sphériques (OMVs) dérivées de la membrane externe (Manabe et al., 2013). Ces vésicules peuvent contenir des protéines périplasmique, cytoplasmique et éventuellement des acides nucléiques et servent à l'infection de cellules hôtes, aux transferts génétiques et peuvent également servir de protection contre des agents antibactériens (Horstman and Kuehn, 2000; Kesty and Kuehn, 2004; Nakao et al., 2012; Renelli et al., 2004; Wai et al., 2003; Yaron et al., 2000). Il a récemment été mis en évidence que la flagelline était associée à la membrane externe bactérienne puisque cette protéine se retrouve de façon importante dans le lumen des OMVs, par ce qui semble être un phénomène d'encapsulation (Manabe et al., 2013). Les résultats d'ITRAQ ont montré que la purification membranaire réalisée contenait également de nombreuses protéines cytosolique, une analyse plus poussée de fractions membranaires purifiées par western blot devrait permettre de préciser la localisation de l'accumulation de cette protéine, et confirmer ou infirmer que cette protéine est associée à la membrane externe bactérienne. L'accumulation de la flagelline pourrait conduire à une sensibilité plus importante de la souche mutante vis-à-vis d'une exposition aux P25-TiO2-NPs. De plus, l'accumulation de la flagelline au niveau de la membrane externe pourrait résulter de la diminution des mécanismes de vésiculation provoqués par la mutation *rfaG*. En effet, il a été mis en évidence que cette mutation pouvait conduire à la diminution des phénotypes bactériens réalisant la vésiculation (McBroom et al.,

2006). Ainsi, il semblerait que la toxicité des P25-TiO2-NPs pour les bactéries Gram négatives, en particulier *E.coli* soit dépendante de la structure de la membrane externe bactérienne, en particulier la charge, de sa composition protéique mais également des protéines présentes et de l'accumulation éventuelle de certaines d'entre elles.

Conclusion

Cette étude a permis de mettre en évidence le rôle prépondérant de la structure, de la composition de la membrane externe et des LPS des cellules bactériennes dans les phénomènes d'interactions et de toxicité lors de l'exposition aux nanoparticules de dioxyde de titane. Cependant, le mécanisme exact de cette toxicité demeure incertain et semble dépendre également du type spécifique de protéines présentes au niveau de la membrane externe. Ainsi, des analyses plus poussées ciblées sur ces protéines intramembranaires devraient permettre de déterminer leurs rôles exacts dans la toxicité du dioxyde de titane pour les microorganismes.

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A.N.[a]	Name, gene name	% Cov[b]	Peptides[c]	3606/Ref[d]	Pval [e]	3596/ Ref[d]	Pval [e]	3601/ Ref[d]	Pval [e]
H0QC15	Glutamine transporter subunit, glnH	28.2	2	84.33	4	83.18	4	8.63	2
C4ZQK1	Flagellar filament structural protein (Flagellin), fliC	38.8	9	54.70	4	0.16	3	0.92	0
H0Q6D7	Chaperone SurA, surA	47.9	8	23.77	4	28.71	4	0.51	1
H0QAS5	Conserved protein, elaB PE=4 SV=1	41.6	2	21.58	4	28.71	4	32.06	4
H0QA10	Oligopeptide transporter subunit , oppA	51.6	12	18.45	4	25.35	4	7.31	1
H0QCD5	Glucans biosynthesis protein G, mdoG	33.5	8	15.70	4	14.26	4	1.02	0
H0QD05	Uncharacterized protein, ydgH	51.9	9	14.45	4	14.32	4	1.94	1
H0QBV1	Protein TolB, tolB	33.3	11	13.24	4	15.63	4	2.82	2
C4ZQJ8	Cystine transporter subunit, fliY	42.9	6	9.95	4	7.87	2	1.39	0
H0Q9N4	Cytochrome bd-II oxidase, subunit I, appC	25.5	6	8.71	4	16.90	4	22.39	4
H0Q7J3	Leucine/isoleucine/valine transporter subunit, livJ	39.8	4	8.05	4	7.18	3	0.32	2
H0Q6I1	Protein translocase subunit, secA	47.6	26	7.91	4	6.28	4	5.15	4
H0Q6K3	Inhibitor of vertebrate C-lysozyme, ivy	33.8	2	7.87	4	15.56	4	1.94	1
H0QC94	Chromosome partition protein MukB, mukB	39.7	21	6.00	4	2.90	4	4.45	4
H0QC81	30S ribosomal protein S1, rpsA	33.6	8	5.40	3	4.02	2	3.82	1
H0Q9A2	Alanine--tRNA ligase, alaS	34.1	9	4.85	3	3.40	3	4.00	2
H0QAM2	Ferric iron-catecholate outer membrane transporter, cirA	29.3	8	0.20	4	0.15	2	0.44	4
H0QA23	Outer membrane protein W, ompW	28.3	4	0.19	3	0.04	4	0.18	4
H0Q9W1	Isocitrate dehydrogenase [NADP], icd	18.5	2	0.17	4	0.47	2	1.01	0
B1XE29	Conserved inner membrane protein, yagU	33.3	5	0.14	4	0.09	4	0.35	2
H0QE42	30S ribosomal protein S13, rpsM	58.5	7	0.13	4	0.18	2	0.09	4
H0QBN0	Iron-enterobactin outer membrane transporter, fepA	35.9	8	0.11	4	0.06	3	0.07	4
H0Q838	Membrane-bound lytic murein transglycosylase A, mltA	38.9	7	0.09	4	0.09	3	0.34	2
H0Q9K1	UPF0145 protein YbjQ, ybjQ	33.6	2	0.09	3	0.15	4	1.57	0
H0Q7E6	Sensory histidine kinase in 2-component regulatory system with OmpR, envZ	19.1	2	0.07	4	0.87	0	0.42	0
H0Q7P0	Multidrug resistance efflux transporter, mdtE	43.1	2	5.52	1	23.66	3	24.43	3
H0Q7P1	Multidrug transporter, RpoS-dependent, mdtF	23.2	15	2.42	2	20.04	4	31.33	4
H0QCI0	Nitrate reductase 1, alpha subunit, narG	29.9	22	3.63	3	15.92	4	17.54	4
H0Q6Y7	DegP protein, degP	34.2	6	6.17	1	13.18	3	0.29	0
H0Q9M9	Hydrogenase 1, large subunit, hyaB	32	12	6.55	2	9.77	4	15.56	4
H0Q8T2	Uncharacterized protein, yfeY	23.6	4	3.89	0	9.59	4	1.69	0
H0Q702	Outer membrane protein assembly factor BamA, yaeT	47.5	31	0.56	0	0.15	4	0.77	0
H0QC56	Fused cysteine transporter subunits, cydC	31.6	6	0.36	0	0.15	3	0.40	0
H0QFJ2	Vitamin B12 transporter BtuB, btuB	52.8	18	0.43	2	0.08	4	0.11	4
C4ZQL2	Flagellar M-ring protein, fliF	15.8	2	0.63	0	0.05	3	0.14	2
C4ZV09	DLP12 prophage; outer membrane protease VII (Outer membrane	46.1	17	0.56	0	0.04	4	0.21	2

	protein 3b) , ompT								
C4ZQL3	Flagellar motor switch protein FliG, fliG	14.2	2	1.43	0	0.03	4	0.04	2
H0QD34	50S ribosomal protein L20, rplT	65.3	7	0.24	2	0.03	3	0.98	0
C4ZT38	Methyl-accepting chemotaxis protein I, serine sensor receptor, tsr	42.1	12	2.75	3	0.03	4	0.03	4
C4ZQH0	Methyl-accepting chemotaxis protein II, tar	44.9	16	1.95	2	0.02	4	0.06	4
C4ZUJ1	Bacterioferritin, bfr	26.6	2	3.06	2	0.99	0	18.03	4
H0Q976	Uncharacterized protein, ygaU	73.8	4	5.08	1	4.11	2	10.14	4
H0Q6V7	Predicted transporter subunit, yadG	28.6	2	0.86	2	0.40	1	9.73	3
H0QC73	Pyruvate formate lyase I, pflB	38.3	16	3.16	4	2.79	3	8.24	4
H0Q9C5	Formate hydrogenlyase complex iron-sulfur protein, hycF	21.7	2	4.39	1	0.72	2	7.59	3
H0Q7G9	Glycogen synthase, glgA	25.6	4	3.31	0	3.28	0	7.05	3
H0QB48	Phosphoenolpyruvate-protein phosphotransferase, ptsI	27.8	8	0.61	0	0.58	0	0.14	3
H0QBT4	Succinate dehydrogenase, flavoprotein subunit, sdhA	31.5	11	0.71	0	1.72	1	0.13	4
C4ZQG9	Methyl-accepting protein IV, tap	19.7	6	0.97	2	0.05	2	0.07	3
H0QB03	Long-chain fatty acid outer membrane transporter, fadL	22.1	2	0.19	0	0.04	2	0.04	4

Supplementary table 1. iTRAQ analysis of significantly deregulated proteins in, at least, one of the strains.

[a]Accession Number from the Swiss Prot database
[b]Coverage of the protein sequence by the identified peptides
[c] Number of distinct peptides that allowed protein identification.
[d] Ratio of median signals from one strain to the strain BW25112 considered as the reference strain. A grey background highlight ratio of significant deregulated protein. The protein is considered significantly deregulated when ratio is above or upper the threshold ratio and with at least 3 associated p-value ≤ 0.05.
[e] Number of pValue ≤0.05

Nos travaux précédents sur les effets des TiO_2-NP (P25 Degussa) sur la souche modèle *Escherichia coli* ont mis en évidence une relation directe entre l'adsorption de ces nanoparticules à la surface des cellules bactériennes et leur toxicité. Cette adsorption des nanoparticules est conditionnée par la nature des déterminants biophysiques des interphases bactériennes (présence/nature/propriétés de structures de type LPS et/ou fimbriae, balance hydrophobe/hydrophile, élasticité mécanique des structures membranaires), mais dépend également des interactions électrostatiques qui vont s'opérer entre les bactéries et les nanoparticules. De plus, la composition du milieu dans lequel elles évoluent (nature et valence des ions, force ionique, pH) va être un paramètre fondamental dans la modulation de ces interactions aux interphases en contrôlant les phases d'approche des nanoparticules vers/depuis ces interphases et en régulant l'adsorption/pénétration des nanoparticules de dioxyde de titane sur/dans les structures périphériques bactériennes. Les tests de viabilité cellulaire réalisés sur une dizaine de mutants d'*E. coli* K12 BW25113 présentant des modifications significatives au niveau de leur surface (longueur/densité des lipopolysaccharides (LPS) et des fimbriaes, présence/absence de flagelle) ont mis en évidence une sensibilité accrue (>3 log UFC) de la souche mutées au niveau du gène *rfaG* par rapport à la souche sauvage BW25113 ou aux autres mutants testés. Le mutant *rfaG* possède des LPS tronqués au niveau de l'heptose II, il présente également une disparition du flagelle, une charge de surface plus importante (plus électronégative), une diminution du nombre de protéines constituantes des porines, et une accumulation de 50 à 500 fois supérieure en flagelline.

La toxicité des nanoparticules de dioxyde de titane est donc fortement dépendante des interactions électrostatiques entre la membrane externe bactérienne et les nanoparticules mais la composition et la topologie de la membrane joue également un rôle prépondérant dans l'expression de ce potentiel toxique. L'expression d'une toxicité pour la cellule peut également être le résultat de l'accumulation de dommage à l'ADN ou intervenant lors de la synthèse ou de l'expression de gènes, c'est pourquoi nous avons décidé d'explorer un autre type d'effet néfaste potentiel, la génotoxicité des nanoparticules de titane. En effet, les données acquises précédemment vont nous permettre de se focaliser sur l'optimisation d'un test de mutagénèse, le test d'Ames, dans le but de mettre en évidence le potentiel génotoxique et mutagène des nanoparticules de titane.

Chapitre III : Etudes de la génotoxicité des nanoparticules

I. Le cas du dioxide de titane: Modifications of the bacterial reverse mutation test reveals mutagenicity of TiO_2 nanoparticles and byproducts from a sunscreen TiO_2-based nanocomposite.

Résumé :

Recommandé par l'Organisation de coopération et de développement économiques (OCDE) le test de détection de mutation reverse bactérien a été récemment utilisé par un certain nombre d'équipes scientifiques dans le but d'évaluer le potentiel mutagène des nanoparticules. Lors de ces essais, le test d'Ames a produit des résultats négatifs alors que les autres tests de génotoxicité effectués sur les mêmes nanoparticules se sont avérés positifs. En effet, lorsque des tests *in vitro* sont réalisés avec des nanoparticules sur des cellules de mammifères, les réponses diffèrent de celle observées avec le test d'Ames.

Les travaux réalisés précédemment ont montré qu'une simple préexposition des bactéries à des nanoparticules de titane dans une solution présentant une faible force ionique, à savoir le NaCl 10 mM, et à un pH (pH= 5,5) inférieur au point isoélectrique des nanoparticules de titane (6,8), permettait de favoriser fortement les interactions électrostatiques entre ces deux éléments. Ainsi, nous avons supposé qu'en favorisant l'établissement des interactions entre les bactéries et les nanoparticules nous pourrions augmenter l'efficacité du test. Cette étude vise à déterminer le potentiel mutagène des nanoparticules de dioxyde de titane (TiO_2-P25, TiO_2-NA et TiO_2-TLB) en utilisant le test d'Ames en version liquide, appelé également test en fluctuation. Ce test utilise les souches de *Salmonella typhimurium* TA97a, TA98, TA100 et TA102. Ces expérimentations ont montré que le test, lorsqu'utilisé de façon conventionnelle, n'est pas adapté à l'étude des nanoparticules. En effet, le milieu d'exposition utilisé dans le test présente une force ionique importante qui minimise les interactions électrostatiques entre les microorganismes et les TiO_2-NPs conduisant le test à produire de faux-négatifs.

En modifiant la procédure de test en fluctuation, nous avons mis en évidence le potentiel mutagène des nanoparticules de dioxyde de titane. La souche de *S. typhimurium* TA 102 s'est également révélée être la plus sensible pour détecter ce potentiel mutagène, laissant à penser que l'effet mutagène implique un stress oxydatif malgré la conduite des expérimentations à l'obscurité afin de ce prémunir des effets photocatalytiques des nanoparticules.

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Modifications of the bacterial reverse mutation test reveals mutagenicity of TiO_2 nanoparticles and byproducts from a sunscreen TiO_2-based nanocomposite

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HIGHLIGHTS

- The Ames test is not suitable for nanoparticle (NP) genotoxicity assessment.
- The Ames test medium prevents electrostatic interactions between bacteria and NPs.
- The Ames test medium strongly promotes the aggregation of NPs.
- Simple pre-exposure step in an adequate medium improve the accuracy of the test.
- Modified Ames test showed mutagenicity of NP-TiO_2 and NP-TiO_2-based nanocomposite.

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ABSTRACT

The bacterial reverse mutation test, recommended by the Organization for Economic Co-operation and Development (OECD) to determine genotoxicity of chemical compounds, has been recently used by several authors to investigate nanoparticles. Surprisingly, test results have been negative, whereas in vitro mammalian cell tests often give positive genotoxic responses. In the present study, we used the fluctuation test procedure with the *Salmonella typhimurium* strains TA97a, TA98, TA100 and TA102 to determine the mutagenic potential of TiO_2 nanoparticles (NP-TiO_2) and showed that, when it is used conventionally, this test is not suitable for nanoparticle genotoxicity assessment. Indeed, the medium used during exposure prevents electrostatic interactions between bacterial cells and nanoparticles, leading to false-negative responses. We showed that a simple pre-exposure of bacteria to NP-TiO_2 in a low ionic strength solution (NaCl 10 mM) at a pH below the nanoparticle isoelectric points (pH 5.5) can strongly improve the accuracy of the test. Thus, based on these improvements, we have demonstrated the genotoxicity of the engineered NP-TiO_2 tested and a NP-TiO_2 byproduct from a sunscreen nanocomposite. It was also shown that strain TA102 is more sensitive than the other strains, suggesting an oxidative stress-mediated mechanism of genotoxicity.

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1. Introduction

Due to the increasing industrialization of many countries and resulting technological advances, environmental pollution has become a serious health issue. Many efforts have been made to protect the environment and human health. A priority area concerns the development of in vitro assays to evaluate the toxicological effects of environmental chemicals and then build prioritization models of in vivo toxicity. Over the years, multiple bioassays have been developed utilizing many organisms. Microbial tests have several advantages over other bioassays, including rapid response times due to the much shorter microbial life cycles, reproducibility of test conditions, amenability to genetic manipulations, increased sensitivity and reduced cost (Davoren, 2005). In addition, microorganisms possess the majority of the same biochemical pathways present in higher organisms, they exhibit significant membrane structure organization and generally elicit toxic responses to many chemicals through mechanisms similar to that of higher organisms (Qureshi et al., 1984).

The bacterial reverse mutation assay is recommended by national and international environmental protection agencies for substance evaluations (e.g. Organization for Economic Co-operation and Development (OECD test guideline 471); and The International Conference on Harmonization (ICH)) (Mortelmans

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and Zeiger, 2000). This test has also been recently approved as one of the two assays recommended by The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) (Kirkland et al., 2011). The bacterial reverse mutation test uses several strains of *Salmonella typhimurium* with mutated histidine synthesis genes. The test principle is based on the fact that reverse mutations caused by exposure to mutagenic compounds can reactivate the ability of mutated bacterial strains to synthesis histidine, thereby allowing them to grow in the absence of this essential amino acid. Several procedures for performing the bacterial reverse mutation test have been described. Among those commonly used are the plate incorporation method, commonly named the Ames test (Ames et al., 1972, 1973a,b), the preincubation method (Maron and Ames, 1983; Aeschbacher et al., 1987), the fluctuation method (Green et al., 1976; Hubbard et al., 1984; McPherson and Nestmann, 1990), and the suspension method (Thompson and Melampy, 1981). Over the years, many validation studies have been performed to determine the sensitivity and correlation of this test with animal carcinogenicity studies. It has been established that there is a high predictivity of a positive mutagenic response in the test for rodent carcinogenicity ranging from 77% to 90% (McCann et al., 1975; Tennant et al., 1987; Zeiger, 1998). To date, there are thousands of research and testing laboratories throughout the world using this assay to screen potentially mutagenic drugs and chemicals. Many companies and regulatory agencies use the results from this assay as part of their short-term toxicological testing programs to determine chemical safety (Felton and Wu, 2003).

Nanotechnology is a relatively new branch of science, heralded as a technological revolution (The White House, 2000). Engineered nanoparticles have rapidly moved from the laboratory to industry and are currently being used in many consumer products. Currently, there are over 1000 products in the consumer marketplace that include nanomaterials (Woodrow Wilson Database: http://www.nanotechproject.org), which is projected to substantially increase in the near future. This phenomenon has aroused great concern about potential human health effects and, on a larger scale, environmental effects (Nel et al., 2006), giving birth to a new biological field known as "Nanotoxicology". The Royal Society and Royal Academy of Engineering first raised this concern in 2003 (The Royal Society and the Royal Academy of Engineering, 2003; The Royal Society, 2004), paving the way for a rapid increase in investigational studies on nanoparticle toxicity; in particular, genotoxicity studies, as many nanoparticles were found to cause chromosomal aberrations, DNA strand breaks, oxidative DNA damage, and subsequent genetic mutations (Singh et al., 2009). In commonly used in vitro (chromosomal aberrations, comet assay, micronucleus) and in vivo mammalian test cell systems, nanoparticles have been largely found to promote positive genotoxic responses, while negative responses have been generally obtained for these nanoparticles with the bacterial reverse mutation test (Doak et al., 2012). It was reported that within 19 published studies, where this test was used for the genotoxicological analysis of nanoparticles, 17 showed negative mutagenicity. The two remaining studies only revealed weak mutagenic effects. Therefore, these studies seemed to have indicated that although the Ames test is excellent for testing chemical mutagenic activity, it does not appear to be suitable for nanoparticles. This might be related to the degree of nanoparticle uptake by bacterial cells, which is likely to be less than in mammalian cells (Singh et al., 2009; Doak et al., 2012). Indeed, bacteria cannot perform endocytosis and their cell wall forms a barrier against simple diffusion of nanoparticles. This lack of uptake could potentially lead to false negative results.

Based on our previous work (Pagnout et al., 2012), we think that another plausible hypothesis that leads to false negative results is the lack of interactions between nanoparticles and bacterial cells due to the use of an inappropriate medium during the exposure. We also think that performing the bacterial reverse mutation test by the fluctuation method instead of the plate incorporation method, with a pre-exposure step in a low-ionic strength solution at a pH value below the nanoparticle isoelectric points (NaCl 10 mM, pH 5.5), could improve these interactions and make the test more accurate for the assessment of the nanoparticle genotoxicity. As a consequence, in the present study, we assessed the mutagenic potential of two engineered TiO_2 nanoparticles and a TiO_2-byproduct derived from a nanocomposite material commonly used in sunscreens with the conventional fluctuation test and with a modified version of this test according to the modification mentioned above. TiO_2 nanoparticles (NP-TiO_2) were used as a model in this study for the following reasons: (i) these nanoparticles are widely used in consumer products (e.g. paints, plastics, paper, ceramics, cosmetics, and sunscreens) with expanded applications over the last decade (Colvin, 2003; Gleiche et al., 2006); (ii) in 2006, TiO_2 was reclassified from *Unclassifiable as to carcinogenicity in humans* (Group 3 carcinogen) to *Possibly carcinogenic to humans* (Group 2B carcinogen) based on sufficient evidence using experimental animals (Ng et al., 2010); (iii) NP-TiO_2 was recently listed by the OECD as one of the priority nanomaterials for immediate testing (OECD, 2008); (iv) NP-TiO_2 nanoparticles are minimally water-soluble and their potential carcinogenic effects cannot be attributed to the release of titanium ions in the medium; and (v) several studies showed no mutagenicity (Warheit et al., 2007; Pan et al., 2010) or very weak mutagenicity (Kumar et al., 2011) caused by NP-TiO_2 with the bacterial reverse mutation test (plate incorporation procedure), whereas NP-TiO_2 has been found to have positive genotoxic responses in other in vitro cellular test systems (Balasubramanyam et al., 2009; Di Virgilio et al., 2010; Osman et al., 2010; Shi et al., 2010).

2. Materials and methods

2.1. Evaluated nanomaterials

TiO_2 nanopowder AEROXIDE® P25 (TiO_2-P25) was provided by Evonik Degussa GmbH (Frankfurt, Germany, Stock # 4168050298). These nanoparticles are described by the supplier as having a primary size of 25 nm with a specific surface area (SSA) of 50 ± 15 m^2/g and a ratio of anatase/rutile forms of 80/20. The TiO_2-P25 stock suspension was prepared by dispersing 100 mg of NP-TiO_2 in 10 mL of sterile ultrapure water (milli-Q water, 18.2 MΩ cm). The resultant suspension was then probe-sonicated (Sonics Vibra-cell 750 W, Sonics & Materials, Inc., Newton, CT, USA; frequency 20 kHz, 3 mm micro tip, amplitude 40%) for 30 min at 4 °C to homogenize and break the larger agglomerates apart (Pagnout et al., 2012).

The second type of NP-TiO_2 used in this study (TiO_2-NA) was provided as a 15% (w/v) stable suspension in acidified water produced by Nanostructured & Amorphous Materials, Inc. (Houston, TX, USA – Stock # 7012WJWR). These nanoparticles are described by the supplier as being 100% anatase, with a primary size ranging from 5 to 30 nm, a SSA of 200–220 m^2/g, and a purity >99.5%. The stock suspension was prepared to 10 g/L by dilution in sterile ultrapure water.

The third nanomaterial used in this study was a byproduct obtained after alteration of a TiO_2-based nanocomposite, namely T-Lite™ SF (BASF, Germany) (TiO_2-TLB), which is commonly used in sunscreens as a UV blocker. The T-Lite nanocomposite consisted of a TiO_2 rutile core (5–10 nm cross-section per 50–200 nm length) arranged together in large clusters, which had an average size of 200 nm. These clusters were embedded in an amorphous layer of aluminum oxide [$Al(OH)_3$] and polydimethylsiloxane (PDMS) (Labille et al., 2010; Auffan et al., 2010). The byproduct, resulting from an accelerated ageing process, was provided by Jérôme Labille (CEREGE Laboratory, Aix-en-Provence, France). Briefly, the alteration process consisted of mixing 100 mg of TiO_2-TLB in 250 mL of ultrapure water. The mixture was magnetically stirred at 690 rpm under a white light (400 W Philips® 114 Master HPI-T Plus) for 48 h. After alteration, this mixture was settled for 48 h and the supernatant containing stable altered TiO_2 nanocomposites (byproducts) was obtained (Labille et al., 2010; Auffan et al., 2010). As previously described by Bigorgne et al. (2011), the quantity of TiO_2 byproducts was measured by filtering an aliquot of suspension (20 mL) through a 25 nm membrane filter and drying filter at 105 °C for 24 h. The concentration of TiO_2 byproducts obtained was adjusted to 100 mg/L with ultrapure water.

2.2. Physicochemical characterization of nanomaterials

The shapes and primary sizes of nanoparticles were determined by transmission electron microscopy (TEM), using a CM 20 Philips electron microscope at 200 kV at the Service Commun de Microscopies Electroniques et de Microanalyses (SCMEM, Nancy, France). Samples were prepared by evaporating a droplet of nanoparticle suspension on a copper grid at room temperature. Size was determined using 100 randomly chosen nanoparticles. Nanoparticle crystalline structures were determined by X-ray powder diffraction (XRD). Electrophoretic mobility and nanoparticle size distribution measurements in aqueous media were performed with a ZetaSizer 3000HS (Malvern Instruments, Worcestershire, UK).

2.3. Bacterial electrophoretic mobility

Bacterial electrophoretic mobility measurements were conducted using a Zetaphoremeter IV (CAD Instrumentations, Les Essarts le Roi, France) in a quartz Suprasil® cell (Heraeus Quarzglas GmbH & Co., Hanau, Germany) at 24 °C from the reflection of a laser beam by bacteria tracked with a charge-coupled device camera. Image analysis software was used to process recorded images in real time to calculate the electrophoretic mobilities from the displacement (migration motion) of bacteria subjected to a constant direct-current electric field (800 V/m). Different cycles were recorded to yield 100 bacterial mobility measurements.

