In Vitro Immunotoxicology of Quantum Dots and Comparison with Dissolved Cadmium and Tellurium

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Received 30 January 2013; revised 13 June 2013; accepted 15 June 2013

ABSTRACT: The increasing use of products derived from nanotechnology has raised concerns about their potential toxicity, especially at the immunocompetence level in organisms. This study compared the immunotoxicity of cadmium sulfate/cadmium telluride (CdS/Cd-Te) mixture quantum dots (QDs) and their dissolved components, cadmium chloride ($CdCl_2$)/sodium telluride ($NaTeO_3$) salts, and a $CdCl_2$/$NaTeO_3$ mixture on four animal models commonly used in risk assessment studies: one bivalve (*Mytilus edulis*), one fish (*Oncorhynchus mykiss*), and two mammals (mice and humans). Our results of viability and phagocytosis biomarkers revealed that QDs were more toxic than dissolved metals for blue mussels. For other species, dissolved metals (Cd, Te, and Cd-Te mixture) were more toxic than the nanoparticles (NPs). The most sensitive species toward QDs, according to innate immune cells, was humans (inhibitory concentration [IC_{50}] = 217 μg/mL). However, for adaptative immunity, lymphoblastic transformation in mice was decreased for small QD concentrations (EC_{50} = 4 μg/mL), and was more sensitive than other model species tested. Discriminant function analysis revealed that blue mussel hemocytes were able to discriminate the toxicity of QDs, Cd, Te, and Cd-Te mixture (Partial Wilk's $\lambda = 0.021$ and $p < 0.0001$). For rainbow trout and human cells, the immunotoxic effects of QDs were similar to those obtained with the dissolved fraction of Cd and Te mixture. For mice, the toxicity of QDs markedly differed from those observed with Cd, Te, and dissolved Cd-Te mixture. The results also suggest that aquatic species responded more differently than vertebrates to these compounds. The results lead to the recommendation that mussels and mice were most able to discriminate the effects of Cd-based NPs from the effects of dissolved Cd and Te at the immunocompetence level. Environ Toxicol 30: 9–25, 2015.

Keywords: Cd-Te CdS; cytotoxicity; phagocytosis activity; lymphoblastic activity; model species

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Contract grant sponsor: Environment Canada's and Health Canada's Chemicals Management Plan Funds.

Contract grant sponsor: the Canada Research Chair in Environmental Immunotoxicology.

Published online 27 July 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/tox.21890

INTRODUCTION

The toxicity of nanoparticles (NPs) is partly the result of physical–chemical properties such as size, shape, porosity, surface reactivity, and composition (Nel et al., 2006; Oberdörster, 2007; Jiang et al., 2009; Oberdörster, 2010). Quantum dots (QDs) are inorganic semi-conductors that have specific optical and physical properties (Hardman, 2006). For example, QDs functionalized with antibodies are enabled to target specific cells such as tumor cells (Ballou et al., 2004). QDs used in this study are composed of cadmium (Cd) and tellurium (Te), which can be released during breakdown in the environment. Although the immunotoxic potential of dissolved Cd is better understood (Coles et al., 1995; Campbell, 2006; Nordberg et al., 2008), the immunotoxicity of QDs and Te is less understood at the present time.

In oysters (*Crassostrea gigas*), dissolved Cd stimulates the formation of reactive oxygen species (ROS), leading to toxicity at high doses (Auffret et al., 2002). In vertebrates, Cd^{2+} increases the production of metallothioneins, which is a well-known detoxification mechanism (Eisler, 1985; Jarup et al., 1998; Klaassen et al., 2009). In addition, Cd^{2+} decreases glutathione levels, and induces a number of ROS such as superoxide anions, hydroxyl radicals, and hydrogen peroxide. Furthermore, dissolved Cd can induce DNA damage and apoptosis (Stohs et al., 2000). However, the toxicity of Te is not well understood. In one study, oxyanion tellurite (TeO_3^{2-}) was toxic to bacteria at 1 μg/mL (Taylor, 1999). Another study by Chasteen et al. (2009) demonstrated that TeO_3^{2-} reduced thiols and catalase activity while increasing ROS levels of *Escherichia coli* (Chasteen et al., 2009).

Recent evidence suggest that QD-induced toxicity cannot be explained solely based on QDs' basic components (Maysinger, 2007). The core and shell composition could also influence toxicity (Sutherland, 2002; Smith et al., 2009; Oberdörster, 2010). At present time, few studies highlighted the comparative toxicity of Cd-Te-based QDs with its Cd and Te components (Yeh et al., 2011). Hence, there is a need to develop screening tools to distinguish the toxic effects of dissolved metals from metals contained in NPs.

The impairment of the immune system contributes to susceptibility to infectious and immune-related diseases (Cabassi, 2007). In the context of environmental health issues, we produced a test battery composed of four test species (mussels, fish, rats, and humans) to compare the different immune system organization and defense against NPs. The blue mussel *Mytilus edulis,* a marine species, depends only on the innate immune system to defend against diseases. In fish, the immune system is similar to mammalian systems, in that it has both adaptative and innate immunity capacity.

The purpose of this study was twofold. First, the comparative *in vitro* immunotoxicity of QDs with respect to dissolved Cd and Te salts including Cd-Te mixture at the same Cd-Te ratio found in QDs was examined to determine if the toxicity of NPs could be explained by their dissolved component. Second, the immunotoxicity of QDs was examined in four species, these species were chosen because they were sentinel species in their own habitat. The immune parameters were observed to determine which species were the most sensitive and most able to discriminate between QDs and dissolved Cd and Te.

MATERIALS AND METHODS

QD Solution

Green NPs of cadmium sulfate/cadmium telluride (CdS/Cd-Te) mixture in a 20-mg/mL stock solution were obtained from Vive Nano Inc. (Toronto, Ont., Canada). The QDs consisted of a CdS shell bordering a Cd-Te core. The CdS shell was in turn linked with sodium polyacrylate, giving a net negative charge on the surface to prevent aggregation (Fig. 1). According to the manufacturer's specifications, 90% of the particles lie in the size ranges of 1–10 nm, the excitation wavelength is <500 nm and the emission wavelength is 538 nm. The NPs were stored at 4°C and were considered stable for at least 6 months. Visual characterization of this QDs was made in a previous article by Bruneau et al. (2013) (see 2.2 Preparation of Qds for microscopical observations, Material and method part).

Characterization of QDs in Culture Media

The stability of the QDs was examined in five different media. These were (1) sterile water; (2) sterile sea water (35 g/L of salts from Instant Ocean® and filtrated on a 0.22-μm diameter membrane); (3) Roswell Park Memorial Institute (RPMI) medium (for mice): RPMI-1640 Medium supplemented with 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated fetal bovine serum (FBS); (4) RPMI medium without bicarbonate (RPMI medium without bicarbonate for trout): RPMI-1640 Medium without bicarbonate supplemented with 10 mM of HEPES, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated FBS; and (5) RPMI AB (for humans): RPMI-1640 Medium supplemented with 10 mM of HEPES, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% of heat-inactivated AB serum. All the cell-culture media and reagents, unless mentioned otherwise, were purchased from Sigma-Aldrich (Ontario, Canada).

To determine the concentration and behavior of the metals contained in the QDs in these media, 2 mL of each medium was placed in 5-mL polypropylene round-bottom tubes, and 95 μL of QDs was added, giving a dilution factor of 1/21. For the determinations of total Cd, the samples were mixed with 7.905 mL of 5% nitric acid (HNO_3) (total volume, 10 mL). After 24 h, the samples were