2.4. The conventional fluctuation test

The bacterial reverse mutation test was performed using *S. typhimurium* strains TA97a, TA98 to detect GC frameshift mutations, strain TA100 for GC base-pair substitution mutations and strain TA102 for AT base-pair substitution mutations, without S9 metabolic activation. These strains were a gift from Bruce Ames (University of California, Berkeley, CA, USA). The genetic integrities of these strains were verified before the tests for histidine/biotin, rfa marker and pKM101 plasmid (Maron and Ames, 1983). The test was conducted in liquid medium in 96-well microtiter plates, according to the fluctuation method (Environment Canada, 1993). Briefly, bacterial strains were grown overnight in Oxoid broth supplemented with the appropriate antibiotics (25 μg/mL Ampicillin for strains TA97a, TA98, and TA100 and 25 μg/mL Ampicillin + 2 μg/mL Tetracycline for strain TA102) at 37 °C while shaking. To a volume of 2.5 mL of the Ames reagent mixture (108 mL of 5.5× concentrated Davis–Mingioli salts) [38.5 g/L K_2HPO_4, 11.0 g/L KH_2PO_4, 5.5 g/L $(NH_4)_2SO_4$, 1.375 g/L sodium citrate, 0.55 g/L $MgSO_4 \cdot 7H_2O$ – autoclaved 15 min at 121 °C], 24 mL of 40% (w/v) D-glucose (6 mL of D-biotin [0.1 mg/mL], 0.3 mL of L-histidine [1 mg/mL] and 12 mL of bromocresol purple [2 mg/mL]), 5 μL of an overnight culture and 17.5 mL of sterile ultrapure water containing nanoparticles (1, 10, 100 mg/L or water as a control) were added, giving final exposure NP-TiO_2 concentrations of 0, 0.875, 8.75, 87.5 mg/L. A 200 μL volume of the mixture was dispensed into wells of 96-well microtiter plates and incubated for 5 days at 37 °C in the dark. Classical positive controls, 9-aminoacridine (10 μg/mL), 2-nitrofluoren (0.4 μg/mL), sodium azide (25 ng/mL) and cumene hydroperoxide (3.5 μg/mL), were used for TA97a, TA98, TA100 and TA102, respectively. Distillated water was used as negative control. Sterility controls (i.e. sterility of the media, sterility of the NP-TiO_2 and sterility of the positive controls) were also included for each test. The test was considered valid only if the positive controls induced a number of positive wells significantly higher than the number obtained with the negative control (spontaneous revertants), and only if the wells of all the sterility controls stayed negative during the experimentation (0 positive well on the 96 tested).

2.5. The modified fluctuation test

Bacterial strains were grown overnight in Oxoid broth supplemented with the appropriate antibiotic (25 μg/mL Ampicillin for TA97a, TA98, TA100 and 25 μg/mL Ampicillin + 2 μg/mL Tetracycline for TA102) at 37 °C while shaking. A volume of 2 mL of these overnight cultures were transferred to 500 mL Erlenmeyer flasks containing 200 mL of Luria–Bertani medium with the appropriate antibiotics and incubated at 37 °C while shaking until they reached an optical density (OD) of 0.5–0.6 at 600 nm (exponential growth phase). The bacterial cells were then centrifuged at 8000 × *g* for 10 min at 4 °C, washed twice with NaCl 10 mM (ultrapure water – pH 5.5), and resuspended in the same saline solution at a final OD of 4.0 at 600 nm. A 200 μL volume of these bacterial suspensions was transferred to 100 mL flasks containing 20 mL of NaCl 10 mM (ultrapure water – pH 5.5) and nanoparticles (1 mg/L, 10 mg/L or 100 mg/L or water as a control). After a short pre-exposure (0.1 h), 10 h or 20 h at 20 °C under agitation (150 rpm), 500 μL aliquots were transferred to 2.5 mL of the Ames reagent mixture (previously described) and 17 mL of sterile distilled water, giving final concentrations of NP-TiO_2 in the fluctuation test of 0, 0.025, 0.25 and 2.5 mg/L. A 200 μL volume of the mixture thus obtained was dispensed into 96-well microtiter plates and incubated for 5 days at 37 °C in the dark. Experiments were performed independently twice and one microplate was used for each concentration. When these experiments gave contradictory results, the experiment was repeated a third time. Classical positive, negative, and sterility controls were also included as previously described in Section 2.4.

2.6. Transmission electron microscopy of bacterial cells

Transmission electron microscopy (TEM) observations were realized to study NPs and bacterial interactions. After exposure, bacterial suspension was centrifuged at 10,000 × *g* for 10 min at 4 °C. Then, the cells were fixed with a 2.5% glutaraldehyde solution, washed twice with ultrapure water, post-fixed with osmium tetraoxide (OsO_4), washed three times with ultrapure water, and then, dehydrated in graded concentrations of ethanol (Strum et al., 1971). The pellet obtained was embedded in Epon and polymerized for 2 h at 38 °C and 2 days at 60 °C. Ultra-thin sections (90 nm) were collected over copper grids and counterstained with lead citrate and uranyl acetate. These sections were then observed with a CM 20 Philips electron microscope at 200 kV at the Service Commun de Microscopies Electroniques et de Microanalyses (SCMEM, Nancy, France).

3. Results and discussion

3.1. NP-TiO_2 characterization

The NP-TiO_2 used in this study has been thoroughly characterized. Analyses confirmed that TiO_2-P25 is a mixture of anatase and rutile forms (~84% anatase and 16% rutile) with an average primary particle size of 23 ± 4.9 nm (Supporting Information Fig. S1). Dynamic light scattering (DLS) measurements revealed that the average hydrodynamic diameter of the nanoparticle stock suspension obtained after dispersion in milli-Q water and probe sonication ranged between 60 and 80 nm (Supporting Information Fig. S1), meaning that the nanoparticles were agglomerated. The isoelectric point of TiO_2-P25 was previously determined to be approximately pH 6.8 (Pagnout et al., 2012).

TiO_2-NA, which was reported to be 100% anatase, was found to be a mixture of anatase and brookite forms (~86% anatase and 14% brookite) with an average primary particle size of 5.7 ± 1.9 nm (Supporting Information Fig. S2). DLS measurements revealed that the stock suspension provided by NanoAmor, Inc. was well dispersed with an average hydrodynamic diameter of the nanoparticles between 5 and 10 nm (Supporting Information Fig. S2). The isoelectric point of the TiO_2-NA was found to be around pH 6.5 (data not shown).

TiO_2-TLB, resulting from an accelerated ageing procedure of the TiO_2-based nanocomposite T-Lite™ SF, was provided and fully characterized by Labille et al. (2010) and Auffan et al. (2010) (the same stock suspension was used for the present study). It was shown that the alteration process removed most of the PDMS layer and produced stable suspensions of TiO_2 byproduct with a size ranging from 100 to 700 nm. ICP-AES measurements did not detect any measurable concentration in solution and Al was clearly detected by TEM-EDS analysis on the altered nanocomposite, indicating that the Al remained mainly under a solid form at the nanocomposite surface. The isoelectric point of the TiO_2-TLB was found to be between pH 7 and 8 (Labille et al., 2010).

3.2. Mutagenicity assay using the conventional fluctuation test

The bacterial reverse mutation test was performed using *S. typhimurium* strains TA97a, TA98, TA100 and TA102 as described in Section 2 to evaluate the genotoxic potential of NP-TiO_2. The test was conducted in liquid medium in 96-well microtiter plates, according to the fluctuation method. The fluctuation test was shown to be more sensitive than the plate incorporation method, probably because of the greater diffusion of tested compounds in liquid medium than in solid medium. However, exposition of *S. typhimurium* strains to TiO_2-P25, TiO_2-NA and TiO_2-TLB induced no positive response (Table 1).

These results confirm those obtained by Warheit et al. (2007) and Pan et al. (2010), both showed negative mutagenicity of the TiO_2 nanoparticles with the bacterial reverse mutation test (plate incorporation procedure, or Ames test), even at high concentrations. They also reinforce the observations made by Doak et al.

Table 1
Number of revertants obtained with the conventional fluctuation test.

	Concentration (mg/L)	*Salmonella typhimurium* strains			
		TA97a	TA98	TA100	TA102
TiO_2-P25	0	35/40	11/9	15/12	60/62
	0.875	33/36	10/11	16/14	59/60
	8.75	35/35	10/8	19/15	61/63
	87.5	32/34	9/10	18/16	60/59
TiO_2-NA	0	39/33	13/10	12/15	61/60
	0.875	40/36	11/12	14/13	63/61
	8.75	39/34	12/11	11/13	65/60
	87.5	32/30	10/9	12/11	60/59
TiO_2-TLB	0	30/36	11/14	19/15	59/63
	0.875	33/34	9/13	16/14	58/62
	8.75	34/33	12/11	17/14	61/63
	87.5	33/35	10/12	16/13	59/60
Positive controls					
9-AA	10	96/96			
2-NF	0.4		92/94		
SA	0.025			95/91	
CH	3.5				96/96

Numbers of revertants statically different (Chi-square analysis (Gilbert, 1980)) from the number of spontaneous revertants obtained with the control are underlined. Experiments were performed independently twice and one 96-well microplate was used for each concentration. Mutagenicity of NP-TiO_2 is considered when the tests were positive twice (results highlighted in gray).

(2012) that many nanoparticles are negative for mutagenicity in the Ames test, whereas they have widely been found to have positive genotoxic responses in other in vitro mammalian cell test systems. Of 19 studies published, in which this test was used for genotoxicological analysis of nanoparticles, 17 reported negative mutagenicity. The remaining two studies only reported very weak mutagenic effects. The authors related this phenomenon to the degree of nanoparticle uptake by the bacterial cells, which is likely to be less than in mammalian cells, due to their incapability to cross the cell wall.

Based on our previous work on the electrostatic interactions between nanoparticles and *Escherichia coli* (Pagnout et al., 2012), we believe that the lack of interactions between nanoparticles and bacteria could make the bacterial reverse mutation test unsuitable for nanoparticle mutagenicity assessment due to the use of an inadequate exposure medium. We also think that a pre-exposure step in a simple saline solution at a pH value below the nanoparticle isoelectric points, which would allow interactions between bacterial cells and nanoparticles, can strongly improve the accuracy of the test. NP-TiO_2 aggregation and electrophoretic mobilities of both bacterial cells and nanoparticles in the exposure medium of the bacterial reverse mutation assay (AM) and in NaCl saline solution (SS) were conducted.

3.3. NP-TiO_2 aggregation in exposure media

Nanoparticle aggregation measurements were performed in both AM and SS (Fig. 1). No statistical difference was found between TiO_2-P25 aggregate sizes in the two exposure media. Their average sizes were found to be approximately 800 nm in AM and 700 nm in SS. Regarding TiO_2-NA, the average size of aggregates was found to be approximately 3800 nm in AM and only 67 nm in the SS. Significant differences between the average aggregate size in the two media were also observed for TiO_2-TLB, which had sizes of more than 1300 nm in the AM and 730 nm in SS.

Nanoparticle aggregation in exposure media can be explained in the framework of the DLVO theory (named after Derjaguin, Landau, Verwey and Overbeek) of colloidal stability, which considers the balance between the repulsive electrostatic interactions and

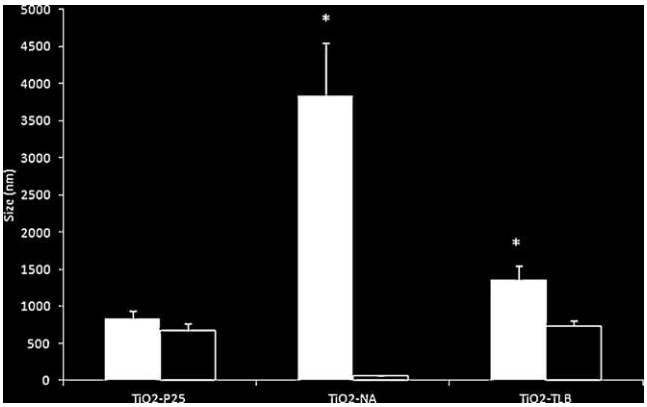


Fig. 1. Average sizes of the NP-TiO_2 aggregates in the Ames medium (black bars) and in the saline solution (white bars) after 5 min. Aggregation experiments were performed independently four times for each NP-TiO_2 in both media. The statistical differences are determined by the Wilcoxon–Mann–Whitney test with a p-value <0.05 (*).

the attractive Van der Waals forces (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). If electrostatic repulsion dominates, the particles repel each other and the nanoparticle suspension is well dispersed. Reversely, if Van der Waals forces dominate, nanoparticles experience mutual attraction and aggregate. The pH, electrolyte concentration, and ion valence determine the strength of electrostatic forces. AM contains polyvalent ions and several organic compounds (glucose, biotin, histidine and bromocresol purple) that can screen charges of the nanoparticles. Moreover, the pH value of AM (pH 7.4) is close to the isoelectric point of NP-TiO_2 (6.8 for TiO_2-P25, 6.5 for TiO_2-NA and between 7 and 8 for TiO_2-TLB). In these conditions, nanoparticles quickly aggregate as soon as they are introduced to AM and then cannot properly interact with bacterial cells during the bacterial reverse mutation assay, potentially leading to the underestimation of their mutagenic potential. Conversely, SS contains exclusively monovalent ions and has a pH value (pH 5.5) further away from the isoelectric points of NP-TiO_2, thereby leading to reduced nanoparticle aggregation compared to AM.

3.4. Bacterial and NP-TiO_2 electrophoretic mobility in exposure media

Electrophoretic mobility of both NP-TiO_2 and *S. typhimurium* strains in AM and in SS are presented in Table 2. Regardless of the medium considered, bacterial strains are always negatively charged. Depending on the strain, electrophoretic mobilities vary from −0.27 μm/s/V/cm (strain TA100) to −2.30 μm/s/V/cm (strain TA98) in AM and from −0.79 μm/s/V/cm (strain TA100) to −4.87 μm/s/V/cm (strain TA97a) in SS. These results are consistent with those previously obtained by several authors showing that at

Table 2
Electrophoretic mobilities of the *Salmonella typhimurium* strains and NP-TiO_2.

		Mobility in the fluctuation test medium (μm/s/V/cm)	Mobility in the saline solution (μm/s/V/cm)
S. typhimurium strains	TA97a	−2.07	−4.87
	TA98	−2.30	−4.74
	TA100	−0.27	−0.79
	TA102	−0.92	−1.63
NP-TiO_2	TiO_2-P25	−2.94	+2.42
	TiO_2-NA	−2.60	+3.19
	TiO_2-TLB	−2.70	+1.99

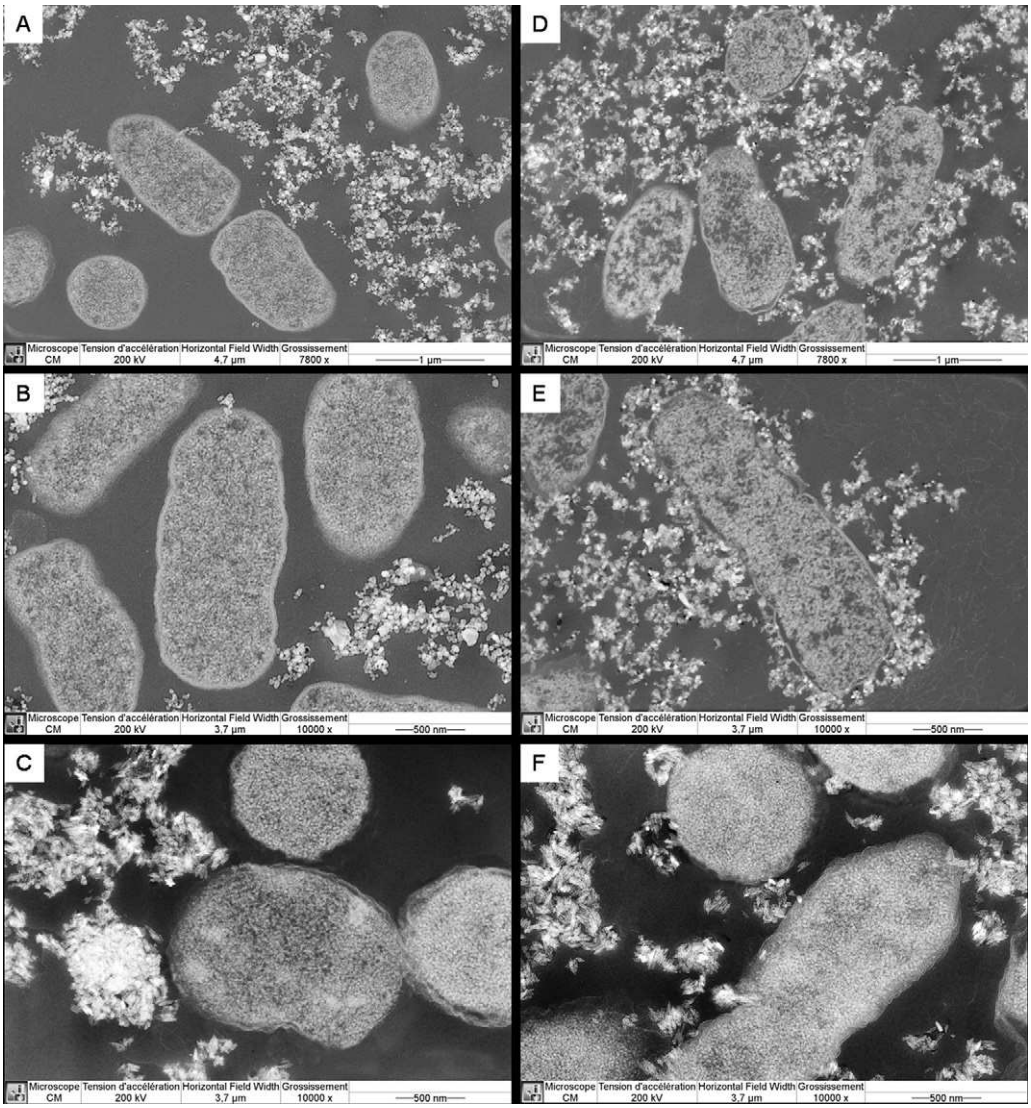


Fig. 2. Transmission electron microscopy images of *Salmonella typhimurium* TA 102 after TiO_2-P25 (A, B, D, and E) and TiO_2-TLB exposure (C and F) in the exposure medium of the bacterial reverse mutation test (A–C) and in the NaCl saline solution (D–F).

environmental pH, most bacterial cell wall surfaces are negatively charged due to the presence of several functional groups (carboxyl, phosphate and hydroxyl) (Blake et al., 1999; Kiwi and Nadtochenko, 2004; Rincon and Pulgarin, 2004).

Conversely, in AM, the NP-TiO_2, which we previously showed aggregated, have strongly negative electrophoretic mobilities (−2.94 μm/s/V/cm for TiO_2-P25, −2.60 μm/s/V/cm for TiO_2-NA and −2.70 μm/s/V/cm for TiO_2-TLB) (Table 2). In contrast, all NP-TiO_2 exhibited net positive charges in SS (+2.42 μm/s/V/cm for TiO_2-P25, +3.19 μm/s/V/cm for TiO_2-NA and +1.99 μm/s/V/cm for TiO_2-TLB).

Consequently, electrostatic forces between negatively charged bacterial cells and nanoparticles in AM would be repulsive; whereas in SS, these forces would be attractive due to the nanoparticle charge inversion, leading to higher interactions. This is supported by the TEM observations showing that nanoparticles strongly adsorb on the surface of the bacterial cell wall in SS, whereas this is not the case in AM (Fig. 2).

3.5. Mutagenicity assay using the modified fluctuation test

The conventional fluctuation test was modified as described in Section 2. A pre-exposure step of 0.1 h, 10 h or 20 h in SS was performed before testing to improve interactions between NP-TiO_2 and bacterial cells. Results presented in Table 3 revealed high mutagenicity of NP-TiO_2 tested with *S. typhimurium* strain TA102. For TiO_2-P25, positive responses were obtained at higher nanoparticle concentrations after 0.1, 10 h or 20 h of pre-exposure in SS. The strongest response was observed with 100 mg/L of TiO_2-P25 after 20 h pre-exposure with 96 positive wells. TiO_2-P25 mutagenicity was also detected to a lesser extent with strain TA98 at 100 mg/L of nanoparticles after 0.1 and 10 h pre-exposure in SS and at 10 and 100 mg/L after 20 h pre-exposure in SS. For TiO_2-NA, mutagenicity was exclusively detected with strain TA102. A stronger response was obtained with 1 and 10 mg/L of nanoparticles after 10 h or 20 h pre-exposure of 96 positive wells. Table 3 also indicated that NP-TiO_2, which had an average size much smaller than TiO_2-P25 in SS (∼67 nm for the TiO_2-NA and ∼700 nm for the TiO_2-P25), induced toxicity in strains TA97a, TA98 and TA102 at higher concentrations (numbers of revertants obtained after exposure lower than the number of spontaneous revertants obtained with the control). For the TiO_2-byproduct (TiO_2-TLB), as for all NP-TiO_2, mutagenicity was detected with strain TA102 at 100 mg/L after 0.1 h pre-exposure, 10 and 100 mg/L after 10 h pre-exposure, and 1, 10 and 100 mg/L after 20 h pre-exposure in SS. To insure that the mutagenicity observed

Table 3
Number of revertants obtained with the modified fluctuation test using the SS as pre-exposure medium.

	Pre-exposure time (h)	Pre-exposure concentration (mg/L)[a]	*Salmonella typhimurium* strains[b]			
			TA97a	TA98	TA100	TA102
TiO_2-P25	0.1	0	43/32/43	12/5	10/14/11	68/41/70
		1	34/35/44	17/2	10/12/8	67/61/69
		10	30/35/33	12/10	5/21/18	71/68/65
		100	35/42/32	43/81	19/10/17	95/71/68
	10	0	23/36	17/7	13/15/18	65/45/63
		1	30/26	13/8	13/5/11	64/57/67
		10	36/35	14/14	11/7/12	66/64/76
		100	36/38	76/59	11/8/15	96/65/94
	20	0	22/27	11/14/12	14/18	68/40/74
		1	28/24	32/22/23	12/13	64/74/82
		10	37/28	63/22/45	19/10	67/57/87
		100	38/22	89/94/88	13/11	96/96/96
TiO_2-NA	0.1	0	37/33	5/21	13/10	68/66/70
		1	41/36	6/27	12/17	84/65/96
		10	31/35	7/18	15/15	92/79/96
		100	26/31	4/10	14/15	96/87/90
	10	0	29/30	21/18	16/31	69/76
		1	32/32	12/11	15/24	96/96
		10	22/23	13/9	10/28	96/96
		100	17/18	1/0	10/17	37/16
	20	0	32/34	7/13	15/17	59/72/64
		1	40/34	9/11	11/17	92/96/96
		10	37/32	4/6	12/18	96/96/96
		100	15/3	4/0	24/14	30/72/66
TiO_2-TLB	0.1	0	25/25/22	6/26	21/10	67/61/66
		1	20/25/31	8/17	12/14	71/68/72
		10	17/35/33	4/24	19/18	81/66/70
		100	14/35/32	6/14	19/14	95/77/58
	10	0	31/24	19/15	33/25	65/68
		1	25/27	17/18	43/22	72/70
		10	37/31	13/17	50/30	78/80
		100	21/31	14/19	43/31	77/82
	20	0	18/25	4/25	16/10	68/63/76
		1	16/36	3/11	14/8	96/70/90
		10	18/35	4/16	19/10	90/70/96
		100	22/31	7/14	40/20	96/84/96
Positive controls						
9-AA		10	96/96/96			
2-NF		0.4		95/96/93		
SA		0.025			85/96/96	
CH		3.5				96/96/96

[a] NP-TiO_2 concentrations in SS, giving final concentrations of 0, 0.025, 0.25 and 2.5 mg/L in the fluctuation test.
[b] Numbers of revertants statically different (Chi-square analysis (Gilbert, 1980)) from the number of spontaneous revertants obtained with the control are underlined. Experiments were performed independently twice and one 96-well microplate was used for each concentration. When these experiments gave contradictory results, the experiment was repeated a third time. Mutagenicity of NP-TiO_2 is considered when the tests showed positive responses at least twice (results highlighted in gray).

with the TiO_2-TLB was not related to other elements present in the byproduct, such as aluminum or PDMS residues released during the nanocomposite alteration process (Labille et al., 2010), the fluctuation test was also performed on the supernatant fraction obtained after centrifugation (10,000 × *g* for 5 min at 4 °C) and filtration using a 25 nm membrane filter. Regardless of the strain used, results showed no mutagenicity of this fraction (data not shown).

An additional set of experiments was also done using the AM instead of the SS as a pre-exposure medium to support the hypothesis of an exposure medium-effect in the NP-TiO_2 genotoxicity observed with the modified fluctuation test, but not with the conventional one. Indeed, the pre-exposure step of the modified fluctuation test is performed in 100 mL flasks at 20 °C under agitation, whereas the conventional test is performed in 96-well microtiter plates at 37 °C without agitation. Results obtained after 20 h of bacterial cell pre-exposure to TiO_2-P25, TiO_2-NA and TiO_2-TLB in the AM showed no positive response (Supplementary data Table S1), supporting the initial hypothesis that a pre-exposure step in a simple saline solution (NaCl 10 mM, pH 5.5) improved the interactions between nanoparticles and bacterial cells and the accuracy of the fluctuation test.