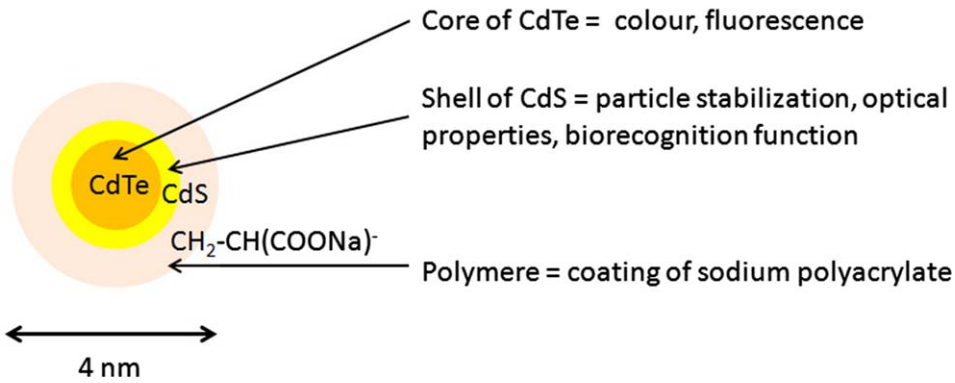

Fig. 1. Structural organization of QDs. QD is composed of a suspension of core–shell CdTe/CdS QDs NPs, stabilized by polyacrylate sodium. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

centrifugated at 2000 × *g* for 20 min to remove denatured proteins. The Cd and Te were analyzed inductively by coupled plasma mass spectrometry (ICP-ES) according to a previously published method by Gagne et al. (2008). Briefly, QD solutions (1 L) were prepared by the dilution (1:1000) of the stock solution. Solutions were analyzed by ICP-ES for the total Cd content. Part of the solutions was passed on a filtration membrane (pore size, 0.45 μm; FHLC04700 de Millipore) and 50 mL was sampled for total Cd determination. Analyses of Cd in the filtered solutions (50 mL) were carried out by ICP-ES (Optima 5300 DV, Perkin-Elmer).

According to the supplier, the concentration of the particle was 20 mM at 2.23 mg/mL of Cd.

Exposure to QDs, Cd, Te Salts, and Cd-Te Mixture

To test the contribution of the toxicity of metals in the QDs, a range of exposure concentrations were used, which were equivalent to the metal content within the QDs. According to the ICP-MS results, the ratio of Cd to Te in the QDs was measured to be 6:1. Hence, a range of cadmium chloride ($CdCl_2$) and sodium telluride ($NaTeO_3$) were prepared for the QDs, and the mixed $CdCl_2$/$NaTeO_3$ was made in this same Cd-Te proportion (Supporting Information Table I). All the dilutions were prepared in deionized sterile water.

Hemolymph and Leukocyte Preparation

For all the species tested in this study, ethical and animal permission were obtained for the process on mouse, fish, and mussel.

Adult mussels (*M. edulis*) were obtained from the Aquarium of Quebec. The hemolymph was collected from the posterior adductor muscle using a syringe with a 23G-gauge needle, and was immediately transferred on ice to avoid hemocyte aggregation (Auffret and Oubella, 1997). An initial count of individual mussel hemocytes and viability was assessed by flow cytometry using the supplier's ViaCount kit, to check for optimal viability before initiating the exposure (Frouin et al., 2007). The results were read with a flow cytometer, and set to exclude fragmented samples (which were easily recognizable by their small size and complexity). Hemocytes were pooled to reach a working solution of 12 mL. Cell viability of pooled hemocyte suspension was determined with flow cytometry using a ViaCount kit. Afterward, a concentration of 5 × 10^5 cells/mL was prepared in polypropylene round-bottom tubes (non-adherent tube), and the volume was adjusted to 500 μL with sea water. A volume of 25 μL of QD suspensions was added during a 21-h period at 15°C. Control tubes were also included (hemocyte suspensions with 25 μL of added distilled water).

Fish obtained from a commercial hatchery (Lachute, Quebec, Canada) were humanely euthanized with 0.1% of MS-222 (Sigma-Aldrich, Ontario, Canada). Nine fish were

TABLE I. Concentrations of Cd in the various media (μg/mL)

Dilution	Total QDs Mass (μg/mL)	Cd			
		SW	SSW	RPMI	RPMI w/o
Prepared stock (1/21)	952. 4	87.1	87.5	117.3	88.6
1/2	476.2	43.1	44.6	92.1	51
1/4	238.1	23.2	23.5	37	27.5
1/8	119.05	11.8	11.8	18.4	13.4
1/16	59.52	6.7	6.5	9.6	6.2
1/32	29.76	3.5	2.9	5.4	3.6
1/64	14.88	1.7	1.6	2.8	2.2
1/128	7.44	0.9	0.8	1.4	0.9
1/256	3.72	0.4	0.4	0.6	0.5
Ctrl	0	0	0	0	0

Abbreviations: SW, sterile distilled water; SSW, sterile sea water; RPMI, completed RPMI medium; RPMI w/o, RPMI medium without bicarbonate.

sacrificed for the exposure experiments. Head kidneys were removed under sterile conditions (laminar flow hood) and homogenized with sterile stainless steel mesh (diameter, 100 mm) in tissue culture medium RPMI-1640 supplemented with 10 U/mL of heparin, 10 mM of HEPES, penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% of heat-inactivated FBS at pH 7.2. The leukocytes were collected by centrifugation on Lympholyte-M gradient (density = 1.085) at 600 × *g* for 20 min at room temperature. Cells were collected from the Lympholyte-M interface and washed two times in phosphate-buffered saline (PBS), then resuspended in RPMI medium without heparin but containing 10% of FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10 mM of HEPES for phagocytosis, viability of macrophages, and lymphoblastic transformation. The ratio of live/dead cells was assessed using trypan blue dye exclusion under a microscope with a haemocytometer (in duplicate).

C57BL/6 female mice were obtained. Mice arrived at the animal facility at 3–4 weeks of age, weighing 12–15 g, and were placed in an exposure room with an automatic 12-h light/dark cycle and fed a standard rodent diet and tap water *ad libitum*. After 2 weeks of acclimation, the mice were killed by CO_2 inhalation. Ten mice were used for *in vitro* QD exposition. Mouse leukocytes were also isolated as described above, with the following changes: the spleens of six mice were aseptically removed and placed in RPMI medium. The cell suspensions were then filtered through nylon wool into Pasteur pipettes, washed two times in RPMI medium, and retrieved by centrifugation at 311 × *g* for 10 min. Cells were resuspended in supplemented RPMI medium. Viability of cells was determined by trypan blue dye exclusion using a light microscope before culturing the cells (in duplicate).

For humans, peripheral blood samples from healthy human volunteers were collected by venipuncture into heparinized tubes (in accordance with the INRS-Institut Armand-Frappier institutional permission). Peripheral blood samples from three specimens were used for the exposure experiment. Monocytes and lymphocytes were separated from whole blood by density gradient centrifugation using a Lympholyte-H (density = 1.077). The recovery of viable lymphocytes and monocytes after centrifugation was ≥70 and 72%, respectively. Cells were collected from the Lympholyte-H interface and washed three times in PBS, then resuspended in RPMI medium containing 10% heat-inactivated AB serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10 mM of HEPES. The count of cells was assessed using a Beckman coulter.

Immunocompetence Assessments

Cell Viability

After exposure to increasing concentrations of QDs, CdCl2, NaTeO3, and Cd-Te mixture, human monocytes (1×10^6 cells/mL), fish, and mouse macrophage (1×10^6 cells/mL), and mussel hemocytes (5×10^5 cells/mL) were assessed for viability by flow cytometry using propidium iodide (PI). Four microliter of PI (100 μg/mL) was added to 500 μL of each cell suspension for 1 min before the measurement. PI fluorescence was analyzed with a flow cytometer equipped with an argon laser excitation ($\lambda = 488 \pm 10$ nm) (FACS Calibur). The PI fluorescence was measured at the FL3 channel at 625 nm with 42 nm bandwidth; 5000 events were registered.