To the best of our knowledge, this study is the first to report NP-TiO_2 mutagenic potential by the use of the bacterial reverse mutation test. The mechanisms by which these nanoparticles can cause mutagenicity have not been clearly elucidated, but several pathways have been examined. Some studies have shown that nanoparticles can potentially cross the bacterial cell wall and locate to the cytoplasm (Sondi and Salpotek-Sondi, 2004; Xu et al., 2004; Brayner et al., 2006; Huang et al., 2000; Kumar et al., 2011). In this case, mutagenicity caused by direct or indirect interaction between nanoparticles and DNA, and/or its regulatory apparatus (primary mechanisms), could be a first hypothesis. However, in our study, mutagenicity was observed even with large-sized or aggregated nanoparticles. Moreover, TEM observations of the *S. typhimurium* strains after exposure did not show the presence of NP-TiO_2 inside the cells (>50 cells observed per bacterial strain)

(Fig. 2). Another hypothesis of indirect genotoxic mechanisms, resulting from reactive oxygen radical induction that may damage lipids, proteins or DNA, is more likely (secondary mechanisms). This idea is supported by the high sensitivity of the TA102 strain in detecting NP-TiO_2 mutagenicity. Indeed, this strain was specifically developed by Levin et al. (1982) to detect a variety of oxidative mutagens. However, even though there is ample evidence for reactive oxygen species (ROS) formation when TiO_2 is exposed to UV light (Matsunaga et al., 1985; Konaka et al., 1999; Hirakawa et al., 2004; Gurr et al., 2005), the ROS production without UV irradiation is less documented and somewhat more controversial. Fenoglio et al. (2009) showed direct evidence that NP-TiO_2 are active in the generation of free radicals, including oxygenated free radicals and carbon-centered radicals, causing cleavage of C—H bonds in a model organic molecules. These reactions can occur even in the dark and could serve as the first step of oxidative damage of biological molecules such as polyunsaturated fatty acids to form harmful aldehydes that can trigger longer periods of damage to proteins and other molecules (i.e. lipid peroxidation). Such redox interactions could in turn generate more free radicals that perpetuate the formation of highly reactive epoxides that could further compromise the integrity of the cell membrane and damage DNA (Lyon et al., 2007; Cabiscol et al., 2000). Gou et al. (2010) recently highlighted this mechanism by analysis of the *E. coli* whole-cell-array stress genes exposed to NP-TiO_2 in the dark. The mechanisms by which lipid peroxidation may be involved in mutagenicity are twofold. The lipid-derived radicals and their breakdown products, such the malondialdehyde (MDA) which is a major dead-end product, can interact with DNA, forming intra- and inter-strand DNA crosslinks and cyclic base adducts. MDA is known to revert frameshift and base-pair substitution mutations in *S. typhimurium* (Marnett et al., 1985; Basu and Marnett, 1983), which could explain the TiO_2-P25 mutagenicity detected with both TA102 and TA98 strains. Lipid peroxidation products are also known to affect the cell indirectly by altering cell metabolism, binding to cellular proteins, inactivating macromolecule synthesis and depleting cellular antioxidants such as glutathione (Howden and Faux, 1996). In regards to mammalian/human cell studies, it was shown that, even in the dark, NP-TiO_2 induces oxidative stress-mediated toxicity in many cell types (Gurr et al., 2005; Wang et al., 2007; Hussain et al., 2010; Shukla et al., 2011; Meena et al., 2012; Saquib et al., 2012) and the lipid peroxidation was also suspected as the underlying mechanism of this genotoxicity. On human bronchial epithelial cell line (BEAS-2B), Gurr et al. (2005) reported that in the absence of photoactivation, the anatase NP-TiO_2 (10 and 20 nm) can induce an increase of the cellular MDA level, oxidative DNA damage, and increase cellular nitric oxide and hydrogen peroxide levels. Shukla et al. (2011) that NP-TiO_2 can induce micronucleus formation, significant reduction in glutathione with a concomitant increase in lipid hydroperoxide and ROS generation in exposed human epidermal cells.

4. Conclusions

The booming demand for nanoparticles has spurred significant public alarm about their possible adverse effects. TiO_2 nanoparticles, like several other nanoparticles, have been listed by the OECD as a priority nanomaterial for immediate testing. Several studies have been published, in which the bacterial reverse mutation test (OECD test guideline 471) was utilized for genotoxicological analysis of nanoparticles. Most have reported negative mutagenicity of nanoparticles, whereas the same nanoparticles have largely been found to have positive genotoxic responses in other in vitro mammalian cell test systems (Doak et al., 2012).

In the present study, we showed that the medium used for bacterial exposure to nanoparticles in the bacterial reverse mutation test is not suitable. Indeed, this medium prevents electrostatic interactions between bacterial cells and nanoparticles and promotes the aggregation of the latter. It was also shown that a simple pre-exposure step in a low ionic-strength solution, at a pH below the nanoparticle isoelectric points (NaCl 10 mM, pH 5.5), could strongly improve the accuracy of the test. Thus, based on these improvements, we highlighted the mutagenicity of the two engineered NP-TiO_2 tested and a NP-TiO_2 byproduct derived from a nanocomposite that is commonly used in sunscreens and purportedly inert.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2012.09.012.

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Supplementary Information

SUPPLEMENTARY INFORMATION FOR: Modification of the Bacterial Reverse Mutation Test Reveals Mutagenicity of TiO_2 Nanoparticles and a byproduct from a TiO_2-Based Nanocomposite used in Sunscreens. S. JOMINI, J. LABILLE, P. BAUDA, C. PAGNOUT

Nanoparticle Characterization.

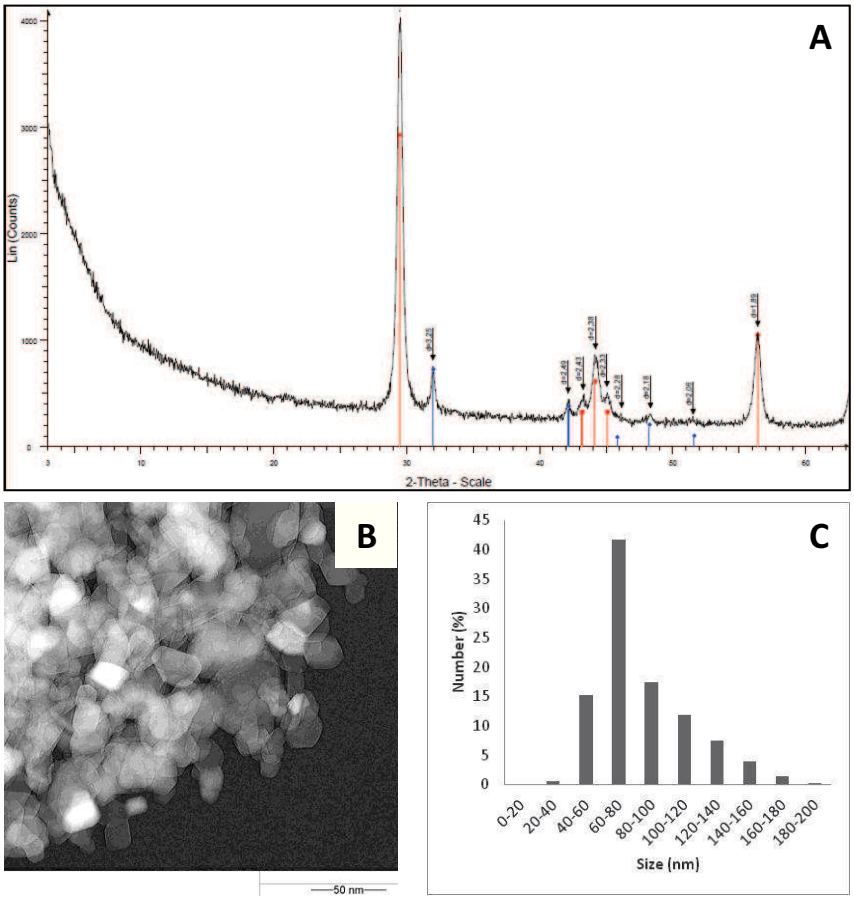


Figure S1: Characterization of the TiO_2-P25 nanoparticles. (A) X-ray diffraction pattern of the initial TiO_2-P25 nanoparticles. Red peaks were assigned to the anatase form and blue peaks to the rutile form; (B) TEM micrograph and (C) Hydrodynamic diameter of the TiO_2-P25 nanoparticles stock suspension after 30 min of dispersion by sonication in ultrapure water.

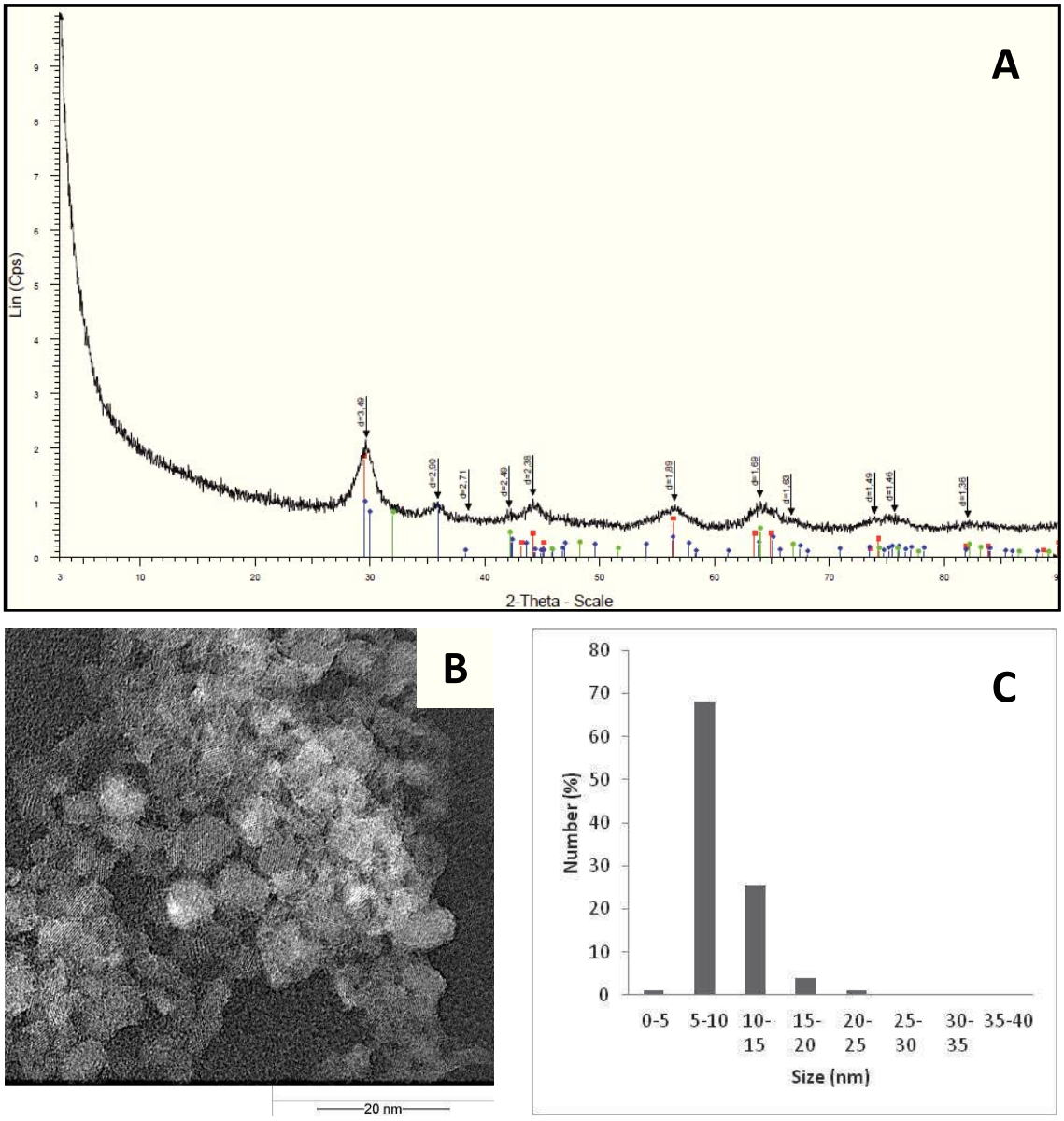


Figure S2: Characterization of the TiO_2-NA nanoparticles. (A) X-ray diffraction pattern of the initial TiO_2-NA nanoparticles. Red, blue and green peaks were respectively assigned to the anatase, brookite and rutile forms; (B) TEM micrograph and (C) Hydrodynamic diameter of the TiO_2-NA nanoparticles stock suspension provided by NanoAmor, Inc.

Table S1. Number of revertants obtained with the modified fluctuation test using the AM as pre-exposure medium (20 h of pre-exposure).

		***Salmonella typhimurium* strains**[b]			
	Pre-exposure concentration (mg/L)[a]	**TA97a**	**TA98**	**TA100**	**TA102**
TiO_2-P25	0	41/39	13/11	12/11	66/61
	1	40/40	12/13	15/9	63/60
	10	42/39	12/10	11/10	65/59
	100	40/41	10/12	9/11	64/61
TiO_2-NA	0	36/35	15/10	14/12	58/60
	1	37/38	13/11	11/14	62/62
	10	37/37	14/10	13/12	60/59
	100	34/33	10/9	10/9	56/57
TiO_2-TLB	0	41/39	13/15	16/19	60/61
	1	39/38	12/14	18/16	61/57
	10	43/36	13/13	17/17	59/58
	100	38/35	13/14	15/16	59/60
Positive controls					
9-AA	10	96/96			
2-NF	0.4		96/94		
SA	0.025			96/96	
CH	3.5				96/96

a. NP-TiO_2 concentrations in AM, giving final concentrations of 0, 0.025, 0.25 and 2.5 mg/L in the fluctuation test.

b. Numbers of revertants statically different (Chi-square analysis (Gilbert, 1980)) from the number of spontaneous revertants obtained with the control are underlined. Experiments were performed independently twice and one 96-well microplate was used for each concentration. Mutagenicity of NP-TiO_2 is considered when the tests were positive twice (results highlighted in grey).

II. Revised procedure of the Bacterial Reverse Mutation Test for genotoxic evaluation of nanoparticles

Résumé

L'évaluation du potentiel mutagène et génotoxique des nanoparticules constitue une préoccupation de première importance au vue de la production et de l'utilisation de plus en plus frénétique de ces particules. Ainsi, l'ensemble de la communauté scientifique s'évertue à déterminer dans quelles proportions ces composées peuvent potentiellement présenter un risque de génotoxicité. Une review récente s'intéressant aux stratégies de tests des nanoparticules in vitro a mis en évidence que le test de détection de mutation reverse bactérien (le test d'Ames) produit principalement des résultats négatifs alors que l'ensemble des autres tests disponibles conduisent à une réponse positive (Doak et al., 2012). De ce fait, la pertinence et l'efficacité de ce test pour l'évaluation du potentiel mutagène des NPs est sujet à d'importantes discussions au sein de la communauté scientifique. Pour pouvoir détecter un effet mutagène par l'intermédiaire de ce test, l'interaction entre la bactérie et le xénobiotique d'intérêt est nécessaire de même que la pénétration de cette substance. Cependant, les organismes procaryotes sont incapables de réaliser l'endocytose et les NPs se révèlent être majoritairement d'une taille trop importante pour être librement transportées au travers des pores de la membrane bactérienne. Malgré cette impossibilité, un pouvoir mutagène peut apparaître. En effet, certaines nanoparticules sont capables de générer des radicaux libres, ou espèces réactives de l'oxygène, produits intrinsèquement ou indirectement lorsque les nanoparticules sont adsorbées sur la surface bactérienne. Ces évènements devraient être détectables par le test d'Ames.

Un autre phénomène pouvant expliquer la faible efficacité du test d'Ames à détecter un potentiel mutagène des nanoparticules est le manque d'interactions entre les nanoparticules et les cellules bactériennes du fait de la forte agrégation des nanoparticules. En effet, lorsque les nanoparticules sont placées dans un milieu riche, les nanoparticules tendent à s'agréger, une diminution de leur potentiel de surface s'opère et des phénomènes de répulsion électrostatique entre bactéries et nanoparticules interviennent car les deux vont présenter une charge de surface négative.

Basé sur nos précédent travaux qui ont montré qu'il était possible d'améliorer la sensibilité du test d'Ames pour la détection du potentiel mutagène des nanoparticules (Jomini et al., 2012), ce chapitre prodigue un certains nombres de recommandations pour l'évaluation

du potentiel génotoxique et mutagène des nanoparticules en proposant un protocole modifié du test d'Ames intégrant les particularités des nanoparticules.

Revised procedure of the Bacterial Reverse Mutation Test for genotoxic evaluation of nanoparticles

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Summary

A recent review of in vitro genotoxicity testing strategies for nanoparticles (NPs) revealed that the conventional *Bacterial Reverse Mutation* test (Ames test) primarily yielded negative results, whereas the other in vitro genotoxicity assays were usually positive. Accordingly, the efficiency of the test for NP evaluation was questioned, as was the NP entrance in bacterial cells. Indeed, prokaryotes are unable to perform endocytosis and NPs are too large to be transported through the pores of the cell wall. However, regardless of whether they have been internalized, the mutagenic potential of free radicals, produced intrinsically or indirectly by NPs adsorbed onto the bacterial cell walls, should be detected by the *Bacterial Reverse Mutation* test.

Another phenomenon that can explain the low test efficiency is the lack of interactions between NPs and cells due to strong NP aggregation in the exposure medium of the assay and the presence of electrostatic repulsive forces between NPs and bacteria, which both carry overall negative charge. This hypothesis is supported by our recent study that revealed the mutagenic potential of NP-TiO_2 using a revised procedure of the *Bacterial Reverse Mutation* test, which improves the NP-cell interactions and the sensitivity of the test.

In the present chapter, we provide several recommendations for the genotoxic evaluation of NPs and propose a revised version of the *Bacterial Reverse Mutation Test* more in line with the specific properties of NPs.

Key words: bacterial reverse mutation assay, Ames test, fluctuation, nanoparticle, mutagenicity, genotoxicity, in vitro, oxidative stress, *Salmonella typhimurium*, *Escherichia coli*.

1. Introduction

The definition of nanoparticles (NPs), or ultrafine particles, continues to be the subject of debate. However, there is widespread agreement that NPs are particles with sizes between 1 and 100 nm that are manufactured specifically for their particular physicochemical properties, compared to bulk material. Engineered nanoparticles are increasingly being produced for use in a wide range of industrial and consumer products, raising concerns regarding their impacts on human and environmental health.

A large number of scientific papers have recently been published to highlight these issues, but given the large number of variables such as the characteristics of NPs tested (different chemical composition, size, shape, surface recovery), suspension/dispersion protocols (sonication, stirring, mixing, use of a dispersing agent or not), toxicological assays used (endpoints, tested cells/species, methods of administration, dose range, exposure periods), and the relevance of these assays for evaluation of NPs, conclusively establishing safety or toxicity of NPs is still a major challenge. Additionally, it is becoming evident that NPs cannot be treated as chemical compounds with regards to their safety as their unique physico-chemical properties introduce an additional level of complexity.

According to the scientific community, more work should be conducted to standardize (i) physico-chemical characterization procedures, (ii) dispersion protocols, (iii) methods for (eco)toxicity evaluation, the existing assays having been designed for evaluation of chemical substances, and (iv) to identify standard reference NPs to correlate test results across laboratories to improve the reliability and reproducibility of data.

The *Bacterial Reverse Mutation Test* is recommended by several regulation agencies (Organisation for Economic Co-operation and Development, Food and Drug Administration, International Conference on Harmonisation) for substance evaluation and, due to its simplicity and relatively low cost, is commonly employed as an initial screening method for genotoxic activity. In this chapter, we present a revised version of this test, taking into account in a more precise way particular properties of NPs, and we provide recommendations for genotoxic evaluation of NPs.

1.1 The Bacterial Reverse Mutation Test and its different procedures

The *Bacterial Reverse Mutation Test* was initially developed by Bruce Ames in the 1970s (1-3). This test evaluates the genotoxicity of compounds by measuring their ability to induce reverse mutations (frameshift mutations or base-pair substitutions) in auxotrophic bacterial strains of *Salmonella typhimurium* (histidine –) or *Escherichia coli* (tryptophan –). The revertant bacteria are then detected by their ability to grow in absence of the amino acid required by the auxotroph strain.

The strains used in the *Bacterial Reverse Mutation Test* present specific features that make them more sensitive for the detection of mutagens such as i) increased cell wall permeability with regard to large molecules due to mutation, which causes partial loss of the lipopolysaccharide barrier (*rfa* mutation), ii) mutation in the bacterial cell system to excise and repair defects in the DNA resulting in the inability to repair damaged/mutated sections (*uvrB* mutation) and iii) for specific strains, a R-factor plasmid pKM101 that increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system (4). Strain TA102 also contains the *hisG428* mutation on the multicopy plasmid pAQ1 to enlarge the number of target sites (Table 1) (4, 5). The OECD guideline for testing of chemicals (OECD No. 471) recommends the use of at least the five following strains in its testing strategy: *S. typhimurium* TA1535, TA1537 (or TA97 or TA97a), TA98, TA100, TA102 (or *E. coli* WP2 *uvrA* or *E. coli* WP2 *uvrA* (pKM101)) (6).

The conventional method for performing the *Bacterial Reverse Mutation Test*, also called the *Plate incorporation method/procedure* or, more generically the *Ames test*, consists of exposing the bacterial suspensions (100 µL of ~10^9 viable cells/mL) to grade concentrations of the test substance (50 µL or 100 µL) in the presence or absence of an exogenous metabolic activation system (500 µL of S9-mix containing an adequate amount of post-mitochondrial fraction of rat liver homogenate). This metabolic system is optionally added to simulate the effects of mammalian metabolism as some compounds are not mutagenic themselves, unlike their metabolic products. These mixtures are immediately supplemented with a soft overlay agar (2.0 mL) and poured into Petri dishes onto minimal medium. After 2 or 3 days of incubation at 37°C, revertant colonies are counted and compared to the background rate of reverse mutation to establish the genotoxicity of the substance. The significance is then determined by several statistical approaches (7, 8). A detailed description and recommendations for performing this test are given in (4, 9-13).

Several other procedures for performing the *Bacterial Reverse Mutation Test* have been developed in past decades, with the main objectives of making the test more sensitive or more suitable for evaluation of substances with specific properties (volatile chemicals or gases, small volume of sample to test, presence of histidine in the test substance…).

The most commonly used variant is the *pre-incubation method/procedure*, in which an incubation step of 20-90 min at 30-37°C in phosphate buffer or S9 mix (500 µL), is introduced prior to adding the soft agar and pouring into Petri dishes as previously described. During the pre-incubation period, tubes are aerated by shaking. The *pre-incubation procedure* is often reported to be more sensitive than the conventional plate incorporation method as short lived mutagenic metabolites have a better chance of reacting with the tester strains than when the incubation mixture is immediately poured into Petri dishes. The *pre-incubation procedure*, fully described in (10), was used in the 80s and 90s for large-scale chemical testing (14-17).

Another popular alternative to the *Plate incorporation method* is the *fluctuation method/procedure*, in which the bacterial growth is entirely performed in liquid medium and not on solid agar medium (18, 19). The medium contains a purple pH indicator (purple bromocresol), which changes color and turns yellow when the pH decreases due to the metabolic process of growing bacteria. After bacterial cell incubation with the test substance for up to 5 days in 96-well microplates, the number of revertants is scored by counting the number of yellow wells. Genotoxicity is then established by comparing the number of yellow wells in treated plates and in the control and significance is established by Chi-square analysis (20). The *fluctuation method* has not been reported to be much more sensitive than the *plate incorporation method*, but it allows testing of higher concentrations of samples (>80% against <2% v/v for the plate incorporation), and is less time consuming in terms of protocol execution (no agar plate to prepare) and reading results (counting 96-wells faster than counting colonies on agar plates). Detailed protocols for the fluctuation procedure have been described in (19, 21, 22).

Other alternative procedures have been developed and used less frequently, such as the suspension method (23) and procedures using desiccators or other sealed vessels to test volatile substances or gases (10, 24, 25).

1.2 NP genotoxicity and evaluation using the Bacterial Reverse Mutation Test

NPs can induce genotoxic effects via physical interactions with genomic DNA or components that determine its integrity (direct primary mechanism), or indirectly by the production of reactive oxygen species (ROS) via active redox cycling on their surfaces, especially on metal-based NPs (indirect primary mechanism), or also through activation of neutrophils and macrophages mediating an inflammatory reactions (secondary mechanism). For review on NP genotoxicity, refer to (26-30).

Over the past decade, the traditional *Bacterial Reverse Mutation Test* (Ames test) has been used by several authors for NP evaluation. In about 20 published studies, the test was always negative or weakly positive, whereas other in vitro genotoxicity assays (e.g., Comet assay, Micronucleus assay…), sometimes performed in parallel with the same NPs, gave mostly positives results (29). Therefore, the efficiency of the *Bacterial Reverse Mutation Test*, and more particularly the degree of NP uptake by cells, was questioned. Indeed, prokaryotes are unable to perform endocytosis and NPs are too large to be transported through the pores of the cell wall (27, 29, 31, 32, 34). Even if some authors are able to locate NPs in the bacterial cytoplasm (33, 34), the ability of NPs to penetrate the double-lipid bilayer of Gram negative bacteria is still the subject of debate. The presence of these NPs inside the cells may be attributed to the precipitation of metal ions released from water-soluble NPs or a forced entry following multiple preparation steps for transmission electron microscopy (centrifugation, dehydration, embedding, slicing).

However, regardless of internalization, the mutagenic potential of free radicals produced intrinsically by NPs adsorbed onto the bacterial cell walls should be detected by the *Bacterial Reverse Mutation Test*, particularly for strains TA102 and TA104, which are especially sensitive to oxidative mutagens (5, 10).

If we closely examine the way in which the traditional *Bacterial Reverse Mutation Test* is carried out, bacterial cells and NPs are mixed together in an aqueous medium containing high salt concentrations and various organic compounds, originating from the nutritive medium in which the cells were grown overnight. This mixture is then supplemented with a soft agar that will quickly solidify and that also contains salts and organic substances. Considering theories on the adhesion of colloidal particles such as the DLVO (35-36), the probability of NPs entering into contact with bacteria is strongly minimized by their own aggregation and their possible interactions with material other than bacterial cells present in the exposure medium.

1.3 Revised procedure of the Bacterial Reverse Mutation Test for NP evaluation

We previously highlighted the paramount role that electrostatic forces plays in the interactions between NPs and bacteria, and the importance of medium characteristics such as pH, ionic strength and electrolyte composition in controlling these forces (37). We also examined the behavior of TiO_2-NPs in complex medium, such as the test medium of the *Bacterial Reverse Mutation Assay*, and revealed their immediate and rapid aggregation as soon as they are introduced (38). It was also shown that TiO_2-NPs, which usually display an isoelectric point (IEP) around pH 7, where also shown to be negatively charged in this medium. Similarly, the *S. typhymurium* strains carry an overall negative charge due to the presence of carboxylic and phosphate groups on their surfaces (39). Accordingly, interaction and subsequent contact between NPs and bacteria are limited by repulsive electrostatic forces between each other, which may explain the negative results commonly obtained by the *Bacterial Reverse Mutation Test* when evaluating the genotoxicity of NPs. This hypothesis is supported by the fact that a simple pre-exposure step in a medium favoring NP-cell interactions before conducting the test leads to the outcome of a positive *Bacterial Reverse Mutation* assay on NP-TiO_2 (38). *S. typhimurium* TA102 was found to be more sensitive than TA97, TA98, and TA100 for the detection of mutagenic events.