Phagocytosis Assay

Phagocytic activity for monocyte macrophages and mussel hemocytes was measured by following the procedure of Brousseau et al. (1998). In brief, 500 μL of adjusted cell concentration was added to 5 mL of polypropylene round-bottom tubes. Cells were incubated with different concentrations of QDs and metals: 90 min for human cells, 60 min for mouse cells, and 3 h for fish and mussel cells. After the incubation, fluorescent latex beads were added to the cell suspensions at a ratio of 100:1 (beads:cell). After an incubation period of 18 h at 15°C, the cell suspensions were overlaid with 4 mL of RPMI medium supplemented with either 3% of bovine serum albumin for mammalian cells or 3.5% of sea salt for marine species (*M. edulis*). The cell suspension was centrifuged at 150 × *g* for 8 min at 4°C. This step permitted removal of free and loosely adhered beads at the cell surface. For mammalian cells, the pellet was resuspended and fixed in 0.5 mL of 0.5% formaldehyde and 0.2% sodium azide (NaN_3) in PBS. For fish and mussel cells, pellets were fixed in a solution of 0.5% formaldehyde in 3.5% of salt water and 0.2% NaN_3. Cell fluorescence was analyzed by flow cytometry with an argon laser ($\lambda = 488 \pm 10$ nm). Latex bead fluorescence was detected with the FL1 channel and a bandwidth filter of 530–42 nm. In total, 10,000 events were acquired. The immunoactivity was defined as the number of cells containing one bead or more (*M*1). The immunoefficiency was operationally defined by the number of cells that were able to ingest three beads or more as explained by Frouin et al. (2010).

Lymphoblastic Transformation

Effects of QDs, $CdCl_2$, $NaTeO_3$, and mixed $CdCl_2$ $NaTeO_3$ mixture on the proliferation of T-lymphocytes under Concanavalin A stimulation (Con A) were assessed by the standard microculture method (Brousseau et al., 1998). Both mice and human cells (5×10^6 cells/well) were cultured at 37°C in a humidified atmosphere with 5% of CO_2, and at 15°C without CO_2 for fish and mussels. The cells were incubated for 66 h (mammals) and 90 h (fish) in 96-well plates under sterile conditions. Lymphoproliferation was evaluated with various concentrations of QDs and metal suspensions (Table I), and using single, predetermined concentrations of Con A (1.25 μg/mL for mice and humans; 20 μg/mL for

TABLE II. Viability of immune cells exposed to QDs and their metallic components (Cd, Te, and Cd-Te mixture)*

Model	Cd Contained in the QDs (μg/mL)	Te Contained in the QDs (μg/mL)	Cd-Te mix Contained in the QDs (μg/mL)	Dissolved Cd (μg/mL)	Dissolved Te (μg/mL)	Dissolved Cd-Te (μg/mL)
Mussel	*55	* 18	*64	–	–	–
	**109	**20	**128			
Trout	*≥7	*≥1	*≥8	*≥60	–	*≥32
	**≥14	**≥2	**≥16			**≥64
Mouse	–	–	–	**≥7	*≥20	**≥4
Human	**≥14	**≥2	**≥16	*≥14	*≥18	**≥4
				**≥28		

[a]For mussels, cells were exposed for 21 h at 15°C. $N = 9$ for QDs, Cd, and Cd-Te, and $N = 16$ for Te. For trouts, cells were exposed for 21 h at 15°C. $N = 11$ for QDs, and $N = 3$ for Cd, Te, Cd-Te. For mice, cells were exposed for 21 h at 15°C. $N = 11$ for QDs and $N = 3$ for Cd, Te, and Cd-Te. For human, cells were exposed for 21 h at 37°C. $N = 3$ for QDs, Cd, Te, and Cd-Te. *$p < 0.05$, **$p < 0.001$.

fish). The cells were pulsed with 0.5 μCi/well of tritiated thymidine (6.7 Ci/mmol) during the last 18 h of incubation. Cells were harvested with a semi-automatic cell harvester and transferred on a fibreglass filter. Incorporated radioactivity was determined using a scintillation counter.