To make the *Bacterial Reverse Mutation* assay more sensitive and accurate for the detection of NP genotoxicity, we suggest that several aspects of the test should be considered.

Pre-exposure medium: It has been shown that in weakly acid solution mainly composed of monovalent ions at low concentration, such as a 10 mM NaCl solution prepared in ultrapure water (pH 5.5-6), NPs with an IEP $\geq$ 6.5 are positively charged and quickly adsorb onto the bacterial cell surface (37, 38). This saline solution can then be used as pre-exposure medium for the assessment of NPs having IEPs $\geq$ 6.5, and may be slightly acidified with HCl for NPs having IEPs below this limit. It should be noted that the phosphate buffer commonly used in the *pre-exposure procedure* is not appropriate for the NP evaluation. Indeed, because this buffer has a pH close to 7.4 and a high concentration of mono/trivalent ions it promotes rapid and strong NP aggregation.

Fluctuation vs. plate incorporation procedure: The *fluctuation procedure* was found to be more sensitive than the *plate incorporation method* for evaluation of NPs. A possible explanation could be the low diffusion efficiency of NPs into the agar gel when compared to

the liquid medium. Several studies demonstrated that particle properties (size, distribution, shape, surface charges) along with the relaxation time of the viscoelastic material each play a role in determining the extent to which NP mobility/transport is reduced within a gel matrix (40-41).

Mid-log vs. early stationary phase of growth: Bacterial cells in the mid-log phase instead of those in the early stationary phase (as recommended in the conventional *Bacterial Reverse Mutation procedure*) are more sensitive for the evaluation of NPs (38). Several factors can explain this phenomenon, such as the intracellular level of the alternative sigma factor RpoS, which accumulates in stationary phase and is responsible for the transcriptional activation of genes involved in stress resistance and DNA repair activity (42, 43); or the cell membrane composition and fluidity, known to change during different bacterial growth phases (44).

Use of S9-mix: Adding the S9-mix fraction to mimic the mammalian metabolic conditions has no reason to be performed during the *Bacterial Reverse Mutation Test* since NPs do not produce metabolites. Moreover, this fraction contains a high protein concentration that may increase the aggregation of NPs and limit NP-cells interactions. As a consequence, we do not recommend performing the test using the S9-mix fraction.

1.4 Other important considerations prior to testing NPs

1.4.1 Nanoparticles to be tested

Characterization*:* To correlate the properties of the NPs to their (geno)toxic potential and ensure that results are reproducible and accurate, characterization of NPs is essential. However, such determination of every possible characteristic of NPs is complex, time consuming, and expensive. Indeed, in addition to the information generally given by the manufacturer (CAS no., structural formulae, molecular structure, composition, purity, morphology, surface chemistry, major commercial uses, method of production, etc), a minimum set of important properties must be addressed in (geno)toxicological studies (Table 2). These include primary size, shape, surface area, chemical composition (purity or additives), crystalline phase, surface chemistry (coating/modification) and, for nanoparticles in suspension (e.g. stock suspension), size distribution (aggregation/agglomeration state) and surface charge (zeta potential) (45). As recommended by the OECD Working Party on Manufactured Nanomaterials (46), other relevant information, characteristics or properties can

be given such as dustiness, water solubility, porosity, K_{ow}, crystallite size, redox potential, radical formation potential, photocatalytic activity, etc.

***Stock suspension preparation**:* The method used to prepare the stock suspension directly impacts the NP degree of dispersion and, consequently, their effects on organisms. Several procedures have been described in the literature, but we recommend the use of the *Protocol for Nanoparticle Dispersion* developed by PROSPEcT in support of the OECD Sponsorship Programme (47), which is applicable to a wide range of NPs. This protocol is derived from the BS ISO 14887 (48) *Sample preparation – dispersing procedures for powders in liquids*. Briefly, a mass of dry powder (200 mg for a final NP stock suspension at 10 g/L) was weighed into a 20-25 ml glass container. The contents of the container were then mixed into a thick paste with a few drops of deionized (DI) water using a pre-cleaned metal spatula with sufficient energy to remove visible aggregates. Next, the paste was suspended in 15 mL DI water and sonicated (ultrasonic probe tip half way down the small vial) with an ultra-sonic probe for 20 s at 90% amplitude to disperse the NP agglomerates. Where required, this sonication time can be extended with an appropriate cooling system. Finally, the sonicated suspension was mixed into the end volume (20 mL for a final NP stock suspension at 10 g/L) of DI water using a glass stirring rod to make the stock. For NPs coated with hydrophobic organic molecules, a similar protocol can be used but with ethanol, DMSO, or another appropriate solvent as a dispersant instead of DI water.

***Storage and Stability**:* When stored, NP stock suspension must be kept at 4-6°C away from light in a glass/polyethylene sealed container. Additionally, the stability of the suspension (agglomeration, metal ion/capping agent released) must be investigated before beginning a new experiment. It is recommended that Dynamic Light Scattering (DLS) or UV/Visible spectrometric measurements in solution be conducted before the test to ensure that the NP characteristics meet those obtained with the freshly prepared suspension. Metal ions and capping agent released in the solution can be measured in the supernatant by ICP-MS after NP removal by (ultra)centrifugation. The speed and time of (ultra)centrifugation are determined by the size of the NPs. We recommend 2 h at 40,000×g for NPs 10–30 nm, 1 h at 20,000×g for NPs 30–60 nm, and 30 min at 14,000×g for NPs 60–100 nm. A second (ultra)centrifugation or a filtration can be performed to ensure that all NPs are removed.

***Sterility**:* The presence of micro-organisms in the NP suspension may affect the accuracy of the test. Careful attention should be paid when attempting to find a suitable method of

sterilization for each NP suspension, and the effects of sterilization on the integrity of the physicochemical properties of NPs need to be investigated.

1.4.2 Controls to be performed

In addition to the conventional controls performed during the genotoxicity assays (dispersant/solvent, sterility, and positive controls), parallel controls must be included when assessing NPs.

***Supernatant control**:* Components other than NPs in the stock suspension, such as impurities remaining from the synthesis process, stabilizers or surfactants added during the dispersion procedure (SDS, PVP, Tween, etc), or even products from the NP alteration (metal ions or capping agents), may adversely affect test results. Accordingly, we recommend an additional control consisting of the supernatant of the NP stock suspension after NP removal by (ultra)centrifugation at the speed/time mentioned above.

***Metal ions/coating control**:* Throughout the assay, metal ions or capping agents may be released from NPs. To ensure that the results obtained from the test are NP-dependant and not related to an artifact of the NP alteration, we recommend additional controls for water-soluble or embedded NPs consisting of the corresponding metal salt in solution ($AgNO_3$, $CuCl_2$, $ZnCl_2$, etc) and the free capping agent (citrate, PVP, PEG, etc).

***NP positive controls**:* Suitable reference NP suspensions with good physical and chemical stability and mutagenic effects detectable by the *Bacterial Reverse Mutation Test* must be found or designed. A small number of reference materials already exist in the field of manufactured NPs, but most of them are certified for size and are used mainly to calibrate instruments which measure particle size. Currently, in nano-genotoxicology no NP positive control has been established or universally accepted for the tests. These reference NPs are necessary in the validation of tests, assessment of test method performances, and laboratory proficiency.

2. Materials

2.1 Equipment and supplies

Note 1: This section does not consider the preparation of the NP stock suspension and the NP characterization. For these purposes refer to (47, 48) and (45), respectively. Moreover, this section does not consider the preparation of frozen working cultures and their long-term storage, or confirmation of tester strain genotypes. For these purposes refer to (4, 10, 13).

Large equipment: Autoclave, laminar flow hood, centrifuge, ultracentrifuge for supernatant control preparation, gyratory and stationary incubators at 37°C and a gyratory shaker at room temperature for pre-incubation, ultrapure and deionized water source, 4°C fridge, –80°C and –20°C freezer.

Small equipment*:* Sterile 5 or 10 mL polypropylene culture tubes, 1.5 mL Eppendorf tubes, 50 mL Falcon tubes, micropipettors (0-1000 µL) and supply of sterile disposal tips, multichannel dispenser for distribution into wells of the microtiter plates, sealed filtration units (0.22 µm pore size) for filter sterilization of stock solutions, balance, vortex, 96-wells microtiter plates.

2.2 Stock solutions and media

Nutrient broth medium: 2.5 g Oxoid nutrient broth No. 2 are dissolved in 100 mL of deionized water and autoclaved at 121°C for 15 min. Medium stored at room temperature in the dark can be used for at least 1 month.

NaCl pre-exposure solution *(10 mM)*: 58.44 mg of NaCl is dissolved in 100 mL of ultrapure water (18.2 MΩ.cm at 25 °C) and autoclaved at 121°C for 15 min. This pre-exposure solution should be slightly acidified with HCl for NPs having IEPs below 6.5.

Sterile water: Ultrapure water (18.2 MΩ.cm at 25 °C) and deionized water are sterilized by autoclaving at 121°C for 15 min and used for the preparation of NP dilutions and for the test procedure, respectively.

Biotin solution *(0.01 % w/v)*: 10 mg of D-biotin are dissolved in 100 mL of deionized water, and the solution is then sterilized by filtration through a 0.22 µm filter. Stock solutions stored at 4°C can be used for at least 6 months.

Histidine solution *(0.1 % w/v)*: 100 mg of L-histidine are dissolved in 100 mL of deionized water, after which the solution is sterilized by filtration through a 0.22 µm filter. Stock solutions stored at 4°C can be used for at least 6 months.

Tryptophan solution *(0.1 % w/v)*: 100 mg of L-tryptophane are dissolved in 100 mL of deionized water and the solution is then sterilized by filtration through a 0.22 µm filter. Stock solutions stored at 4°C can be used for at least 6 months.

Ampicillin solution *(5 % w/v)*: 500 mg of ampicillin are dissolved in 10 mL of deionized water, after which the solution is sterilized by filtration through a 0.22µm filter. Stock solution stored at -20°C can be used for at least 4 months.

Tetracycline solution *(0.5 % w/v)*: 50 mg of tetracyclin are dissolved in 10 mL of ethanol. The solution need not be sterilized. Stock solution stored at -20°C can be used at least 4 months.

Glucose solution *(40% v/v)*: 40 mg of D-Glucose are dissolved in deionized water to a final volume of 100 mL. The solution is then sterilized by filtration through a 0.22µm filter. Stock solution stored at 4°C can be used at least 6 months.

Davis-Mingioli salts *(5.5×)*: 38.5 g of K_2HPO_4, 11.0 g of KH_2PO_4, 5.5 g of $(NH_4)_2SO_4$, 1.375 g of Tri-sodium citrate and 550 mg of $MgSO4.7H_2O$ are added and dissolved in the order listed in 600 mL of deionized water. The final volume is then adjusted to 1 L with deionized water, after which the solution is autoclaved at 121°C for 15 min. Stock solution stored at room temperature can be used for at least 6 months.

Purple bromocresol solution *(0.2 % w/v)*: 300 mg of purple bromocresol are dissolved in 150 mL of deionized water and the solution is then autoclaved at 121°C for 15 min. Stock solutions stored at 4°C can be used for at least 6 months.

Test reagent mixture: A volume of 108 ml of Davis-Mingioli salts (5.5×*)* is supplemented with 24 mL of D-glucose (*40% w/v*), 6 mL of D-biotin *(0.01 % w/v)*, 0.3 mL of L-histidine *(0.1 % w/v)* (for tester strains *S. salmonella*) or tryptophan (*0.1 % w/v)* (for tester strains *E. coli*) and 12 mL of bromocresol purple solution *(0.2 % w/v)*. This mixture must be prepared the day of the assay and immediately before the test and should not be stored.

Positive controls: Positive controls are dissolved under sterile conditions in dimethyl sulfoxide at the following concentrations: 9-aminoacridine (6 mg/mL), 2-nitrofluorene (160 µg/mL), sodium azide (50 µg/mL), cumene hydroperoxide (2 mg/mL), 4-nitroquinolone-1-oxide (100 µg/mL). These stock solutions stored at -20°C can be used at least 1 year.

3. Methods

Note 2: idem Note 1

3.1. Preparation of bacterial strains.

Bacterial overnight cultures are started from frozen glycerol stocks by scraping a small portion of frozen cells and inoculating into liquid Oxoid nutrient broth No. 2 supplemented with appropriate antibiotics (Table 1). Ampicillin was added to a final concentration of 25 µg/mL and tetracycline to 2 µg/mL. Cultures are then grown overnight in the dark at 37°C in a rotary shaker and used the next morning to inoculate 50 mL of the same fresh medium in 250 mL Erlenmeyer flasks. These new cultures are incubated as described above until reaching an absorbance density (OD_{600nm}) between 0.4 and 0.6 (mid-log phase of growth), at which point the Erlenmeyer flasks are removed and the bacterial suspensions transferred into individual 50 mL Falcon tubes. Cells are then washed twice by centrifugation (10 min at 7,000×g at room temperature) and resuspension in 50 mL of NaCl pre-exposure solution. Cells are finally redispersed in an adequate volume of NaCl pre-exposure solution to obtain a final absorbance density (OD_{600nm}) of 5.0.

3.2. Preparation of the NP concentration range.

The concentration range is prepared from the NP stock suspensions in sterile 1.5 mL Eppendorf tubes by serial dilutions (1:1, 1:10, 1:100, and 1:1,000) in sterile ultrapure water. These ratios can be modified depending of the concentration of the initial NP stock suspension (usually 10 g/L) and potential toxicity of NP evaluated (refer to the toxicity assessment of NPs section). Dilutions must be prepared immediately before use in the pre-incubation procedure and cannot be stored for future assays.

3.3. Pre-incubation procedure.

A total of 200 µL of the bacterial suspension prepared as described above was transferred to 100 mL flasks containing 20 mL of the NaCl pre-exposure solution. Next, 200 µL of NPs at various concentrations were added and the flask was incubated for 30 min at 20°C in the dark under orbital agitation (150 rpm). The time of incubation could be extended or reduced depending of the NP toxicity.

3.4. Mutagenicity test.

After the pre-exposure step, flasks are removed and aliquots of 500 µL are transferred into 50 mL Falcon tubes containing 2.5 mL of the test reagent mixture. A volume of 17 mL of sterile distilled water is then added to the tubes, giving a final NP dilution factor of 4,000 (based on the concentration described above). A total of 200 µL from these mixtures is subsequently dispensed into 96 well microtiter plates, after which the plates are sealed in plastic bags and incubated at 37°C for 5 days in the dark.

Various controls are run concurrently, such as (i) negative controls corresponding to sterile distilled water or other dispersants used in the preparation of the NP stock suspension (DMSO, ethanol…), (ii) positive control substances to confirm the reversion properties of the strains (Table 1), (iii) supernatant controls prepared as described in the *Controls to be performed* section, (iv) in case of genotoxic evaluation of water-soluble or coated NPs, the corresponding metallic salt or the capping agent, and (v) all appropriate sterility controls: medium, NPs, reference mutagens used as positive controls, etc.

3.5. Interpretation of results

A yellow well is considered positive and a purple well negative. The test is considered valid if after 5 days of incubation (i) all wells in the sterility control plates are still purple, (ii) the results from the negative control plates are as expected (spontaneous revertants) and (iii) between 90 and 96 wells in the positive control plates are yellow. NPs tested are considered mutagenic if the number of positive wells is significantly greater in treated plates than in both negative control plates, the supernatant control, and when performed, in the metallic salt/capping agent treatments. Results obtained were tested for significance by the Chi-square analysis using Table 3 (20). Based on the number of positive wells obtained with the negative control (column Control), this table gives the minimum number of positive wells that must be obtained from the treated plates (column Exposed) to be statistically significant at $\alpha = 0.05$.

3.6. Toxicity assessment of the NPs

Toxicity is generally indicated by a reduction of positive wells in the treated plates relative to the negative control plates. It is also possible to carry out an initial toxicity test to determine the most appropriate concentration range to assay. In the case of NP toxicity, only the higher tested dose should exhibit some toxicity.

3.7. Safety precautions

In addition to the safety precautions that must be taken in laboratories when using potentially mutagen/carcinogen substances and micro-organisms, even with the non-pathogenic tester strains used in the *Bacterial Reverse Mutation* assay, specific recommendations have to be taken into account when handling NPs.

All manipulation of NP powders has to be carried out in a glove box, glove bag, chemical fume hood, or other airborne contaminant control system. NP in suspensions may be handled on the lab bench with appropriate personal protective equipment such as goggles, anti-static gloves and a lab coat. The *Bacterial Reverse Mutation* assay has to be performed under sterile conditions in a well-ventilated area.

3.8. Disposal of wastes

All materials contaminated with micro-organisms have to be autoclaved. Wastes containing NPs and/or mutagen compounds, such as positive controls, must be collected and managed as hazardous waste.

4. References

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Table 1. Specificity of *Salmonella* and *Escherichia* tester strains recommended by the OECD guidelines.

Bacterial strains	Histidine mutation	Reversion event	Additional mutations			Spontaneous revertants[a]	Positive controls[b]
			LPS defect	Repair defect	Plasmid (resistance)		
S. typhimurium							
TA1535	hisG46	Base-pair substitutions	*rfa*	*uvrB*	-		Sodium azide
TA1537	hisD3076	Frameshifts	*rfa*	*uvrB*	-		
TA97	hisD6610	Frameshifts	*rfa*	*uvrB*	pKM101(Amp^r)	3-10	9-aminoacridine (10µg/mL)
TA98	hisD3052	Frameshifts	*rfa*	*uvrB*	pKM101(Amp^r)	3-10	2-nitrofluorene (0.4µg/mL)
TA100	hisG46	Base-pair substitutions	*rfa*	*uvrB*	pKM101(Amp^r)	10-30	Sodium azide (25ng/mL)
TA102	hisG428	Transitions/transversions	*rfa*	*uvrB*	pKM101(Amp^r) + pAQ1(Tet^r)	60-70	Cumene hydroperoxide (3.5 µg/mL)
E. coli							
WP2 uvrA	trpE	Transitions/transversions		*uvrA*		3-15	
WP2 uvrA (pKM101)	trpE	Transitions/transversions		*uvrA*	pKM101(Amp^r)	3-15	4-nitroquinolone-1-oxide (0.1 µg/mL)

[a]Spontaneous revertants expected in the 96-well control plate with the fluctuation procedure

[b]Positive controls used without S9 mix

Table 2. Common techniques for measuring NP characteristic relevant for (geno)toxicity studies.

Techniques	NP powder	NP suspension	Liquid dispersant	Measured parameters	Sensitivity	NP state during analysis
Scanning Electron Microscopy (SEM)	+	+		Size Shape Agglomeration/aggregation	~2 nm – 1 µm	On a surface
Transmission Electron Microscopy (TEM)		+		Size Shape Agglomeration/aggregation	~ 0.2 nm – 1 µm	On a surface
Dynamic Light Scattering (DLS)		+		Size Agglomeration/aggregation Dispersion stability	~ 1 nm – 7 µm	Dispersed in liquid
Brunauer, Emmett and Teller (BET)	+			Surface area	~ 5 nm – 10 µm	Dry powder
Zeta-potential		+		Surface charge Agglomeration/aggregation Dispersion stability	~ 5 nm – 1 µm	Dispersed in liquid
Scanning Mobility Particle Sizer (SMPS)	+			Size	~ 5 nm – 2 µm	Aerosol
X-ray Photoelectron Spectroscopy (XPS)	+	+		Surface chemistry/ contamination	ppt range	On a surface
Energy Dispersive X-ray	+	+		Surface chemistry/	ppm range	On a surface

Spectroscopy (EDX)				contamination		
Secondary Ion Mass Spectrometry (SIMS)	+	+		Surface chemistry/ contamination	ppb range	On a surface
Fluorescence spectroscopy		+	+	Photocatalytic activity	variable	Dispersed in liquid
Redox potential		+	+	Surface chemistry/ contamination	ppm	Embedded
UV-vis spectroscopy		+	+	Photocatalytic activity		Dispersed in liquid
Ion exchange chromatography			+	Solubility/dissolution		
pH			+			

Table 3. Significance thresholds with α = 0.05 (20).

Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
0	3	25	36	50	62	75	84
1	5	26	37	51	63	76	85
2	7	27	39	52	64	77	86
3	9	28	40	53	65	78	87
4	10	29	41	54	66	79	87
5	12	30	42	55	67	80	88
6	13	31	43	56	68	81	89
7	15	32	44	57	68	82	90
8	16	33	45	58	69	83	90
9	17	34	46	59	70	84	91
10	19	35	47	60	71	85	92
11	20	36	48	61	72	86	93
12	21	37	49	62	73	87	93
13	22	38	50	63	74	88	94
14	24	39	51	64	75	89	94
15	25	40	52	65	76	90	95
16	26	41	53	66	77	91	96
17	27	42	54	67	78	92	96
18	28	43	55	68	78	93	96
19	30	44	56	69	79	94	
20	31	45	57	70	80	95	
21	32	46	58	71	81	96	
22	33	47	59	72	82		
23	34	48	60	73	83		
24	35	49	61	74	83		

Les résultats obtenus au sein des chapitres 2 et 3 ont mis en évidence l'importance des interactions électrostatiques gouvernées par l'électrohydrodynamique et la composition de la membrane externe bactérienne dans l'expression et la détection d'un potentiel toxique et génotoxique des nanoparticules de dioxyde de titane sur les bactéries. Ces données laissent à penser que ces effets néfastes des TiO_2-NPs observés sont susceptibles d'impacter les communautés bactériennes. Les nanoparticules de dioxyde de titane vont pouvoir exercer leurs potentiels toxique sur les différents types bactériens présent au sein des communautés pouvant aboutir à des modifications de structure et éventuellement à l'altération de processus fonctionnels. En effet, la littérature a mis en évidence des sensibilités différentes des types bactériens lors de l'exposition aux nanoparticules (exemple : bactéries Gram + et Gram -) (Beranová et al., 2014; Ciobanu et al., 2012; Negi et al., 2013).

Ainsi, dans un contexte de rejet ponctuel de nanoparticules dans l'environnement, nous avons fait le choix de suivre l'évolution d'une communauté bactérienne d'eau douce naturelle provenant de la rivière Moselle après exposition à différentes concentrations de nanoparticules de dioxyde de titane. Nous avons fait le choix de prélever l'eau de la Moselle et de travailler au laboratoire afin de contrôler les conditions d'exposition sur une durée suffisamment longue pour atteindre des effets à ce niveau d'organisation biologique. De plus, dans les nanoparticules sont à l'heure actuelle encore émergente dans l'environnement, conduisant à des pollutions insuffisantes pour réaliser une évaluation *in situ*. Cela permet également de s'affranchir des effets de multi-contaminations qui existent dans le milieu naturel. Afin d'obtenir une réponse globale, nous avons décidé de suivre l'évolution des bactéries planctoniques et sessiles exposées aux nanoparticules de dioxyde de titane). De plus, nous avons suivi l'évolution des taxons de ces deux types de communauté afin de voir quels sont les groupes les plus affectés.

Chapitre IV : Effets des nanoparticules de dioxyde de titane sur les communautés bacteriennes

Impact of manufactured TiO_2 nanoparticles on planktonic and sessile bacterial communities

Résumé

La production et l'utilisation croissante des nanoparticules conduit inévitablement à leurs rejets dans l'environnement et plus particulièrement dans le compartiment aquatique (Gottschalk et al., 2013). Du fait de ces rejets, les communautés bactériennes aquatiques naturelles vont être exposées aux nanoparticules et des effets potentiellement néfastes pour ces communautés bactériennes peuvent donc survenir.

Pour étudier l'impact de ces rejets, un échantillon d'eau de la Moselle a été exposé pendant deux semaines à différentes concentrations de P25-TiO_2-NPs et l'évolution des communautés bactériennes libres et fixées a été suivie. Les quantités de bactéries présentes dans la colonne d'eau tendent à augmenter (183% pour la plus forte concentration) alors que les bactéries sessiles diminuent fortement (88%) pour la concentration la plus importante en nanoparticules (100 mg/L). La composition des communautés bactériennes a ensuite été suivie. Les ordinations NMDS des profils de PCR-DGGE montre clairement que les P25-TiO_2-NPs ont un impact sur les assemblages des bactéries sessiles lorsque la concentration est la plus importante. Pour ce qui est des communautés planctoniques, les analyses des librairies de clones révèlent une diminution du nombre de séquences, confirmé par l'indice de Shannon. Grâce à la comparaison RDP, on observe une forte diminution des *Proteobacteria* et ce particulièrement au sein des *Betaproteobacteria* malgré l'augmentation des *Oxalobacteraceae* qui ne compense pas la perte de diversité obtenue au sein des *Betaproteobacteria*. Pour ce qui est des *Bacteroidetes,* une augmentation de la diversité est visualisée, principalement au niveau des *Cytophagaceaes,* et dans une plus forte proportion grâce à *Emtiticia*. A l'inverse, lorsque le biofilm, dans son stade "juvénile", est exposé à 100 mg/L de P25-TiO_2-NPs, la diversité bactérienne augmente, avec un nombre d'OTUs passant de 31 pour le témoin à 38 pour le biofilm exposé. Cependant, au sein de ce biofilm, une diminution importante des Betaproteobacteria est observée, principalement due aux *Burkholderiales* (*Leptothrix* et *Comamonadaceae*). Ces observations sont confirmées par les comparaisons LIBSHUFF.

A notre connaissance cette étude est la première s'intéressant à l'impact d'un rejet de nanoparticules de dioxyde de titane sur des communautés bactériennes aquatiques naturelles d'eau douce.

Impact of manufactured TiO_2 nanoparticles on planktonic and sessile bacterial communities

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Abstract

We investigate the effects of various P25-TiO_2-NPs concentrations on natural freshwater bacterial communities. Exposition to 100 mg/L of P25-TiO_2-NPs increase the amount of planktonic bacteria while a sharp decrease of sessile bacteria occurs after 2 weeks. The PCR-DGGE profiles clearly showed modifications of sessile bacterial assemblages for each concentration and exclusively for the highest concentration in planktonic bacterial assemblages. NP exposure conducts to decreasing OTUs richness confirmed by diversity index. For planktonic community, diversity slightly decreases (36 *vs*. 33 OTUs) due to the loss of *Comamonadaceae* and increase in the *Oxalobacteraceae*, *Cytophagaceae* (especially *Emtiticia*) occurs. For sessile bacteria exposed to 100 mg/L of P25-TiO_2-NPs, diversity increase (31 *vs*. 38 OTUs). An important decrease happened in Betaproteobacteria due to the *Burkholderiales* (*Leptothrix* and *Comamonadaceae*) confirmed by LIBSHUFF comparison. These data encourage TiO_2-NP accumulation and possible adverse effects assessment against bacteria in environmental food web.

Keywords: nanoparticles, community, diversity, structural impact, interactions.

Introduction

New technologies and industrial processes based on nanoparticles lead to their release in the environment, especially, in aquatic ecosystem (Benn et al., 2010; Colvin, 2003; Dunphy Guzman et al., 2006; European Commission, 2004; Gao et al., 2008; Hsu and Chein, 2007; Lecoanet and Wiesner, 2004; Nowack and Bucheli, 2007; USEPA, 2007; von der Kammer et al., 2010). Titanium dioxide nanoparticles (TiO_2-NP) are the most widely used nanomaterial (Mueller and Nowack, 2008) with an actual production comprised between 38,000 to 64,000 MT/y for the US (Hendren et al., 2011; Weir et al., 2012) and, according to market estimation, around 2 million tons per year worldwide for the period of 2012-2018 (www.transparencymarketresearch.com). It is assumed that they could totally replace bulk material around 2025 (Robichaud et al., 2009). Nowadays, TiO_2 based-nanomaterials represent over 185 products (www.nanotechproject.org, 2014) and are widely used in consumer products with expanded applications over the last decade (Colvin, 2003; Gleiche et al., 2006). The wide use of TiO_2-NP have created a great concern for environmental impact (Clift et al., 2011; Jomini et al., 2012; Nel et al., 2006; Service, 2005) because TiO_2-NPs may represent the nanoparticles with the highest environmental concentrations in the future

(Gottschalk et al., 2009) since it was first described as potentially reaching natural water after paints applied on building facades released (Kaegi et al., 2008). That's why TiO_2-NPs were recently listed by the OECD as one of the priority nanomaterials for immediate testing (OECD, 2008b). These concerns were confirmed by their recent detection in wastewaters, sewage sludge, soils and surface waters (Biswas and Wu, 2005; Gottschalk et al., 2009; Jośko and Oleszczuk, 2013; Klaine et al., 2008). Moreover, TiO_2-NP could be released by intentional overflow discharge in wastewater treatment plant (WWTP) during heavy rain (Kiser et al., 2012; Mueller and Nowack, 2008). Nowadays, predictive environmental concentrations values in water are 0.7-16µg/L (Battin et al., 2009; Gottschalk et al., 2009; Mueller and Nowack, 2008), but these concentrations can reach much higher values. Indeed, concentration has been measured in WWTP influent at 185 µg/L and even 600µg/L for Ti in runoff of new façade (Kaegi et al., 2010, 2008; Kiser et al., 2009) and even approximately 5 mg/L was reach when including the aggregates in WWTP (Kiser et al., 2009). This suggests that much higher concentrations will be expected in the future than those currently found or predicted by models. In this context, it is of first importance to assess the potential effects of TiO_2-NP on freshwater bacterial communities.

Titanium is considered immobile and insoluble element (Van Baalen, 1993) and TiO_2-NP effects cannot be attributed to the titanium ions released because they strongly resist dissolution and are highly insoluble under almost all environmental conditions (Cornelis et al., 2011; Judy and Bertsch, 2014). By reaching natural water, these nanoparticles may interact with microorganisms possibly causing damages. Indeed, microorganisms are the basis of food web and play a major role in ecosystem functioning. We can hypothetized that free-living bacteria and attached bacteria may have different response to TiO_2-NP exposure due to the protective mechanism of encapsulation present for attached bacteria (Battin et al., 2009). Moreover, it was noticed that biofilm are a major receptor for TiO_2-NP and may contain concentrations of several hundreds of µg/kg (Battin et al., 2009; Yeo and Nam, 2013). If nanoparticles could potentially affect diversity and structure of microbial communities this could inturn, have several impacts on overall ecosystem health and function (Nogueira et al., 2012).

In this context, these study objectives were to assess the TiO_2-NPs impacts on bacterial community and diversity for both planktonic and sessile bacteria (early stage of biofilm). To this end, a microcosms experiment was realized with increasing concentrations of TiO_2-NPs and, at the end of exposure, total amounts of bacteria were determined. Moreover, it is important to note that TiO_2-NPs present semiconducting properties resulting in photocatalytic

potential when exposed to light with sufficient energy (Benabbou et al., 2007). In order to avoid the photocatalytic effect of TiO_2-NPs, all experiments were conducted under dark condition. Denaturing gradient gel electrophoresis technique (DGGE) analyses were performed to assess the effects of increasing TiO_2-NP concentrations on bacterial planktonic and sessile assemblages. Amplified 16S rRNA sequences of clone libraries were compared between control and water exposed to the highest concentration of TiO_2-NPs for sessile and planktonic bacterial community.

Materials and Methods

Experimental design

Water from the Moselle River was collected the 20 February 2011 upstream of the Metz city (Northeastern France) (N 49°6'7.0662"; E 6°7'4.8786") and used for TiO_2-NPs exposure of bacterial communities in a laboratory experiment. To this end, we used TiO_2 nanopowder AEROXIDE® P25 (P25-TiO_2-NPs) (Evonik Degussa GmbH, Frankfurt, Germany, Stock # 4168050298) and stock suspension was prepared as previously described by Pagnout et al., 2012. Taking into account evolutions for TiO_2-NPs released and environmental concentrations, we used relevant and high dose of P25-TiO_2-NPs. In two litter flasks, one liter of water from the Moselle River were supplemented with or without P25-TiO_2-NPs stock suspension to obtain a control and 3 levels of TiO_2 concentrations (0, 1, 10 and 100 mg/L with 4 replicates each). Sets of 8 Isopore polycarbonate membrane filter (0.2µm GTBP Millipore Corporation, Billerica, MA, USA, 2013) were placed in each bottle for the determination of biofilm bacterial biomass (DAPI staining) and community structure, respectively. Bottles were agitated at 80 rpm during two weeks under dark condition.

Water physicochemical characteristics

The pH of water was measured with a pH meter microprocessor (pH 3000, WTW) and conductivity was assessed using a Metrohm Herisau Conductometer E518 (Herisau, Switzerland) at 25°C. Concentrations of Ca^{2+}, Mg^{2+}, K^+ and Na^+ were determined by Atomic Absorption Spectrophotometry (Aanalyst 100; Perkin Elmer and Varian SpectrAA-300). Concentrations of Cl^-, SO_4^{2-}, NO_2^- and NO_3^- were determined by ion chromatography (Dionex 1500i with an AS 4 A SC column; Sunnyvale, USA). NH_4 and PO_4^{2-} concentrations were acquired by UV/Vis. spectrophotometric methods (Analytikjena, SPECORD 205, PO Box

932, Wembley, HA0 9EH, UK) and Volatile Suspended Solid and Total Suspended Solid where determined by gravimetric analysis. Physicochemical characteristics of water are summarized in Table I.

Table I: Physico-chemical characterization of the Moselle water site.

Location	pH	Conductivity	NH_4	NO_2	NO_3	Cl	SO_4	PO4	VSS	TSS	Ca	Mg	Na	K
		($\mu S\ cm^{-1}$)	($mg\ N\ l^{-1}$)	($mg\ N\ l^{-1}$)	($mg\ N\ l^{-1}$)	($mg\ l^{-1}$)	($mg\ l^{-1}$)	($mg\ P\ l^{-1}$)	(%)	($mg\ l^{-1}$)	($mg\ l^{-1}$)	($mg\ l^{-1}$)	($mg\ l^{-1}$)	($mg\ l^{-1}$)
N 49° 6' 7.0662" E 6° 7' 4.8786"	8.1	1520	0.338	0.051	2.160	470	70	0.044	26.8	11.2	223	11.3	112	7.3

Nanoparticles physicochemical characteristics

P25-TiO_2-NPs are a mixture of anatase and rutile forms (~ 84 % anatase and 16 % rutile) with an average primary particle size of 23 ± 4.9 nm. Dynamic light scattering (DLS) measurements revealed that the average hydrodynamic diameter of the nanoparticle stock suspension ranged between 60 and 80 nm, this size corresponding to an agglomerated state. The determined isoelectric point (measured on a Zetasizer NanoZS (Malvern Instruments,UK)) was approximately of pH 6.8 (More information in Pagnout et al., 2012). To determine the behavior of these nanoparticles in the Moselle water, sized measurements were acquired with a Malvern Mastersizer Hydro200SM granulometer (Malvern Instruments SA, Orsay, France). Concretely, Moselle river water was filtered and sterilized on 0.22 µm nylon filter and then placed in the granulometer column. The measurements were realized every 10 minutes during 40 minutes until stabilization and realized in triplicats (Figure 1).

Determination of the bacterial biomass

For the characterization of planktonic bacteria, 10 mL of water was taken to stain and measure the total amount of bacteria. Staining of total cells was done with 4,6,diamidino-2-phenylindol (25 $\mu g.ml^{-1}$) (DAPI, Sigma-Aldrich, St. Louis, USA) for 30 min and incubated for 1 h at room temperature in the dark. After that, stained samples were filtered onto a Isopore polycarbonate filter with a pore size of 0.2 µm (Millipore Corporation, Billerica, USA) according to Hobbie et al., (1977). Cell numbers were determined using an epifluorescence microscope (Olympus model BX41) with the appropriate filter cube for DAPI (absorption 350 nm, emission 450 nm). For sessile bacteria, filters introduced at the beginning of the experiment were retired and stained with 200 µL of DAPI directly dropped onto the

filter. They were then washed with 10 mL of physiological water and bacterial accounts were determined as described above for planktonic bacteria.

DNA extraction and 16S rRNA amplification

A volume of 250 ml by sample was filtered on 0.22 µm nylon membrane and the filter was then washed twice with 5 ml of physiological water (0.9% NaCl). The latter solution was centrifuged at 10000 g for 3 min and the pellet was stored at - 80°C until DNA extraction. For each replicate, the four filters disks were pooled into a composite sample and total DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Bacterial universal primers 341F-GC2 (5′CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGCCTACGGGAGA GCAG-3′) and 907R (5′-CCGTCAATTCMTTTGAGTTT-3′) were used for partial PCR amplification of 16S rRNA genes (Muyzer et al., 1998, 1993). PCR mixture (100 µl) contained 6 U of Taq DNA Polymerase (5 PRIME, Hamburg, Germany), 1× Taq buffer (5 PRIME), 200 µM of each dNTP, 0.5 µM of each primer and 20 ng of extracted DNA as template. The PCR consisted of 5 min at 95°C, followed by 20 cycles of 30 s at 95°C, 30 s at 65–55°C (touchdown −0.5°C per cycle), 35 s at 72°C, and by 10 cycles of 30 s at 95°C, 30 s at 55°C and 35 s at 72°C, followed by 10 min final extension. The amplification products were subjected to quality control on 1% (w/v) agarose gel.

DGGE analysis

The PCR products were separated with the DCODE mutation detection system (Bio-Rad, Hercules, CA). A volume of 10 µL per sample was loaded onto 7% (w/v) polyacrylamide gels in 1× Tris–acetate– EDTA (TAE) buffer with a denaturing gradient ranging from 40 to 60% (40% (v/v) formamide and 7 M urea corresponding to 100% denaturant). The gels were run in 1× TAE buffer at 100 V and 60 ◦C for 16 h, stained with SYBR Green I and imaged with a STARION FLA-9000 scanner (Fujifilm Life Sciences FSVT, Courbevoie, France).

Cloning and sequencing

Fresh 16S rRNA PCR products of control and sample exposed to 100 mg/L of TiO_2-NP were cloned using the TOPO TA Cloning Kit (Invitrogen, Cergy Pontoise, France) and 96 individual clone colonies by sample were sequenced by GATC Biotech (Konstanz, Germany) using the T3 vector primer. The sequences were checked and only quality sequences were

kept for phylogenetic analyses. The sequences used for phylogenetic analyses were deposited in GenBank under accession numbers KJ818489 to KJ818847.

Data analysis

For DAPI staining analysis, 10 randomly chosen areas on a filter were counted and mean value and standard deviation were calculated for both planktonic and sessile bacteria. The variation between each condition was determined with Kruskal-Wallis statistical test. There were a statistically significant difference between sample when the *p*-value was lower than 0.05.

GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium) was used to normalize and compare DGGE profiles. To this end, an internal control samples was made. A tolerance in the band position of 2% was applied. A cluster analysis of DGGE pattern profiles was performed based on the Bray Curtis coefficient. A correlation matrix was then generated for each comparison and non-metric multidimensional scaling (NMDS) was used to analyzed the data with PAST software since it has been shown to be a powerful robust tool for the analysis of community (Minchin, 1987). Analysis of similarity (ANOSIM) was performed using PAST software (Hammer et al., 2001) to characterize differences between samples. The R-value was statistically significant at a *p*-value lower than 0.05 and an R-value of 0 indicates no differences whereas an R-value of 1 indicates completely dissimilar groups.

Default settings of CLUSTALW were used to realize multiple sequence alignments (Larkin et al., 2007). DNAdist was used in the PHYLIP package to make distance matrices (Felsenstein, 2005). Operational taxonomic units (OTUs) were defined at a 97% sequence similarity cut-off in the DOTUR software (Schloss and Handelsman, 2005). The diversity at 97% similarity cut-off was evaluated by using the Shannon $H' = -\sum_{i=1}^{Sobs} p_i \times ln(p_i)$ and Simpson $\frac{1}{D} = \frac{1}{\left[\sum n_i \times \frac{n_i - 1}{N \times (N-1)}\right]}$ indices (Table II). The obtained sequences were identified by comparison with sequences from the Ribosomal Database Project (Cole et al., 2009) and each OTU was affiliated to the phylum level. Comparisons of 16S rRNA gene sequence libraries between exposed samples and control were assessed using the LIBSHUFF program. According to Singleton et al., 2001, we obtained statistically significant differences with a confidence of 95% at a *p*-value equal or lower than 0.025.

Results

Size characterisation of P25-TiO_2-NPs

When the highest concentration of P25-TiO_2-NPs was injected in the Moselle water, there was a fast and strong aggregation of these nanoparticles (Figure 1). Indeed, after 10 minutes in water, formed aggregate were stabilized at a sized of 3.5 µm. At the opposite, when nanoparticles were injected at a concentration of 10 mg/L, the aggregation was slightly slower and stabilization occured later, after 30 minutes and with an aggregate size of 9.3 µm. For 1 mg/L of P25-TiO_2-NPs, the concentration was below the detection limits of the granulometer.

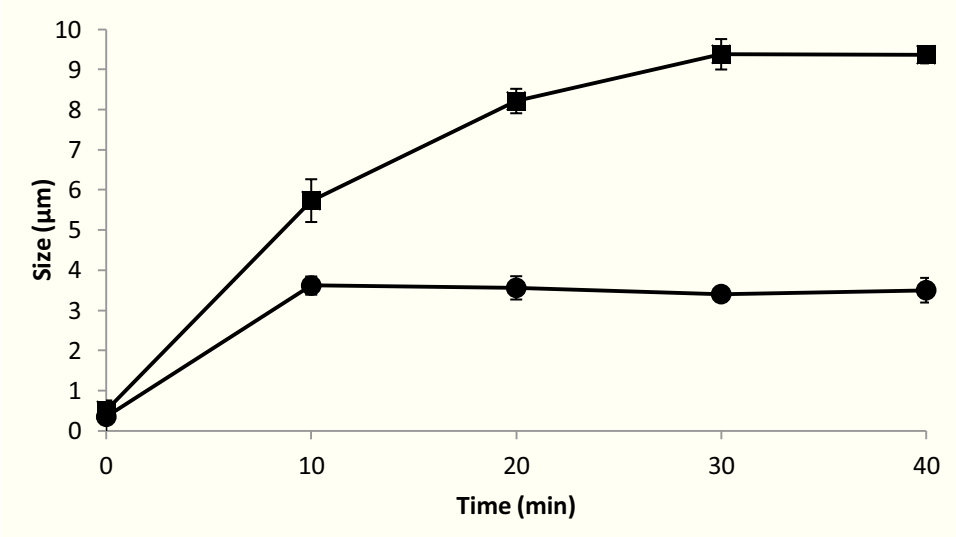


Figure 1: Size evolution measurements in the Moselle water of 100 mg/L of TiO_2-NP (black sphere) and 10 mg/L of TiO_2-NP (black square).

Determination of bacterial biomass

When exposed to increasing concentrations of P25-TiO_2-NPs, total amount of bacteria present in the Moselle water increased after 2 weeks, particularly for the highest concentration of P25-TiO_2-NPs. Indeed, at the beginning of the experimentation, the total amount of bacteria in each flask was 2.6×10^6 bacteria/mL. After 2 weeks of exposure, bacterial concentration decreased slightly for the control to 1.2×10^6 bacteria/mL (corresponding to 63% of decrease), for 1 and 10 mg/L of P25-TiO_2-NPs (respectively 1.1×10^6 and 1.24×10^6 bacteria/mL). For the sample exposed to 100 mg/L of P25-TiO_2-NPs, total amount of bacteria increased to reach 3.4×10^6 bacteria/mL (increase of 31%) (Figure 2A). In contrast to what has been observed for planktonic bacteria, when exposed to 100 mg/L of P25-TiO_2-NPs, the

number of sessile bacteria decreased from 7.5×10^3 bacteria/mL for the control to 8.8×10^2 bacteria/mL (Figure 2B). The flasks exposed to 1 and 10 mg/L of P25-TiO_2-NPs remain stable in comparison to the control (with respectively 7.8×10^3 and 6.5×10^3 bacteria/mL).

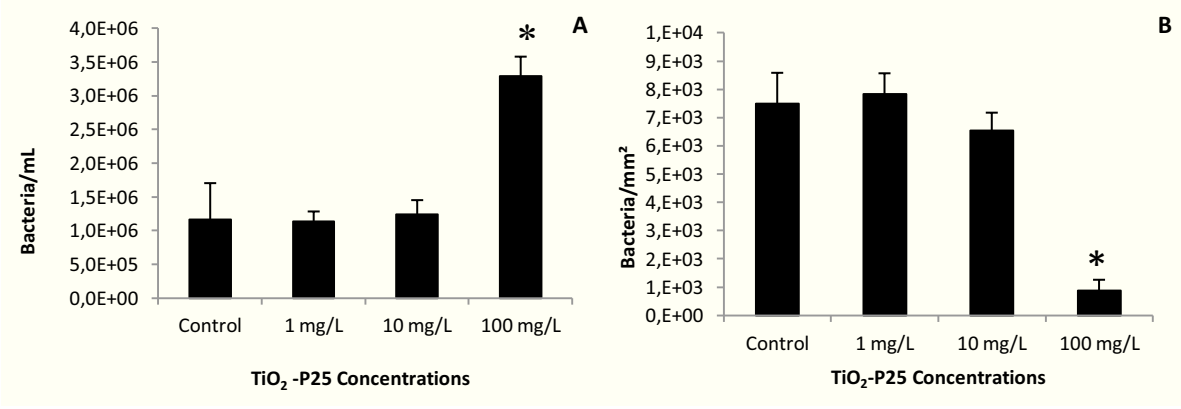


Figure 2: Evaluation of planktonic (A) and sessile (B) bacterial biomass with DAPI staining after 2 weeks of exposure to TiO_2-NP. Significant statistical differences between samples are indicated with a level of significance of $p<0.05$ with Kruskal-Wallis test.

Bacterial Community assemblage

Regarding NMDS ordinations of PCR-DGGE fingerprint profiles for planktonic bacteria (Figure 3A), there is an important difference in the sample exposed to 100 mg/L of P25-TiO_2-NPs in comparison to the control and the two lowest concentrations of nanoparticles. This was confirmed by the ANOSIM analyses performed on the DGGE profiles, where we have a large difference between the highest concentration and all the other treatments ($R=0.833$, $p<0.002$). Planktonic bacterial communities exposed to the two lowest P25-TiO_2-NPs concentrations appeared few different from the control (R values of 0.25, for 1 and 10mg/L, respectively, $p<0.002$).

For sessile bacteria, NMDS ordination showed that bacterial communities from samples exposed to titanium dioxide nanoparticles differs from controls in relation to the x-axis, revealing a significant change in the community structure (Figure 3B). This result was confirmed by the ANOSIM analysis performed on the DGGE profiles that gave an R-value of 0.61 ($p=0.0035$). Moreover, communities exposed to 100 mg/L of P25-TiO_2-NP was also different from those of the samples exposed to 1 mg/L of NP-TiO_2 ($R=0.9375$, $p=0.0035$). For the two other concentrations of P25-TiO_2-NPs the effects were less pronounced, communities from these samples being few different from those of the controls ($R=0.33$ and 0.03 for 1

mg/L and 10mg/L, respectively, p=0.0035). Nevertheless, despite the non-statistical difference with the control, the samples exposed to 1 and 10 mg/L of P25-TiO_2-NPs present a different distribution from the control and the sample exposed to the highest concentration. Indeed, the flasks containing 1 mg/L of P25-TiO_2-NPs move slightly upon the y axis but the flasks containing 10 mg/L of P25-TiO_2-NPs have a totally exploded disposition. The replicates both move on the x and y axis suggesting that an important modification occurs after this exposition.

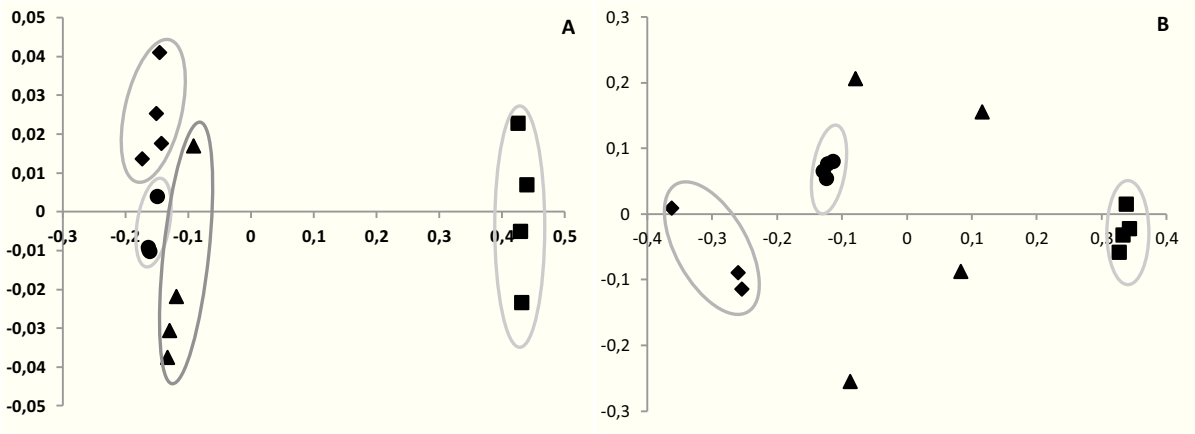


Figure 3: NMDS plots of DGGE bacterial planktonic (A) and sessile (B) bacterial communities after 2 weeks of exposure to 1 mg/L of TiO_2-NP (black circle), 10 mg/L of TiO_2-NP (black triangle), 100 mg/L of TiO_2-NP (black square) and control (black diamond).

Bacterial Community diversity assessment by clone libraries:

To identify differences in bacterial community composition between the Moselle water control and the flask exposed to to 100 mg/L of P25-TiO_2-NPs after 2 weeks, PCR amplifications of 16S rRNA genes were performed.

At 97% sequence similarity cut-off, 36 distinct *operational taxonomic unit (*OTUs) were found for the planktonic bacteria control (Table II), whereas 33 OTUs were obtained for the water exposed to P25-TiO_2-NP. A lower bacterial diversity was also observed in the exposed samples according to Shannon (H') (3.11 vs 2.75, for control and for P25-TiO_2-NPs) and Simpson indices (1/D) (17.63 vs 9.08 for control and for P25-TiO_2-NPs). These changes in community composition operate within different bacterial phyla (Figure 4). We observed an

important decrease of the sequences affiliated to *Proteobacteria,* and most especially to the *Betaproteobacteria*. This decline mainly concerned the Comamonadaceae (17 OTUs) (mainly Comamonas) which represented 29 sequences (32.2%) in the control and only 4 sequences (4.4%) in the exposed sample. A large increase was also significantly notable mainly due to the *Oxalobacteraceae* which represented 0 sequences (0%) in the control and 18 sequences (100%) in the exposed sample that does not offset the loss of diversity. The *Bacteroidetes* also increased due to the *Cytophagaceae* which represented 2 sequences (15.4%) in the control and 28 sequences (58.1%) in the exposed sample and, especially in this groups, the gender *Emticicia* who represent only 1 sequences in the control and 28 sequences in the exposed sample (1.1% *vs* 31.1% of the sequences for this 4 OTUs).

The diversity of sessile bacterial community has a different evolution. A total of 31 OTUs was obtained for the control and 38 OTUs for the sample exposed to 100 mg/L of P25-TiO_2-NPs. This was confirmed by Shannon (2.31 vs 3.03 for control and for P25-TiO_2-NPs sample) and Simpson indices (4.14 vs 10.63 for control and for P25-TiO_2-NPs sample) corresponding to bacterial community diversity modifications. An important decreased occurs in the Betaproteobacteria, mainly due to the *Burkholderiales* especially the *Leptothrix*, which is representing 43 sequences (48.3%) in the control and 26 sequences (28.8%) in the exposed sample. It's also coming from the decrease in the *Comamonadaceae* who represent 18 sequences in the control and 12 sequences in the exposed sample (20.2% *vs* 13.3% of the sequences for these 17 OTUs).

For both, planktonic and sessile bacterial community, the LIBSHUFF comparisons of 16S RNA gene sequence libraries revealed significant differences between controls and exposed samples ($p=0.007$ and $p=0.003$ for planktonic and sessile bacterial communities, respectively).

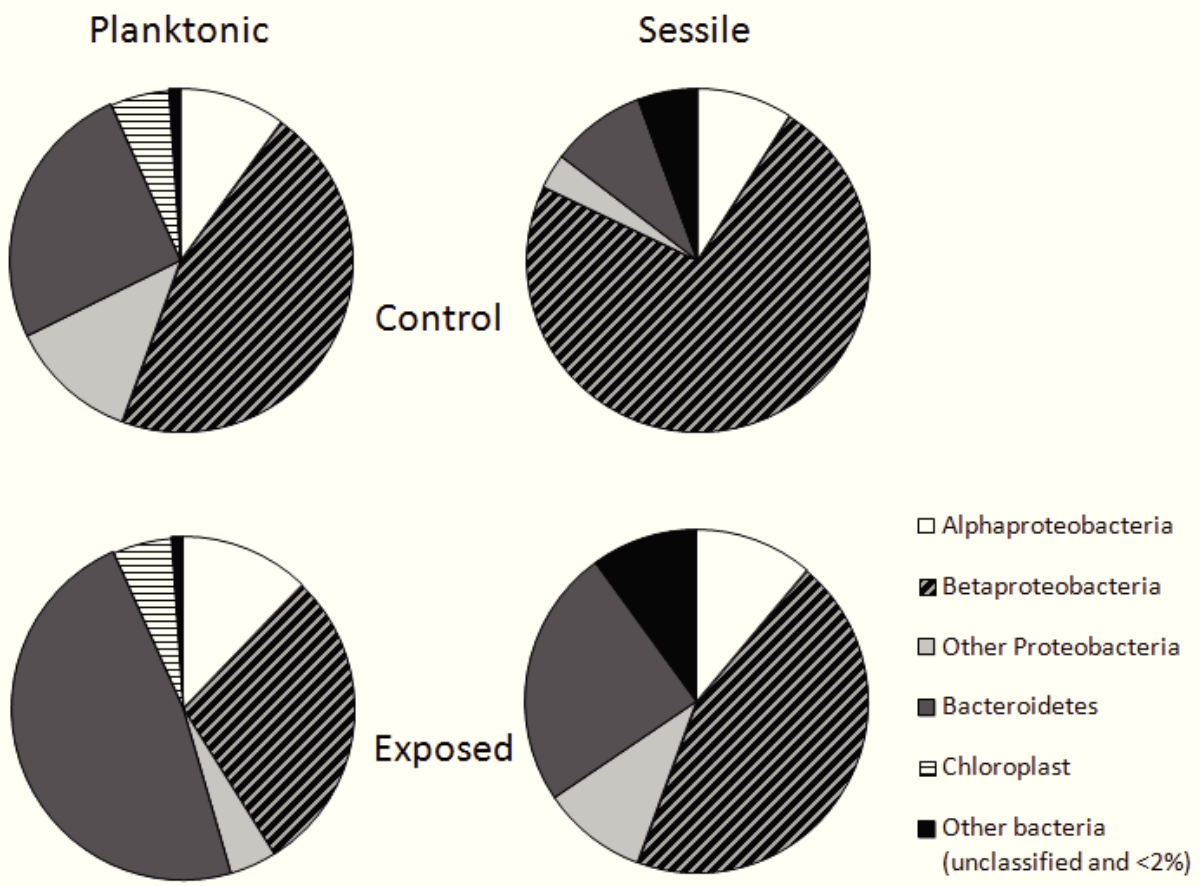


Figure 4: Bacterial planktonic and sessile community composition after 2 weeks of exposure to 100 mg/L of titanium dioxide nanoparticles and control. The RDP comparison was realized with a confidence threshold of 95%.

Table II: Sequences diversity, OTU richness and diversity indices (at 97% sequence similarity) based on 16S rRNA gene clone libraries for water exposed to 100 mg/L of TiO_2-NP.

	Planktonic		Sessile	
	Control	Exposed	Control	Exposed
No. of sequences	90	90	89	90
No. of OTUs	36	33	31	38
H'	3.11	2.75	2.31	3.03
1/D	17.63	9.08	4.14	10.63

Discussion

This study investigated the impact of P25-TiO_2-NPs on bacterial community coming from the Moselle river. When exposed to increasing concentrations of P25-TiO_2-NPs under dark condition, structural modifications of bacterial community occur, for both planktonic and sessile bacteria.

After 2 weeks of exposure, the total amount of planktonic bacteria decreased by 63% in the control and increase by 31% for the flask exposed to 100 mg/L of P25-TiO_2-NPs. When considering the flasks after 2 weeks, the amount of bacteria increases by 183% after P25-TiO_2-NPs exposure in comparison to the control. For sessile bacteria, the decrease equal 88% in comparison to the control. Regarding the aggregation state, P25-TiO_2-NPs aggregates size increased. These large aggregates could therefore afford a support of growth for many microorganisms that would likely explain increasing number of planktonic bacteria (DAPI observations, data not shown) similar to what happen in drinking water distribution system (G. Liu et al., 2013). Particle-associated bacteria represent a small number in water (Brazos and O'Connor, 1996) but can be an important support for bacterial growth (Camper et al., 1986). The amount of sessile bacteria was lower in the highest P25-TiO_2-NP treatment in comparison to the other treatments and in control. It was previously noticed for biofilm formation in the presence of TiO_2-NPs (Maurer-Jones et al., 2013) and in WWTP where TiO_2-NP accumulated in the biosolids (Kiser et al., 2009) where the highest dose lead to the lowest amount of bacteria. Biofilms are negatively charged in natural aquatic environment due to extracellular polymeric substances (EPS) (Ferry et al., 2009; Flemming et al., 2007; R. Handy et al., 2008) corresponding to a polymeric matrix (Barnes et al., 2010; Harrison et al., 2007). Moreover, accumulation in the biofilm can occur (Battin et al., 2009) and there is a possibility that P25-TiO_2-NP just like Au-NPs can conduct to morphological change in biofilm through is action of destabilization and dispersion (Raftery et al., 2013; Stojak et al., 2011).

If accumulation of TiO_2-NP occurs in sessile bacteria and later in biofilm, we can assume that the observed modifications came from contact between nanoparticles and bacteria. This was also observed for Ag NPs who lead to mechanical injury on an arctic soil microbial community (N. Kumar et al., 2011). Bactericidal effect of TiO_2-NP have been theorized to came from the production of hydroxyl radicals (Cho et al., 2004b; Markowska-Szczupak et al., 2011; Văcăroiu et al., 2009), only produced from surface in contact with water

(Linsebigler et al., 1995) and were demonstrated to exhibit oxidative stress-mediated toxicity in different cell types with light irradiation (Gurr et al., 2005; Hussain et al., 2010; Meena et al., 2012; Saquib et al., 2012; Shukla et al., 2011; Wang et al., 2007). Nevertheless, toxic effects may occur without UV illumination as suggested by several study (Gurr et al., 2005; Long et al., 2007; Pagnout et al., 2012; Sayes et al., 2006; Theogaraj et al., 2007). Fenoglio et al. (2009) demonstrate that TiO_2 rutile ultrafine nanoparticles exhibited a comparable reactivity in the dark than under illumination (Fenoglio et al., 2009). In our previous work, the production of reactive oxygen species as a possible mechanism of genotoxicity against bacterial cells under dark conditions was previously highlighted (Jomini et al., 2012) reinforcing this idea. Moreover, it had been evidenced in the case of ZnO, TiO_2 and Ag nanoparticles that interactions with microorganisms could enhance membrane permeability increasing cell death without light illumination (Adams et al., 2006; Gogniat et al., 2006; Li et al., 2008; Liu et al., 2008; Maness et al., 1999). Another study focusing on the whole-cell-array stress genes expression in *E. coli* highlight that under dark condition, TiO_2-NPs induced oxidative stress, cell membrane damages and DNA adverse effects (Gou et al., 2010) which can explain the decreased observed in our experimentation for sessile bacteria.

Titanium dioxide nanoparticles impact the structure of the community for both planktonic and sessile bacteria (Figure 3). For sessile bacteria, structure of the community was impact for each concentration whereas it only happens at 100 mg/L of P25-TiO_2-NP for the planktonic bacteria. At the opposite, the analysis of an indigenous river water bacterial community diversity and structure showed no effect of ZVI-NP (Barnes et al., 2010). For Ag-NPs, exposition to 0.01 to 1 mg-Ag/L impact the bacterial community structure, diversity and significantly reduced bacterial metabolic activity (Das et al., 2012) very quickly for the high dose (0.1 and 1.0 mg-Ag/L) and tended to be less severe for the low exposure concentrations (0.01 and 0.02 mg-Ag/L). Resistance and even stimulation of bacterial community may be due to the presence of metal gene resistance in some bacteria or could be due to higher sensitivity of the most represented bacterial phylotypes (Das et al., 2012) which can also be the case in this study (Figure 2A). As observed with TiO_2-NPs, Ag-NP showed a significant dose and shape-effect relationship on bacterial community structure (Doiron et al., 2012).

The impact of nanoparticles is measured not only on the structure of the community but also on bacterial diversity highlighted by designed and non-specific diversity indices reflecting environmental disturbance (Gray and Delaney, 2008; Washington, 1984). At a concentration of 100 mg/l of P25-TiO_2-NP, bacterial diversity is reduced in the water column while it is rising for the sessile bacteria. Ag-NP showed a significant dose and shape-effect relationship

on bacterial abundance in a microcosm study (Doiron et al., 2012). Decreased in the number of OTUs was observed after exposition to 5 µg/L (41 OTU vs. 44 in the control) and 50 µg/L (28 OTU vs. 44 in the control) of Ag-NPs. Species richness modifications occurs quickly and signs of recovery after 48 hours are notable even at the highest concentration. The decrease in bacterial diversity was also highlighted by Ge et al., (2011) and Nogueira et al., (2012) who demonstrated that TiO_2, ZnO nanoparticles and gold nanorods reduced diversity of soil microbial community. Fabrega et al., (2011) demonstrated that Ag-NPs can cause adverse effect towards natural marine biofilm by reducing biofilm succession, development and species distribution. Ag-NPs may have potential long-term adverse effects on community structure and function. However, these effects may result from the bactericidal action of silver (Durán et al., 2007; Jain and Pradeep, 2005; Martínez-Castañón et al., 2008; Ruparelia et al., 2008; Shahverdi et al., 2007). Changes in diversity and structure of bacterial community may have implications on ecosystem functioning (Nogueira et al., 2012). Indeed, when exposed to high concentration of P25-TiO_2-NP, ratio between *Betaproteobacteria/Bacteroidetes* shifts for both planktonic and sessile bacteria (Figure 4). Increasing amount of *Emtiticia* species in the *Bacteroidetes* groups is an important part of this shift. This specie was recently identified as an oligotrophic bacteria, strictly aerobic and non-motile (Liu et al., 2008; Saha and Chakrabarti, 2006). As gender of the *Bacteroidetes* phylum, this bacteria may participate to the degradation of biopolymers and nutrient cycling (Cottrell and Kirchman, 2000).

On earth, the sessile bacteria and biofilms represent over 99% of the microorganisms (Vu et al., 2009) and are responsible for driving the biogeochemical cycling maintaining the biosphere (Davey and O'toole, 2000; Makin and Beveridge, 1996). Interactions and accumulation of TiO_2-NP on and in biofilms may have a serious and adverse impact on all the food web. Yeo and Nam, (2007) observed that bioconcentration factors in biofilm can reach a concentration of more than 95.6 µg/kg during microcosm experiments when exposed to 1.8 mg/L of TiO_2-NP after 17 days and even 2374.3 µg/kg in the case of TiO_2-nanotubes. When focusing on gold nanorods contamination of natural rivers in a mesocosm study, biofilm, representing only 0.5% of the total biomass, recovered 60% of Au-NP amount (Ferry et al., 2009). This accumulation is a key parameter to take into account because planktonic bacteria and biofilm are the major food source for heterotrophic flagellates, ciliates and invertebrates in most aquatic systems (Hall and Meyer, 1998; Huws et al., 2005; Lowell et al., 1995). Biofilms are grazed by various organisms (Iriberri et al., 1995; Lawrence et al., 2002; Sanderson et al., 1989; Scholz and Boon, 1993) and could be the basis of TiO_2-NP migration into higher trophic level. Protozoa were impact by morphological changes in biofilm by

affecting their development (Burns and Walker, 2000; Mojtahid et al., 2011) and nanoparticles accumulation in biofilm may reinforce these adverse effects. It had been shown that migration of TiO_2-NP from biofilms to mudsnail can reach 7% for only 17 days (Yeo and Nam, 2013). By impacting biofilm composition, nanoparticles may completely modified biofilm ability to modulate gene expression and lead to a much larger grazing by higher organisms (Raftery et al., 2013) altering the different function of biofilm and affecting higher trophic level development and stability (Schmitt et al., 1995; Sheldon and Walker, 1997; Stojak et al., 2011).

Conclusion

In conclusion, this microcosm-based experiment allowed us to highlight the effects of titanium dioxide nanoparticles in the dark on both planktonic and sessile bacterial community coming from the Moselle River. To our knowledge, it's the first study highlighting the diversity modification in natural water exposed to P25-TiO_2-NPs under dark conditions. We showed that a concentration of 100 mg/L P25-TiO_2-NPs lead to an increase in the planktonic population unlike the sessile bacteria where a sharp decreased is observed. This concentration changes the structural patterns of the bacterial community in both systems. Moreover, the exposition to 100 mg/L P25-TiO_2-NPs impact the bacterial diversity, mainly leading to a shift in the *Proteobacteria/Bacteroidetes* ratio. This study suggests that the main target of nanoparticles in natural water may be the sessile bacteria and later the biofilm by impacting both structure and diversity of the bacterial community. Our results encourage further studies on the effects of TiO_2-NP on diversity indices and community structure. Knowing that concentrations found in the environment will continue to increase due to the extensive use of titanium dioxide nanoparticles; we can expect to observe much localized area with high concentration of P25-TiO_2-NPs. For example, it could be the case of WWTP, major receptor for effluents containing TiO_2-NPs from accidental leaks (Boxall et al., 2007), manufacturers (Gottschalk et al., 2009) and home-using applications (Weir et al., 2012). It also promotes the need to investigate the effect of NP-TiO_2 accumulation in biofilm on higher trophic levels in order to identify potential risks to the entire food chain in the context of risk assessment.

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Conclusion et Perspectives

Conclusion

L'objectif de ces travaux de thèse était d'évaluer les interactions entre les bactéries et les nanoparticules et de déterminer quels pouvaient être les effets au niveau cellulaire, populationnel et communautaire dans un contexte d'évaluation des risques. Les travaux réalisés ont ciblé les nanoparticules de dioxyde de titane, cancérogène possible pour l'homme (groupe 2B), listées de ce fait par l'OCDE comme nanoparticules devant être immédiatement évaluées (OECD, 2008). Pour l'environnement, les nanoparticules de TiO_2-NPs sont les plus produites, les plus utilisées et conduisent à la plus forte exposition (Keller et al., 2013). Lorsque ces travaux ont été initiés en 2009, l'étude des nanoparticules et leurs effets sur les bactéries étaient une thématique émergente pour la communauté scientifique, conduisant à un nombre restreint de données écotoxicologiques dont les données variaient d'une étude à une autre. Ces variations provenaient de pratiques non optimales lors de la préparation et l'utilisation des nanoparticules mais également d'un certain manque de standardisation et de répétabilité des protocoles de tests. Nous avons pu identifier, au sein de notre équipe, les paramètres générant des variations (protocoles de tests, préparation des nanoparticules telle que la sonication, temps de sonication, intensité de sonication, durée d'exposition des organismes, phase de croissance de ceux-ci...) mettant en évidence la nécessité de standardiser nos protocoles. Ainsi, il nous est apparu primordial de réaliser une caractérisation plus poussée des nanoparticules et de fixer un protocole de préparation et d'exposition des bactéries aux nanoparticules que nous avons gardé pour l'ensemble de nos travaux. Ces nanoparticules possèdent également une très grande stabilité et sont extrêmement peu solubles dans les conditions environnementales (Cornelis et al., 2011; Judy and Bertsch, 2014). Cette résistance à la dissolution est une caractéristique primordiale dans nos études puisque l'ensemble des effets observés résultent d'interactions physique et ne sont aucunement liés à des phénomènes de dissolution et de toxicité ionique. De par cette particularité, il nous est apparu que la mise en contact intime entre nanoparticules de dioxyde de titane et bactéries est indispensable pour que des effets puissent survenir. C'est pourquoi nous avons centrer nos travaux sur la détection des interactions entre microorganismes et nanoparticules que nous avons supposé être à la base de la toxicité pour les bactéries.

Notre première étude a montré que les interactions entre les bactéries et les nanoparticules dépendaient majoritairement des charges électrostatiques présentes à la surface des bactéries

et des nanoparticules. Ainsi, les interactions électrostatiques attractives entre des nanoparticules chargées positivement et des bactéries chargées négativement conduisent à l'adsorption des nanoparticules à la surface des bactéries et à une toxicité. A l'inverse, lorsque les deux éléments sont chargés négativement, il y a répulsion électrostatique et aucune toxicité des nanoparticules. La composition du milieu électrolyte dispersant (nature des ions et force ionique) permet de moduler la toxicité des nanoparticules en écrantant les charges de surface qui sont également dépendantes du pH de la suspension. Ces observations indiquent que dans l'environnement, il est probable que des interactions de type électrostatique s'opèrent entre les bactéries et les nanoparticules de titane en fonction de la force ionique des milieux aquatiques et de leur pH, laissant à penser qu'un risque existe pour les bactéries. De plus, la matière organique présente dans la masse d'eau va conditionner l'état des nanoparticules (agrégation, coating,…) ce qui peut également moduler les effets vis-à-vis des bactéries. Cependant, dans des cours d'eau faiblement minéralisés à pH acide (cours d'eau de tête de bassin versant), les interactions électrostatiques sont beaucoup plus probables, pouvant engendrer de la toxicité pour les bactéries présentes. Cette toxicité peut se cumuler avec les effets d'autres stress (pH, qualité et quantités des nutriments,…) impactant plus intensément les organismes présents. Cependant, ces interactions électrostatiques ne peuvent expliquer à elles seules la toxicité et l'adsorption des nanoparticules de dioxyde de titane à la surface des bactéries, c'est pourquoi nous nous sommes intéressés à la nature de l'interphase entre bactéries et nanoparticules.

Pour ce faire, nous avons utilisé des mutants d'*E. coli* dont les déterminants biophysiques de la membrane externe sont modifiés du fait de délétions géniques. Le mutant JW3606, possédant une mutation du gène *rfaG* et une absence de flagelle du fait de cette délétion, se révèle être le plus sensible aux effets des nanoparticules de titane (1000 fois plus sensible que la souche témoin). La composition et la topologie de la membrane externe bactérienne sont les paramètres les plus importants dans la génération de toxicité contrairement à la longueur des LPS qui ne semble pas avoir d'effet. De plus, cette souche présente une électronégativité plus importante qui peut conduire à des cinétiques et des phénomènes d'adsorption de nanoparticules plus importants, générant ainsi une toxicité plus importante. L'analyse comparative des protéomes membranaires d'*E. coli* a mis en évidence une accumulation de la protéine flagellaire et une diminution du nombre de protéines impliquées dans les porines. Nous supposons que la flagelline serait associée à la membrane externe rejoignant ainsi les observations de Manabe et al., 2013. La toxicité des nanoparticules semble provenir de la

combinaison du phénotype de type "deep-rough" et de la délétion spécifique sur le gène *rfaG* puisque l'ensemble des souches mutantes est sensible à l'exposition aux nanoparticules.

Ces deux premières études ont montré que la toxicité des nanoparticules de dioxyde de titane à l'encontre des bactéries dépend de la charge de surface de chacun mais aussi de la membrane externe bactérienne. En effet, l'établissement d'interactions aux interphases bactéries/nanoparticules ne signifie pas forcément qu'une toxicité va survenir, celle-ci va dépendre de la composition et de la topologie de la membrane externe, avec une certaine modulation du fait de la présence/absence et de la longueur des appendices présents. Il est probable que la modification de la topologie de la membrane externe bactérienne induit une électronégativité de la surface plus importante, favorisant l'adsorption et donc la toxicité. De plus, la toxicité est modulée par le milieu environnant qui est en fait le premier inhibiteur des interactions aux interphases. Ainsi, dans l'environnement, si la composition du milieu aquatique ne permet pas l'établissement d'interactions aux interphases entre les bactéries et les nanoparticules, la toxicité de celles-ci va être négligeable. Les concentrations auxquelles vont être confrontées les bactéries sont bien entendu un paramètre primordial, mais, à l'heure actuelle, les concentrations retrouvées dans l'environnement sont trop faibles pour présenter un risque de toxicité directe.

Les interactions entre nanoparticules et bactéries conduisant, dans un milieu à faible force ionique, à la détection d'un potentiel toxique, ces interactions peuvent également conduire à l'expression d'un potentiel génotoxique.

Afin de mettre en évidence ce potentiel mutagène, le test d'Ames a été mis en place. Nous avons constaté que le milieu normalisé du test conduit à un écrantage des charges de surfaces et à des phénomènes d'agrégation conduisant à de faux négatifs. En réalisant une étape de pré-exposition dans du NaCl 10mM, nous avons maximisé les interactions entre bactéries et nanoparticules à pH 5,5, conduisant à la détection du potentiel génotoxique des nanoparticules de dioxyde de titane. La souche de *S. typhimurium* TA 102, sensible aux espèces réactives de l'oxygène, s'est révélée être la plus sensible nous indiquant que les TiO_2-NPs sont capables de générer des ROS même sans illumination UV rejoignant les constatations de plusieurs travaux (Adams et al., 2006; Gogniat et al., 2006; Li et al., 2008; Liu et al., 2007; Maness et al., 1999).

A notre connaissance, il s'agit de la première étude utilisant les souches de *S. typhimurium* TA 97a, 98, 100, 102 mettant en évidence le potentiel mutagène des TiO_2-NPs. Les nanoparticules sont adsorbées sur la membrane bactérienne sans pénétration au sein du compartiment cellulaire indiquant que ce mécanisme de génotoxicité est un mécanisme

indirect. Nous pouvons donc supposer que l'effet des ROS ne peut intervenir que lorsque les nanoparticules sont adsorbées sur la surface des bactéries. Ces observations renforcent les résultats des deux premières études, indiquant que la toxicité et la génotoxicité des nanoparticules de dioxyde de titane sont des phénomènes de surface gouvernés par les interactions aux interphases avec les bactéries. Il semblerait qu'il y ait un phénomène de diffusion des ROS du site de production en surface vers le cytoplasme de la cellule ou les effets sur l'ADN vont avoir lieu. Seul des interactions aux interphases entre des bactéries et des nanoparticules conduit à la détection de génotoxicité, car, dès lors que les nanoparticules sont agrégées dans le milieu, le potentiel génotoxique décroit fortement, voir est supprimé.

Ayant identifié un potentiel toxique et génotoxique des TiO_2-NPs, nous nous sommes donc interessés aux répercussions pour les communautés bactériennes d'un échantillon d'eau de la Moselle. Nous avons constaté une augmentation des quantités de bactéries libres pouvant résulter de la présence d'agrégats de nanoparticules offrant un support de croissance pour les bactéries. Nous observons une inversion de la prévalence entre les *Proteobacteria* et les *Bacteroidetes* qui subissent une augmentation de diversité principalement au niveau des *Cytophagaceae,* et particulièrement grâce à l'espèce *Emtiticia*. Les *Bacteroidetes* sont connues pour être des organismes opportunistes, ce qui peut expliquer cette augmentation. A l'inverse des bactéries planctoniques, les quantités de bactéries sessiles diminuent mais la diversité augmente malgré une diminution importante des *Betaproteobacteria*. La structure de cette communauté est modifiée même à la plus faible concentration. Ces variations quantitatives et qualitatives laissent à penser que la déposition des nanoparticules de dioxyde de titane impacte plus fortement et préférentiellement le développement des bactéries sessiles. On pourrait penser que l'agitation des bouteilles de test conduit à la circulation des nanoparticules sous forme agrégées, pouvant éventuellement conduire à des phénomènes abrasifs. En plus du dépôt de nanoparticules sur les surfaces permettant le développement de ce biofilm précoce, le phénomène oxydatif engendrant la toxicité peut ralentir le développement de celui-ci. L'augmentation de diversité pourrait résulter de l'action des nanoparticules sur les bactéries les plus représentées, laissant ainsi une « niche » de développement pour des espèces dont le temps de génération est plus long ou présentant des systèmes de résistance plus développés face à ce type de stress. L'analyse de la composition des communautés bactériennes indique clairement que les TiO_2-NPs ont un impact sur les assemblages bactériens. Cependant, ces travaux visaient à obtenir une estimation de la gamme de concentrations en nanoparticules pouvant exercer des effets toxiques en ne se basant pas

sur des données quantitatives réelles, c'est pourquoi les concentrations utilisées sont assez importantes. En effet, les quantités actuellement retrouvées dans l'environnement sont faibles, de l'ordre de plusieurs dizaines de µg/L pour les eaux de surface (Gottschalk et al., 2009; Kaegi et al., 2008; Kiser et al., 2009; Mueller and Nowack, 2008) mais, d'après les modèles prédictifs (accroissement de la production, de la vente et de l'utilisation), les quantités retrouvées dans l'environnement risques de fortement augmenter et de se rapprocher des doses utilisées.

Cette thèse nous a donc permis d'étudier les effets des nanoparticules de dioxyde de titane sur les bactéries à différents niveaux d'organisation, de la cellule jusqu'aux effets sur les communautés bactériennes. Il s'avère que les TiO_2-NPs présentent un impact avéré sur les organismes bactériens aux concentrations utilisées. En effet, au niveau cellulaire tout d'abord, avec une génération supposée de ROS ayant un effet délétère sur l'ADN. Nous avons également constaté le rôle primordial des membranes externes bactériennes, de leurs compositions, leurs topologies, leurs appendices et leurs charges de surfaces, conduisant à la détection de différentes formes de toxicité. Les impacts observés au niveau cellulaire conduisent à des modifications à plus large échelle. Ainsi, les interactions entre les nanoparticules de TiO_2 et les surfaces bactériennes vont conduire à la modification des quantités, de la structure et de la diversité des communautés bactériennes. Dans un contexte d'évaluation des risques, les nanoparticules de dioxyde de titane peuvent présenter un risque pour les bactéries et les communautés qu'elles constituent, mais ces risques sont très fortement modulés par le pouvoir complexant tampon que peut jouer l'environnement aquatique. Cependant, si des changements interviennent sur ces communautés, même subtiles, ils peuvent engendrer des dysfonctionnements au sein des fonctions écologiques assurées par les bactéries. Ces travaux ont montré que le contact aux interphases entre bactéries et nanoparticules constitue le préalable indispensable à un effet toxique. L'ensemble des effets délétères mis en évidence lors de l'exposition des bactéries à des nanoparticules de dioxyde de titane pourraient également conduire à modifier et à impacter les niveaux trophiques dépendant des bactéries et de ce fait, l'ensemble de l'écosystème, de manière d'autant plus importante que l'ensemble des expérimentations ont été réalisées à l'obscurité, s'affranchissant ainsi des effets photocatalytiques qui peuvent également être présents.

Perspectives

Ces travaux de thèse ont permis de mettre en évidence les effets potentiels des nanoparticules de dioxyde de titane sur les bactéries dans un contexte d'évaluation des risques et de caractérisations des mécanismes d'interactions entre les bactéries et ces nanoparticules ouvrant ainsi de nouvelles perspectives de recherche. Afin de poursuivre et d'affiner la compréhension des mécanismes de toxicité des nanoparticules, ces perspectives peuvent être envisagées.

Nous avons mis en évidence le potentiel mutagène des nanoparticules de dioxyde de titane à l'obscurité. Les radicaux hydroxyles OH° et le peroxyde d'hydrogène H_2O_2 sont les espèces radicales principalement générées lors de la formation de ROS. Cependant, la faible distance de diffusion et le temps de demi-vie des radicaux hydroxyles laisse à penser que les radicaux de peroxyde d'hydrogène seraient plutôt les responsables de ces effets intracellulaires. Il conviendrait de confirmer que ce soit bien le peroxyde d'hydrogène qui est généré en cherchant à le détecter et à le quantifier. Pour ce faire, la technique de chimiluminescence qui détecte un signal lumineux produit par l'émission de photon d'une molécule excitée retournant vers un état énergétique moins important après une réaction d'oxydation peut être mise en place. Cette technique permet de détecter différentes espèces réactives en fonction des sondes luminescentes choisies (Uy et al., 2011). Lorsque des molécules scavengers spécifiques sont utilisées, les ROS sont piégées par ces molécules conduisant à une diminution de l'émission lumineuse en chimiluminescence, ce qui permet d'identifier et de quantifier les espèces réactives en présence (Carre, 2013). En complément de cette technique, il conviendrait de réaliser une mesure plus fine par la technique de résonance paramagnétique électronique (EPR) couplé à de la capture de radicaux (spin trapping) (détection indirecte). Elle permet la détection du rayonnement paramagnétique produit par des électrons non appariés sur des sondes ayant subi les effets des ROS (Kohno, 2010). Cette technique utilise un composé diamagnétique (spin trap) qui va réagir avec les espèces réactives de l'oxygène dont la durée de vie est très courte afin de former des radicaux plus persistants (spin adduct) (Hirakawa et al., 2001; Pierzchala, 2010). En fonction des sondes utilisées, il sera alors possible de déterminer le type de ROS produits mais aussi les concentrations de chacune.

Lors des expérimentations sur les mutants bactériens, nous avons également mis en évidence l'accumulation de la protéine FliC au niveau de la membrane externe de la souche mutante JW3606. Il serait intéressant d'évaluer quelle est l'étape limitante dans l'exportation de cette protéine lors de l'assemblage du flagelle dans le but de mieux comprendre le mécanisme d'accumulation observé. Il conviendrait de vérifier si la même toxicité vis-à-vis de cette souche serait obtenue sans l'accumulation de la flagelline. On pourrait construire un double mutant présentant à la fois la délétion au niveau de *rfaG* et une délétion de *fliC* qui inhiberait totalement la synthèse du flagelle et voir si la toxicité des nanoparticules varie. De plus, il pourrait être intéressant de travailler avec un double mutant d'*E. coli* présentant une mutation sur *rfaG* mais un flagelle actif afin de vérifier le rôle de celui-ci dans la toxicité. Les propriétés nanomécanique de la membrane externe des souches bactériennes délétées pourraient être caractérisées après exposition aux nanoparticules à la fois lorsque celles-ci sont présentes et après lavage.

Concernant les communautés bactériennes, nous avons observé les effets déstructurants du P25-TiO_2-NP sur une communauté bactérienne aquatique d'eau douce naturelle. Cependant, nous avons utilisé des doses de nanoparticules assez importantes pour des temps d'exposition moyennement longs. Il serait judicieux de mettre en place le même set d'expérimentations en utilisant cette fois des concentrations beaucoup plus faibles mais en exposant les communautés bactériennes naturelles pour une durée beaucoup plus longue. Nous pourrions également étudier l'effet abrasif des nanoparticules de dioxyde de titane sur un biofilm monocouche ayant colonisé un substrat de croissance dans un système d'agitation similaire à celui mis en place pour nos expérimentations. Une expérimentation plus longue pourrait être couplée à des mesures de processus fonctionnels tel que la minéralisation du carbone, et de suivre en « temps réel » les modifications intervenant au sein de ces microcosmes. De plus, il pourrait être intéressant de réaliser une étude de transcriptomique par puce à ADN afin de vérifier si des gènes de résistance aux métaux sont plus exprimés à la fin de l'expérimentation par rapport au début de celle-ci. Les approches expérimentales réalisées au laboratoire sont à compléter par des observations et des analyses *in situ*, comme par exemple l'analyse et le suivi spatio-temporel de coraux exposés à des nanoparticules de titane suite au relargage provenant de crèmes solaires.

Une étape complémentaire intéressante à mettre en place serait l'analyse d'une chaîne trophique dont la base serait bactérienne. En effet, de nombreux organismes se nourrissent sur des biofilms ou de bactéries libres présentes dans la colonne d'eau. Il conviendrait ainsi de suivre la bioaccumulation des nanoparticules au sein d'une chaîne trophique composée

d'organismes bactériens, dont la consommation serait réalisée par des protozoaires, tel que *Tetrahymena pyriformis*. Eux-mêmes seraient alors ingérés par des daphnies (*Cerodaphnia dubia*) qui elles serviraient de nourriture à de jeunes alvins. Cette expérimentation permettra de suivre la bioaccumulation des nanoparticules de titane au sein des différents organismes composant cette chaîne trophique tout en évaluant les effets potentiellement néfastes vis-à-vis de chacun des organismes précités.

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Annexes

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Short Communication

Hemocyte responses of *Dreissena polymorpha* following a short-term in vivo exposure to titanium dioxide nanoparticles: Preliminary investigations

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HIGHLIGHTS

- Phagocytosis inhibition at TiO_2 NP exposure concentrations of 0.1 and 1 mg/L.
- Internalization of TiO_2 NP in freshwater mussel hemocytes.
- Increased phosphorylation level of p38 and ERK 1/2 after in vivo exposure to TiO_2 NP.

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ABSTRACT

The widespread use of titanium-based nanoparticles and their environmental release may pose a significant risk to aquatic organisms within freshwater ecosystems. Suspension-feeder invertebrates like bivalve molluscs represent a unique target group for nanoparticle toxicology. The aim of this work was to investigate the short-term responses of *Dreissena polymorpha* hemocytes after in vivo exposure to titanium dioxide nanoparticles (TiO_2 NP). For this purpose, freshwater mussels were exposed to P25 TiO_2 NP at the concentrations of 0.1, 1, 5 and 25 mg/L during 24 h. Viability, phagocytosis activity and mitogen activated protein kinase (MAPK) phosphorylation level of ERK 1/2 and p38 in hemocytes extracted from exposed mussels were compared to those from control specimens. Results demonstrated an inhibition of the phagocytosis activity after exposure to TiO_2 NP at 0.1 and 1 mg/L. Similar trends, albeit less pronounced, were reported for higher concentrations of NP. Transmission electron microscopy showed for the first time the internalization of TiO_2 NP into *Dreissena polymorpha* hemocytes. Besides, exposure to NP increased the ERK 1/2 phosphorylation levels in all treatments. Concerning the phosphorylation level of p38, only exposures to 5 and 25 mg/L of NP induced significant p38 activation in comparison to that of the control. Finally, these short-term effects observed at environmentally relevant concentrations highlighted the need for further studies concerning ecotoxicological evaluation of nanoparticle release into an aquatic environment.

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1. Introduction

Nanotechnology represents one of the most rapidly expanding field of applied science and technology since funds allocated for research and development substantially increase each year, e.g. from $ 270 million invested by the US Government in 2000 to $ 849 million in 2004 (Roco, 2003; Nowack and Bucheli, 2007). The industrial production may encompass more than 58,000 t of engineered nanomaterials in the next decade, presumably leading to the enhancement of environmental exposure to nanoparticles (NP) (Daughton, 2004; Nowack and Bucheli, 2007). As an illustration, the aquatic environment may be contaminated not only by atmospheric deposition or industrial runoff but also by urban applications (Harley, 2008).

Several studies have highlighted the influence of the nature, size, surface area and concentration of NP on their adverse effects on terrestrial organisms with particular emphasis on human health. For instance, Goulaouic et al. (2008) showed that carbon black NP induced a proinflammatory response in human immune system cells through a modification of cytokine secretion. On the contrary, little is known about the behavior, fate and accumulation of NP in aquatic ecosystems and the issue of their potential ecotoxicological effects must

Abbreviations: CB, carbon black; ERK 1/2, extracellular signal regulating kinase 1 et 2; MAPK, mitogen activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NP, nanoparticle; PAH, polycyclic aromatic hydrocarbon; PEC, predict effect concentration; P-ERK 1/2, phosphorylated ERK 1/2; P-p38, phosphorylated p38; ROS, reactive oxygen species; TEM, transmission electron microscopy; TiO_2 NP, titanium dioxide nanoparticle; XRD, X-ray powder diffraction.

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be clarified. In particular, assessment of the sublethal effects of NP exposure on aquatic invertebrates is still in its infancy (Moore, 2006; Mouchet et al., 2008). For the latter, contrary to terrestrial organisms that ingest and inhale NP, there is another pathway for direct contaminant diffusion, i.e. through epithelial tissues such as gills followed by endocytosis (Moore, 2006; Oberdörster, 2004). Taking into account the ubiquitous geographical distribution and the suspension-feeding mode of some macro invertebrates, many researchers consider bivalve shellfishes like mussels as "suitable sentinel organisms" for the aquatic monitoring of the effects of emerging pollutants—including NP (Sauvé et al., 2002; Baun et al., 2008; Canesi et al., 2004, 2010a; Canty et al., 2007). Recently, Canesi and her collaborators showed that marine mussel (*Mytilus galloprovincialis*) exposure to NP such as TiO_2, C60 fullerene and SiO_2 increased production of reactive oxygen species (ROS) and nitric oxide (NO) by circulating hemocytes (bivalve immune system cells). Their results suggested that the assessment of immunological parameters in marine bivalves could be informative for a better understanding of ecotoxicity of in vivo exposure to NP in sea water.

A large variety of nanoparticles have been produced during the last decades, with TiO_2 NP which are among the first nanomaterials made readily available commercially (Menard et al., 2011). Because of their unique physicochemical properties (photocatalysis, inertness, opacity, resistance to fading), TiO_2 NP have been commonly used in sunscreens, plastics, paints, papers and many other household applications (Mueller and Nowack, 2008). However, despite their wide occurrence in manufactured products and their environmental release, there is still a lack of information regarding their toxicological impact on freshwater ecosystems.

The present study intended to address the specific issue of early biological effects of in vivo exposure to TiO_2 NP within the overall framework of nanoparticle ecotoxicity evaluation. Taking into account that invertebrate hemocytes are responsive to various environmental contaminations in a short time period (Canty et al., 2007; Canesi et al., 2010a; Wootton et al., 2003), the sublethal effects of TiO_2 NP on immune system cells of the zebra mussels (*Dreissena polymorpha*) after 24 h in vivo exposure were investigated. For this purpose, hemocyte viability was first evaluated, followed by several cellular parameters: phagocytosis activity and phosphorylation levels of the mitogen activated protein kinase (MAPKs) ERK 1/2 and p38, which are components of kinase-cell signaling pathways playing a key role in the activation of the immune system.

2. Material and methods

2.1. TiO_2 NP exposure concentrations

There is scarce information relating to measurement of TiO_2 NP concentrations in surface waters. Typical in situ concentrations of nanostructured TiO_2 in headworks of wastewater treatment plants have been reported in the range of 1.4 mg/L of TiO_2 NP (with peak concentrations up to 5 mg/L) whereas the average concentration in the tertiary effluent was 0.06 mg/L (with peak concentrations up to 0.11 mg/L) (Kiser et al., 2009). However, Battin et al. (2009) stated that higher TiO_2 NP concentrations in the range of 5 mg/L in final effluents are most likely to represent a realistic scenario in the case of overflow events. Moreover, once released into water, nanoparticle aggregation and partition to suspended particulate matter would probably occur and subsequent precipitation of agglomerates would lead to their progressive accumulation in sediments (Boxall et al., 2007). As benthic suspension feeding organisms, zebra mussels will be exposed to aggregated nanoparticles in significant concentration levels over time. Thus, in order to cover a wide range of contamination levels that may be reported for polluted environments, bivalves were exposed to P25 TiO_2 NP concentrations of 0.1, 1, 5 and 25 mg/L (chosen as a threshold value for highly exposed areas).

2.2. Preparation and characterization of TiO_2 NP suspensions

TiO_2 nanopowder AEROXIDE® P25 was purchased from Evonik Degussa GmbH (Frankfurt, Germany, Stock # 4168050298). According to the manufacturer, these nanoparticles were characterized by a primary size of 25 nm with a specific surface area (SSA) of 50 ± 15 m^2/g and a ratio of anatase/rutile forms of 80/20. For this study, a stock suspension of P25 TiO_2 (10 g/L) was first prepared by mixing 100 mg of TiO_2 NP with 10 mL of sterile ultrapure water (milli-Q water, 18.2 MΩ·cm). This mixture was sonicated during 30 min at 4 °C (Sonics Vibra-cell 750 W, Sonics & Materials, Inc., Newton, CT, USA; frequency 20 kHz, 3 mm micro tip, 40% amplitude) in order to break the larger agglomerates and to homogenize the resulting suspension (Pagnout et al., 2012). The four suspensions of TiO_2 NP used for bivalve exposure (0.1, 1, 5 and 25 mg/L) were prepared using artificial freshwater reconstituted with Milli-Q grade water and Evian mineral water (50/50 v/v).

Taking into account that TiO_2 has very low aqueous solubility in ambient temperature and pressure (Antignano and Manning, 2008), Ti in freshwater was expected to occur solely in solid phases (not in an ionic form).

The shape and primary size of TiO_2 NP were assessed by transmission electron microscopy (TEM), using a CM 20 Philips electron microscope at 200 kV (Service Commun de Microscopies Electroniques et de Microanalyses, Nancy, France). Samples were prepared following the evaporation of a droplet of nanoparticle suspension on a copper grid at ambient temperature. Sizes were determined using 100 randomly chosen nanoparticles. Nanoparticle crystalline structures were elucidated by X-ray powder diffraction (XRD). Finally, electrophoretic mobility and nanoparticle size distribution in aqueous media were determined with ZetaSizer 3000HS (Malvern Instruments, Worcestershire, UK).

2.3. Sampling, maintenance and in vivo exposure of mussels

Adult specimens of zebra mussels (*D. polymorpha*), 20–25 mm long, were collected from a gravel pit reference site located in Northern France (Moulins-lès-Metz). They were maintained in the dark and acclimated 7 days in aerated water (20 °C) in polypropylene tanks with daily feeding (freeze dried *Spirulina* sp. powder, TETRA). Then, they were transferred into 2 L glass beakers filled with 1 L of artificial freshwater. All mussels (30 organisms) were exposed during 24 h, and 4 concentrations of TiO_2 NP suspensions were tested (0.1, 1, 5 and 25 mg/L) with a parallel control group (mussels in freshwater only). Exposures were conducted in static conditions. However, due to the tendency of TiO_2 NP to agglomerate in water and in order to avoid particle settlement in the bottom of beakers, the media were stirred for all the duration of exposure.

2.4. Hemolymph extraction and determination of hemocyte density

The method of extraction followed the one developed by Canesi et al. (2010b). Briefly, it was extracted from the posterior adductor muscle using a 1 mL sterile syringe provided with a 23 G 1 in. needle and kept at 4 °C. Hemocyte density was determined by counting live cells using trypan blue (0.4% in physiological solution; v/v) exclusion test according to the method used by several authors on hemocytes in culture (Cao et al., 2007; Quinn et al., 2009).

2.5. MTT assay for cell viability

Cytotoxic effects following TiO_2 NP exposure of organisms were assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tétrazolium bromide) assay. The MTT assay for cell viability followed the method developed by Mosmann (1983) and was adapted to measure the viability of invertebrate hemocytes (Domart-Coulon et al., 2000)

with slight modifications. The method is based on the ability of viable cells only to reduce MTT (yellow in solution) to formazan (purple crystal). After exposure of mussels to TiO_2 NP (0, 0.1, 1, 5, and 25 mg/L) and hemolymph extraction, hemocytes were incubated with 100 µL of 5 mg/mL MTT solution for 4 h at 37 °C in a 96-well microtiter plate. Then, 100 µL of a lysis buffer (20% Sodium Dodecyl Sulfate in N, N-dimethylformamide; w/v) were added to each well in order to lyse cells and formazan crystals. Absorbance was read at 570 nm using a microplate spectrophotometer (BioTek PowerWave XS; VWR, Strasbourg, France). Hemocyte viability was expressed as percentages of the value reported for control cells (extracted from non exposed bivalves).

2.6. Phagocytosis assay

The phagocytosis activity of hemocytes was determined using 1 µm fluorescent latex beads (Latex beads Amine-modified Fluorescent yellow-green L1030; SIGMA). After mussel exposure and cell extraction, a volume corresponding to 10^6 cells was centrifuged for 5 min at 150 *g* and taken up in 1 mL of phosphate buffer saline (PBS) 0.15×. Then, 1 mL of cell suspension was deposited on glass slides, previously sterilized and coated with polylysine solution (0.3 mg/mL diluted in PBS 0.15×) in a 12-well plate. After 3 h of incubation at 16 °C (to allow cell adhesion), non-adherent cells were removed by rinsing with PBS 0.15×. Then, 1 mL of microbead solution diluted in PBS 0.15× (final *ratio* 1:100) was added in each well and the preparation was incubated for 4 additional hours at 16 °C. After washing with PBS 0.15× to remove the excess of non-phagocytosed microbeads, cells were fixed with 1 mL of paraformaldehyde (4% solution diluted in PBS 0.15×) for 10 min. Finally, the slides were washed 3 times with PBS 0.15× and mounted on glass slides with one drop editing solution (Aqua Polymount, Polyscience). Slide observations were performed with a fluorescence microscope (Eclipse E800; Nikon) and the number of hemocytes (among 300 observed cells) that had phagocytosed at least one microbead was counted. The percentage of cells realizing phagocytosis was calculated as follows: (number of cells that had ingested at least one bead ∗ 100) / number of total counted cells.

2.7. Confocal laser scanning microscopy (CLSM)

Phagocytosis of FITC-latex beads by *D. polymorpha* hemocytes were monitored with a laser scanning microscope (LSM 510; Carl Zeiss) equipped with an EC Plan-Neofluar 40× lens (numerical aperture 1.3).

2.8. Transmission electron microscopy of hemocytes

Hemolymph was extracted from mussels exposed to 1 mg/L of TiO_2 NP. Hemocytes were recovered after a centrifugation period of 10 min at 10 000 *g* at 4 °C. Cells were fixed with a 2.5% glutaraldehyde solution, washed twice with sterile ultrapure water, post-fixed with osmium tetraoxide (OsO_4), washed three times with sterile ultrapure water, and dehydrated in graded concentrations of ethanol (Sturm et al., 1974). The pellet obtained was embedded in Epon and was allowed to polymerize during 2 h at 38 °C and 2 days at 60 °C. Ultra-thin sections (90 nm) were then collected over copper grids and counterstained with lead citrate and uranyl acetate. The sections were finally observed with a CM 20 Philips electron microscope at 200 kV (Service Commun de Microscopies Electroniques et de Microanalyses, Nancy, France).

2.9. Western blotting

After hemolymph extractions from exposed mussels, cells were incubated in lysis buffer (Tris base 20 mM, NaCl 137 mM, Nonidet 1% and glycerol 10%) containing 1 mg/mL of protease inhibitor cocktail (antipain, aprotinin, chymostatin, E64 and leupeptin) (Sigma-Aldrich) during 45 min at 4 °C. Cell lysates were cleared by centrifugation at 14,000 *g* for 1 min. Before sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), protein concentrations in total lysates were determined using the bicinchoninic acid method with bovine serum albumin as a standard (BC Assay protein quantitation kit; Interchim). Protein aliquots (20 µg) were applied to a 12% SDS-PAGE. Equivalent amounts of proteins, checked by controlling the amounts of β-actin in each sample, were used to allow for comparisons between samples. After transfer onto nitrocellulose membranes (hybond-ECL; Amersham), blots were blocked with 4% dried milk solution overnight and incubated for 2 h with the following primary antibodies: anti-phosphorylated ERK1/2 antibodies (1:2000 dilution, reference 9106; Cell Signalling Technology), anti-phosphorylated p38 MAPK antibodies (1:2000 dilution, reference 9216; Cell Signalling Technology) or anti-beta actin antibodies (1:4000 dilution, reference 3700; Cell Signalling Technology). Then, membranes were washed and incubated for 1 additional hour with the anti-mouse IgG HRp-linked secondary antibody (1:2000, reference 7076; Cell Signalling Technology). In some experiments, membranes were stripped of antibodies (Restore Western Blot Stripping Buffer; Thermo Fisher Scientific Inc.) and probed again with a different one. Bands were finally visualized using a substrate kit (Immun-Star™ HRP Chemiluminescence Kits; Bio-Rad) according to the manufacturer's instructions and registered with a ChemiDoc™ XRS apparatus (Bio-Rad). Quantitative results were obtained using the Image Lab acquisition software (Bio-Rad).

2.10. Statistical analyses

Results were expressed as means of three experiments in triplicate ± SD. After verification of the normality with the Kolmogorov–Smirnov test and of variance homogeneity with the Levene test, data were analyzed by the z-test with a $P \leq 0.05$ significance using the SigmaStat v.3.5 software.

3. Results and discussion

3.1. Nanoparticle characterization

The P25 TiO_2 NP used in this study was thoroughly characterized (Fig. 1). Analyses revealed that TiO_2 NP was a mixture of 84% anatase and 16% rutile forms (Fig. 1A) with an average primary particle size of 23 ± 4.9 nm (Fig. 1B). Dynamic light scattering (DLS) measurements of the initial TiO_2 NP stock suspension resulting from dispersion in Milli-Q water and probe sonication showed that the average hydrodynamic diameter of NP varied from 60 to 80 nm (Fig. 1C), indicating that nanoparticles were partially agglomerated. Additional DLS measurements of the TiO_2 NP suspensions prepared for bivalve exposure revealed that the average hydrodynamic diameters in those cases increased with NP concentrations: below detection limit for 0.1 mg/L of TiO_2 NP, 837 ± 32 nm for 1 mg/L of TiO_2 NP, 1007 ± 79 nm for 5 mg/L of TiO_2 NP and 2330 ± 94 nm for 25 mg/L of TiO_2 NP. Aggregation of TiO_2 NP is generally observed when entering aquatic systems and increases with ionic strength and pH values near the zero point of charge (Domingos et al., 2009; Jiang et al., 2009). Aggregated nanosized particles are usually less mobile, leading to progressive sedimentation and enhancement of their bioavailability to suspension feeder organisms (Farré et al., 2009). On the contrary, the adsorption of natural organic matter including humic acids onto the surface of nanoparticles has been shown to increase the stabilization of nanosized TiO_2, influencing their transport within the water phase on long distances (Domingos et al., 2009). However, other complex interactions may occur between agglomerated nanoparticles and surrounding media. As an illustration, Chen

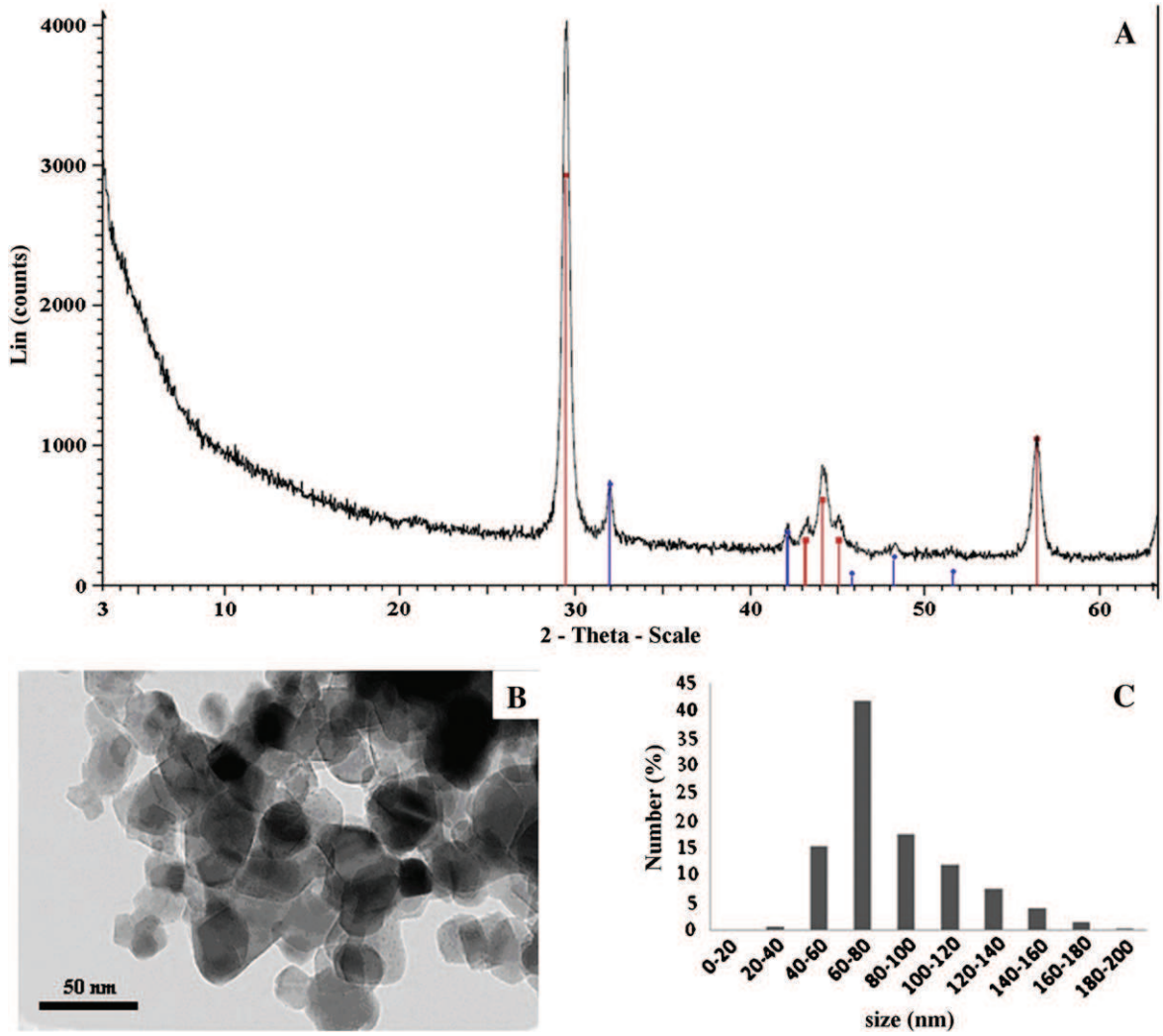


Fig. 1. Characterization of the TiO_2-P25 nanoparticles. (A) X-ray diffraction pattern of the initial TiO_2-P25 nanoparticles. Red peaks were assigned to the anatase form and blue peaks to the rutile form; (B) TEM micrograph and (C) hydrodynamic diameter of the TiO_2-P25 nanoparticle stock suspension after 30 min of dispersion by sonication in ultrapure water.

and Elimelech (2007) showed that enhanced aggregation of C60 fullerene was observed in the presence of organic matter at higher concentrations of $CaCl_2$, presumably due to bridging mechanisms between Ca^{2+}, humic acids and nanoparticles. According to Lei et al. (1996), particles equal to or greater than 1.5 μm in diameter are retained with nearly total efficiency by *D. polymorpha*. Thus, higher retention of nanoparticle aggregates by the mussel gills was likely to occur in our experimental conditions with increasing concentration of TiO_2 NP in exposure media, potentially promoting their uptake by ingestion.

3.2. Cell viability

The necessary prerequisite before any cytological investigation was to ensure that the four different concentrations of TiO_2 NP selected for bivalve exposure had no adverse effect on hemocyte viability. For this purpose, the rapid MTT assay was chosen and performed on hemocytes immediately following hemolymph extraction. Cell viability was respectively in the range of $98 \pm 0.7\%$, $95 \pm 5\%$, $98 \pm 1.9\%$ and $98 \pm 2\%$ of the control for the different exposure concentrations of 0.1, 1, 5 and 25 mg/L TiO_2 NP. No significant effect of the tested NP concentrations was observed on hemocyte viability in accordance with other experiments with the same type of nanoparticles (Warheit et al., 2007; Lee et al., 2008), thus allowing for further cytological investigations.

3.3. Phagocytosis capacity

Immune response of invertebrates like bivalves mainly consists of innate immunity at the cellular level involving phagocytosis by hemocytes, ROS production and secretion of various humoral factors (Pipe and Coles, 1995; Franchini and Ottaviani, 2000; Bettencourt et al., 2009), whereas additional adaptive mechanisms may be described for vertebrates (Fournier et al., 2004). Therefore, sublethal effects of nanoparticle exposure were first evaluated on the capacity of hemocytes to phagocytose. Cells extracted from mussels exposed to TiO_2 NP were analyzed for their phagocytosis activity of FiTC-latex beads using fluorescence microscopy (Fig. 2A). Results indicated that 63% of hemocytes that originated from non-exposed mussel were able to realize the phagocytosis of at least one FiTC-latex bead (data not shown). Furthermore, bivalve exposure to decreased concentrations of NP induced significant inhibition of phagocytosis activity (Fig. 2B). The most important decreases (38.4 and 24.1% of phagocytosis rate compared to hemocytes from non-treated mussels) were reported for mussels exposed to the lowest concentration of NP (respectively 1 and 0.1 mg/L), followed by 19.8 and 14.3% of inhibition for concentrations of 5 and 25 mg/L TiO_2 NP (Fig. 2B). A

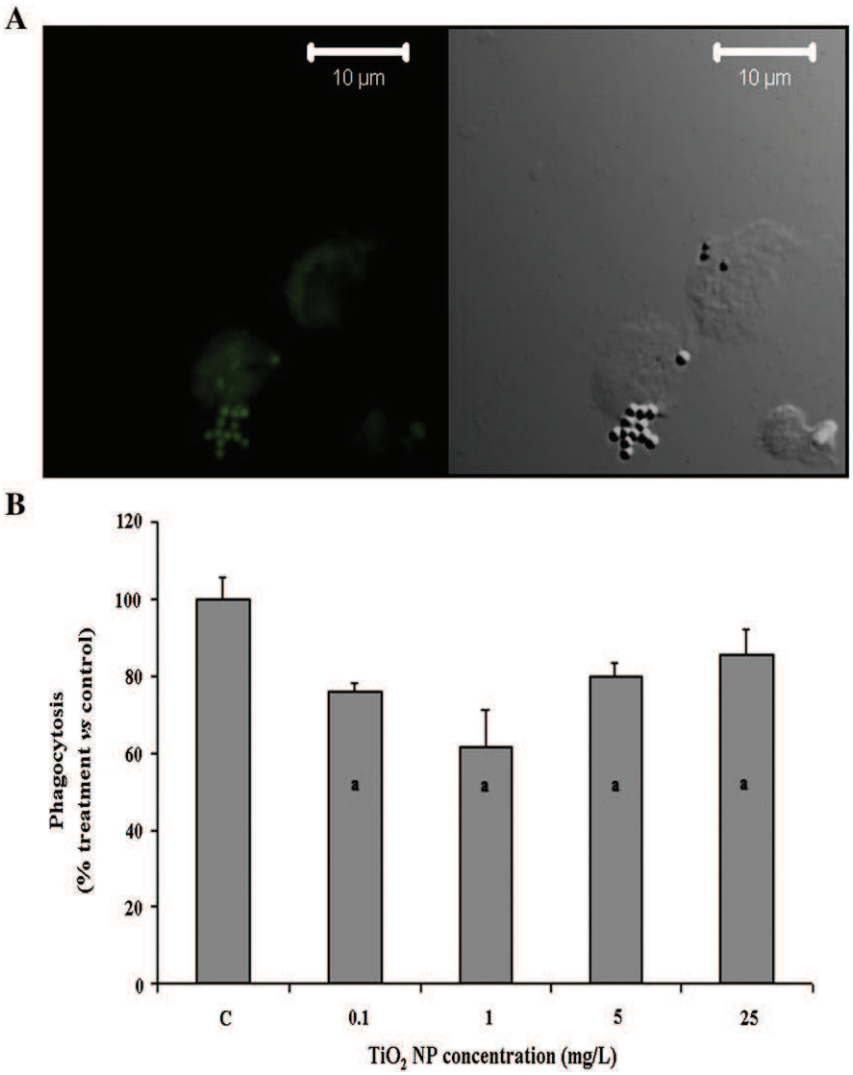


Fig. 2. Effects of 24 h exposure of bivalves to TiO_2 nanoparticles (0.1, 1, 5 and 25 mg/L) on hemocytes phagocytosis capacity. Panel A represents optical fluorescent microscopy analysis of phagocytosis of 1 µm latex beads by hemocytes extracted from mussels exposed to 1 mg/L of TiO_2 nanoparticles (scale bar represents 10 µm). Panel B represents effects of TiO_2 nanoparticles on phagocytosis of latex beads by hemocytes. (Data normalized for the control) (Values are mean ± SD; n = 3). a: vs control ($p > 0.05$).

dose-dependent decrease of phagocytosis is often observed following exposure to various pollutants such as polycyclic aromatic hydrocarbons (PAH) (Wootton et al., 2003; Hannam et al., 2010), heavy metals (Brousseau et al., 2000) and pharmaceutical compounds (Binelli et al., 2009). In this study, the phagocytosis inhibition was not dose-dependent. Data were consistent with those reported by Gagné et al. (2008) who evaluated the toxicity of CdTe quantum dots on the immune system of the freshwater mussel *Elliptio complanata*. Indeed, inhibition of the phagocytosis activity of *E. complanata* was more important at a concentration of 4 mg/L than at 8 mg/L of CdTe NP water suspension. According to the authors, such a phenomenon could be at least partially explained by nanoparticle aggregation at high concentrations. DLS measurements of NP suspensions in our experimental conditions tended to confirm a similar hypothesis for TiO_2 NP: practically three fold higher hydrodynamic diameters of NP were reported at 25 mg/L of TiO_2 NP (2330 ± 94 nm) than at 1 mg/L of TiO_2 NP (837 ± 32 nm). Nevertheless, the fact that phagocytosis inhibition was not linearly correlated with TiO_2 NP concentration in water suspensions may also suggest that there is an intermediate TiO_2 NP aggregate size (in the range of hydrodynamic diameter of 840 nm) that more effectively "frustrates phagocytosis" whereas other cytological parameters (viability, MAPK phosphorylation level) are less affected.

3.4. Localization of TiO_2 NP after mussel exposure to the contaminants

In order to examine the putative link between phagocytosis inhibition and potential intracellular uptake of nanoparticles, attempts were made to localize TiO_2 NP in hemocytes extracted from mussels exposed to 1 mg/L of NP. To our knowledge, relatively few studies have described internalization of TiO_2 NP and for most of them after in vitro exposure of cells. For instance TiO_2 NP internalization has been reported into fibroblasts (Allouni et al., 2012) as well as iron oxide nanoparticles into cancer cells (El-Dakdouki et al., 2012). In the present work, the observations by transmission electron microscopy showed that TiO_2 NP (indicated by white arrows, Fig. 3A) was internalized into hemocytes of *D. polymorpha*. The nature of nanoparticles was confirmed by EDS spectra (Fig. 3B). This finding should be examined more closely using biomarkers of cellular damages and complementary assay techniques in order to establish a causal link between NP internalization and phagocytosis activity inhibition. However, to the best of our knowledge, no TiO_2 NP internalization has been previously reported in hemocytes of zebra mussels after in vivo exposure. As reported by Canesi et al. (2012), nanoparticle aggregates captured onto gills during in vivo exposure may be transported into the digestive gland through a succession of physiological processes involved in the bivalve feeding

mechanism. Nanoparticles can be translocated from digestive gland to hemolymph and reach hemocytes as shown with polystyrene microspheres in the blue mussel *Mytilus edulis* (Browne and Dissanayake, 2008). At the hemocyte level, TiO_2 NP internalization may occur through endocytic pathways leading to the endosomal and lysosomal compartments or else via cell-surface lipid raft associated domains termed caveolae (Moore, 2006).

3.5. Activation levels of MAPKs: ERK1/2 and p38

MAPK signaling pathway is known to play a key role in the immune response of invertebrates (Bettencourt et al., 2009). In bivalves, it has been shown to be involved in the dissemination of hemocytes, ROS production and phagocytosis (Canesi et al., 2002; Humphries and Yoshino, 2003). Indeed, in *Mytilus* hemocytes (as well as in mammalian macrophages), several components of intracellular signaling pathways play a key role in the activation of the immune response: for example, the stress-activated p38 MAPK was activated in mussel hemocytes by bacterial challenge and inflammatory cytokines (Canesi et al., 2002, 2005, 2008; Betti et al., 2006). Persistence of this phenomenon was associated with phosphatidyl serine externalization, indicating apoptotic processes and cell damages (Betti et al., 2006). In parallel, several studies showed that immuno-challenge of mammalian macrophages by cell-free lipopolysaccharide (LPS) or bacteria is often associated with activation of the highly conserved extracellular-signal regulated (ERK) pathway, which can either positively or negatively regulate certain immune responses like phagocytosis (Weinstein et al., 1992; Sanghera et al., 1996; Ruckdeschel et al., 1997; Procyk et al., 1999; Monick et al., 2000; García-García et al., 2001). In order to evaluate if an in vivo exposure to TiO_2 NP could also affect the MAPK signaling pathway of *D. polymorpha* immune cells, the phosphorylation levels of ERK1/2 and p38 MAPK in hemocytes extracted from exposed mussels and the control ones were compared (Figs. 4 and 5). For this purpose, western blot analysis of total protein extracts using specific antibodies directed against the phosphorylated forms of ERK1/2 (P-ERK1/2) and p38 MAPK (P-p38) were done.

Results showed that exposure of mussels to TiO_2 NP (0.1, 1, 5 and 25 mg/L) induced increases of ERK1/2 phosphorylation level which were respectively 40 ± 3.6, 48 ± 4.5, 24 ± 2.7 and $34 \pm 2.3\%$ higher than the ERK1/2 phosphorylation level of control cells (Fig. 4). Besides, for the same exposure conditions, no variation was observed for p38 phosphorylation level at the lowest NP concentrations of 0.1 and 1 mg/L (Fig. 5), whereas exposures to 5 and 25 mg/L of TiO_2 NP induced respectively 33 ± 3.3 and $73 \pm 3\%$ increases in phosphorylation level compared to control (Fig. 5). These results were similar to those of Canesi et al. (2008, 2010a) who reported increases of p38 activation level in hemocytes of *M. galloprovincialis* exposed to CB, TiO_2, SiO_2 and C60 fullerene NPs suggesting that TiO_2 NP can also induce an inflammatory response in freshwater bivalve hemocytes. As previously stated during the TiO_2 NP water suspension characterization, higher aggregate sizes at elevated concentrations of TiO_2 NP are likely to enhance aggregate uptake and ingestion by mussels, thus allowing for a type of concentration dependent modulation of MAPK phosphorylation. Nevertheless, the observed increases in p38 activation level only for high concentrations of TiO_2 NP (5 and 25 mg/L) indicated that p38 phosphorylation was less sensitive to an exposure to this type of contaminant than ERK 1/2.

4. Conclusion and perspectives

The short-term in vivo exposure to TiO_2 NP was demonstrated to induce several changes on functional parameters of *D. polymorpha* hemocytes. Despite the absence of any adverse effect on hemocyte

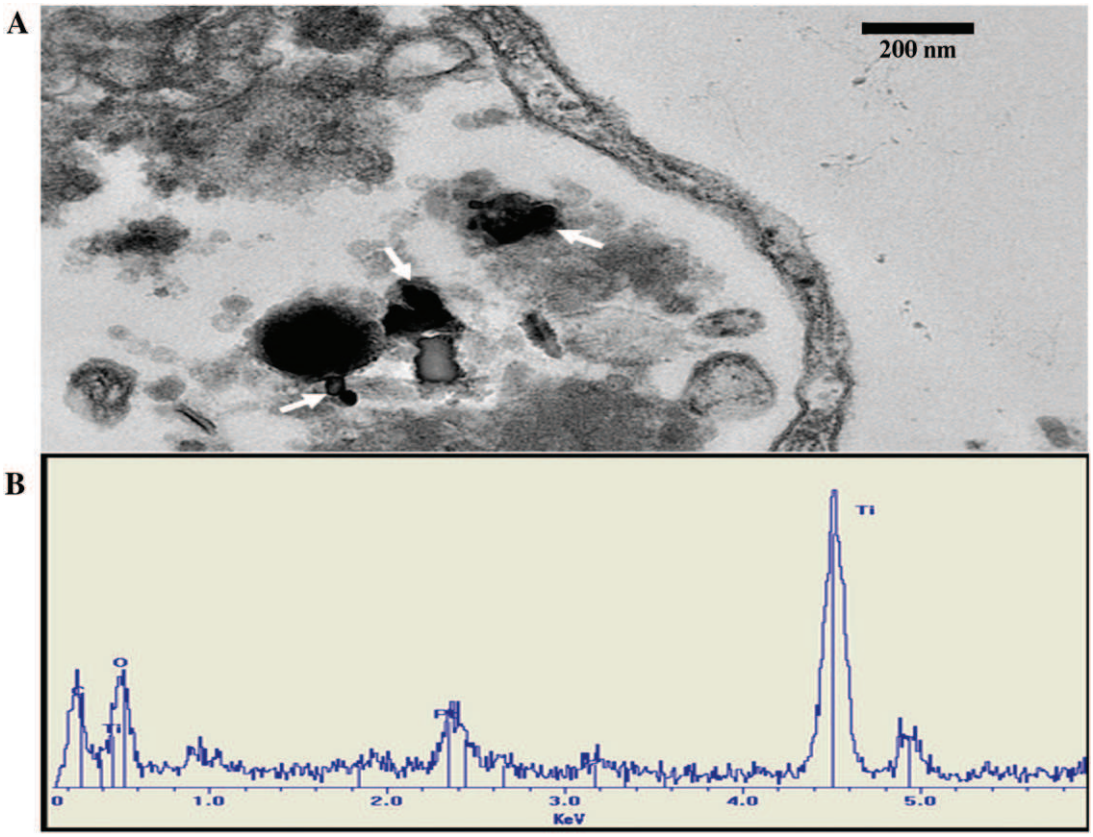


Fig. 3. Internalization of TiO_2 nanoparticles by hemocytes after bivalve exposure. A: SEM image of *Dreissena polymorpha* hemocyte extract from 1 mg/L TiO_2 NP in vivo exposed mussels; white arrows show TiO_2 NP internalized in the hemocyte. B: EDS spectra of internalized particles.

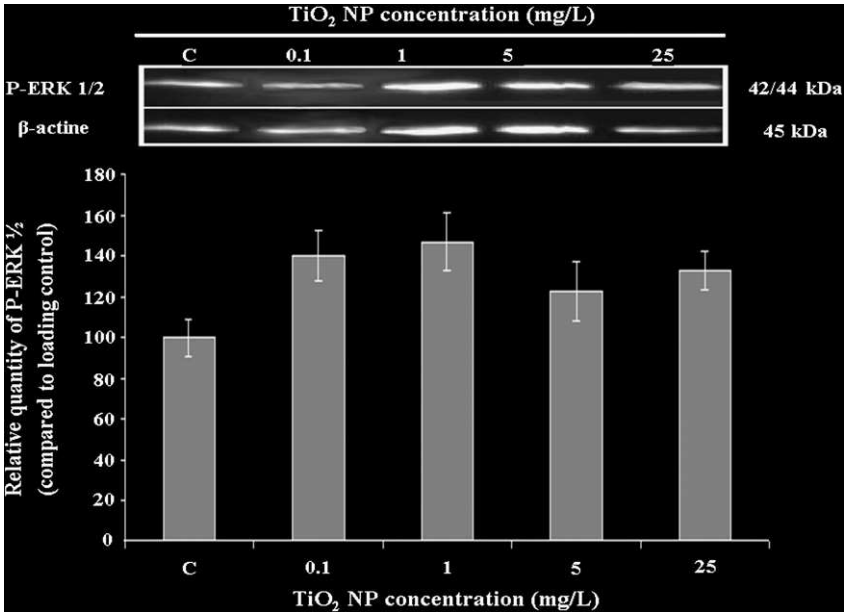


Fig. 4. Effects of 24 h exposure of bivalves to TiO_2 nanoparticles (0.1, 1, 5 and 25 mg/L) on the ERK 1/2 phosphorylation level. A: Whole hemocyte lysates (20 μg) extracted from bivalves exposed (or not) to TiO_2 NP were separated by SDS-PAGE (12%) and immunoblotted with anti-P-ERK 1/2 antibody as described in the "Materials and methods" part. The same blot was stripped with control antibody (anti-β-actin). B: Blots were analyzed using the Quantity One software. Relative quantifications of P-ERK 1/2 were performed in comparison with β-actin. Typical experiments out of 4 similar graphs represent the increase of ERK 1/2 phosphorylation between control cells and the different treatments. Values are means ± SEM (n = 4).

viability, a non-linear response in which phagocytosis was inhibited at the lower concentrations of nanoparticles (0.1–1 mg/L) could be reported. From a mechanistic point of view, a putative link between phagocytosis inhibition and the size of NP aggregate (in the range of hydrodynamic diameter of 840 nm) may be formulated. For the first time, the internalization of TiO_2 NP aggregates into *D. polymorpha* hemocytes was observed, reinforcing this hypothesis. Moreover, mussel exposure to TiO_2 NP was associated with increases in the phosphorylation level of ERK1/2 and p38 MAPK, which are components of kinase-cell signaling pathways playing a key role in the activation of the immune system. Further research is required in order to determine whether the recovery of immunological and functional parameters may occur following in vivo exposure to TiO_2 NP and what kind of physiological impact long-term exposure may have relating to the immune system of freshwater bivalves.

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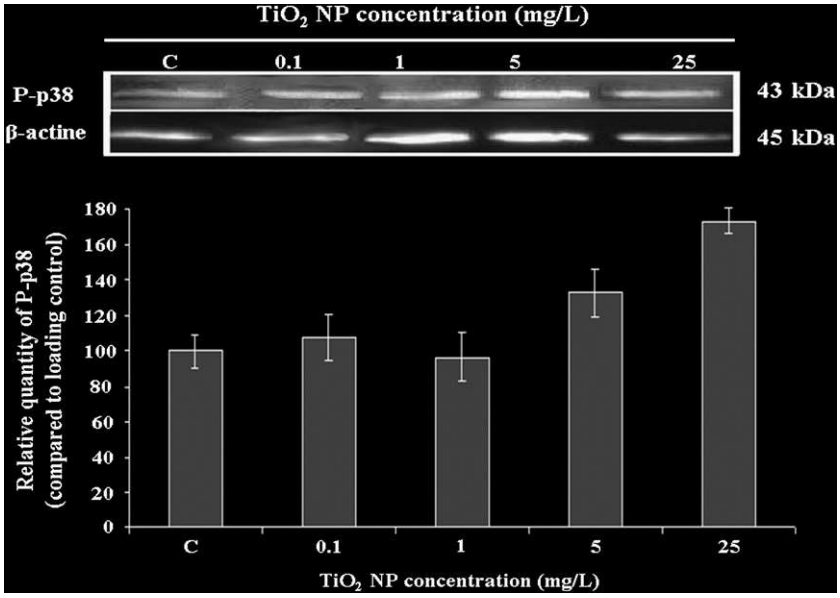


Fig. 5. Effect of 24 h exposure of bivalves to TiO_2 nanoparticles (0.1, 1, 5 and 25 mg/L) on the p38 phosphorylation level. A: Whole hemocyte lysates (20 μg) extracted from bivalves exposed (or not) to TiO_2 NP were separated by SDS-PAGE (12%) and immunoblotted with anti-P-p38 antibody as described in Materials and Methods. The same blot was stripped with control antibody (anti-β-actin). B: Blots were analyzed using the Quantity One software. Relative quantifications of P-p38 were performed in comparison with β-actin. Typical experiments out of 4 similar graphs represent the increase of ERK 1/2 phosphorylation between control cells and the different treatments. Values are means ± SEM (n = 4).

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Résumé : Les nanoparticules ont soit une origine naturelle soit une origine anthropique. De par l'évolution technologique, l'homme produit des quantités croissantes de nanoparticules susceptibles de se retrouver dans l'environnement. Afin de prévenir les risques inhérents à ces rejets, pour la santé humaine ou l'environnement, il est nécessaire de caractériser au mieux les effets potentiels des nanoparticules et d'identifier les mécanismes qui régissent leurs interactions avec les organismes exposés. Dans ce contexte, le premier objectif de ces travaux était de mettre en évidence les mécanismes gouvernant les interactions entre bactéries et nanoparticules et de documenter l'influence de ces interactions sur la toxicité et génotoxicité des NPs pour les bactéries. Le second objectif était de déterminer en quoi cette toxicité et cette génotoxicité pouvait impacter les organismes bactériens au niveau communautaire. Les résultats ont montré que les interactions électrostatiques attractives entre les bactéries et les TiO_2-NPs conditionnaient l'adsorption des nanoparticules à la surface des bactéries et conduisaient à la détection d'une toxicité modulées par les électrolytes présents dans la solution. De plus, les déterminants biophysiques de l'interphase bactérienne, et particulièrement la longueur des LPS et le type de protéines affleurant à la surface de la membrane externe, sont des paramètres de premier ordre dans l'apparition d'un potentiel néfaste pour les microorganismes. En prenant en compte ces interactions, nous avons mis en évidence le potentiel mutagène des TiO_2-NPs. Un effet toxique et génotoxique ayant été constaté, les communautés bactériennes ont ensuite été étudiées. Il a été mis en évidence que les TiO_2-NPs modifiaient la composition, la structure et la prévalence des communautés bactériennes libres et fixées sous forme de biofilms précoces d'une communauté aquatique naturelle d'eau douce. Ces travaux mettent en évidence l'impact potentiel des TiO_2-NPs sur les organismes bactériens dans un contexte d'évaluation des risques et indiquent que les nanoparticules pourraient impacter les communautés microbiennes et pourraient présenter un risque pour le bon fonctionnement de l'écosystème.

Mots-clés: Nanoparticules, TiO_2, toxicité, génotoxicité, communauté, bactéries

Abstract : Nanoparticles have either natural or anthropogenic origin. By technological change, man produces increasing amounts of nanoparticles likely to end in the environment. To prevent inherent risks to human health or environment from these releases, it is necessary to characterize the best potential effects of nanoparticles and to identify the mechanisms governing their interactions with exposed organisms. In this context, the first objective of this work was to highlight the mechanisms governing interactions between nanoparticles and bacteria and document the influence of these interactions on toxicity and genotoxicity of NPs for bacteria. The second objective was to determine how this toxicity and genotoxicity could impact bacteria at community level. Results showed that electrostatic attractive interaction between bacteria and TiO_2-NPs conditioned adsorption of nanoparticles on bacterial surfaces and led to the detection of toxicity modulated by electrolytes in solution. In addition, the biophysical determinants of bacterial interphase, particularly the length of LPS and protein type flush with the outer membrane surface, are key parameters in adverse potential of NPs for microorganisms. Taking into account these interactions, we highlighted the mutagenic potential of TiO_2-NPs. Toxic and genotoxic effect was found, leading to study the effects on bacterial communities. It has been demonstrated that TiO_2-NPs altered the composition, structure and prevalence of planktonic and sessile communities of an aquatic natural freshwater. These studies highlight the potential impact of TiO_2-NPs on bacteria in a risk assessment context and suggest that nanoparticles may impact microbial communities and could present a risk to the ecosystem functioning.