Data Analysis

Differences of Cd/Te ratio were observed in different media, and differences between the biomarker (viability and phagocytic activity) were examined using one-way analysis of variance (ANOVA). When the results were significant ($p < 0.05$), a post hoc Tukey test was used to determine the differences between the groups. When the data were not normal, Kruskal–Wallis ANOVA was used instead. For all the tests, significance was set at $p < 0.05$. Correlation tests were performed for two purposes: (1) to study the relationship between the immune parameter response (viability and phagocytosis) of immune cells exposed to QDs and their metallic contents; and (2) to compare interspecies difference for each immunocompetence. Correlations were used to test for relationships between variables (e.g., biomarkers and contaminant concentrations). These analyses, thus, served to detect associations among parameters and species.

Discriminant function analyses (DAs) were also performed to examine the global response patterns of immunocompetence between the QDs, Cd, and Te salts, and Cd-Te mixture in each test species. The roots in the DA analysis "refer to the eigenvalues that are associated with the respective canonical function" (Statsoft Electronic Statistics Textbook). Each symbol on the DA states the kind of contaminant (blue circle: QDs, red square: dissolved Cd, green diamond: dissolved Te, pink triangle: dissolved Cd-Te mixture). All the statistical analyses were conducted with STATISTICA (version 7). The calculation of the 50% inhibitory concentration (IC_{50}) parameter was carried out with Microsoft® Excel 2007. The IC_{50} was then calculated using linear regressions ($y = ax + b$) where y is the value of 50% of the control and x is the IC_{50}.

RESULTS

Elemental Analysis

The amount of Cd in the QD stock solution was 110 μg/mL and the amount of Te was 18 μg/mL, giving a mass Cd/Te ratio of 6.1 and a combined Cd-Te concentration of 128 μg/mL. The nominal concentrations of the NP were confirmed by ICP-ES for the exposure experiments.

The Cd measured in each media was constant for sterile water and sea water (Table I). According to our results, the correlations between the theoretical Cd concentration (110 μg/mL) and the measured Cd concentration for all the media were highly significant ($r = 0.94$–0.99), which indicates that the increase in total Cd followed the exposure concentration.

Viability

For mussels, hemocyte viability was significantly decreased at 55 μg/mL of Cd contained in the QDs (Table II). For dissolved Cd ($CdCl_2$), no significant difference in viability was observed compared to the control. Hemocyte viability was unchanged with either dissolved Te ($NaTeO_3$) or the dissolved Cd-Te mixture ($CdCl_2$ and $NaTeO_3$). Correlation analysis for cell viability in mussels revealed significant correlations between the QDs and the dissolved Te ($r = 0.93$; $p < 0.05$), and between QDs and Cd-Te mixture ($r = 0.91$; $p < 0.05$). No correlation was found between the QDs and the dissolved Cd (Table III).

For rainbow trout, macrophage viability was significantly decreased at 7 μg/mL of Cd contained in the QDs, at 60 μg/mL of dissolved Cd ($CdCl_2$), and was not affected by dissolved Te (Table II). However, macrophage viability during exposure to the Cd-Te mixture ($CdCl_2 + NaTeO_3$) was significantly decreased at 32 μg/mL, indicating enhanced Cd toxicity in the presence of TeO_3^-. Correlation analysis of trout cell viability revealed significant trends between the QDs and the

TABLE III. Correlation between QDs and their metallic components ($p < 0.05$) for viability, immunoactivity, immunoefficiency

Viability	Mussel	Trout	Mouse	Human
Cd	0.09	**0.87***	0.73	**0.83***
Te	**0.93***	0.25	0.68	**0.97***
Mix	**0.91***	**0.94***	**0.77***	0.55
Immunoactivity				
Cd	**0.97***	0.08	−0.47	0.60
Te	0.74	−0.44	**−0.78***	**0.95***
Mix	**0.79***	0.55	**−0.79***	0.64
Immunoefficiency				
Cd	**0.95***	−0.03	−0.07	0.66
Te	−0.49	0.11	0.66	0.68
Mix	**0.86***	0.25	−0.12	0.67

*The significant correlations.

dissolved Cd ($r = 0.87$; $p < 0.05$), and between QDs and Cd-Te mixture ($r = 0.94$; $p < 0.05$) (Table III). For mice, macrophage viability was unchanged with the exposure to QDs, but it was significantly decreased at 7 μg/mL for dissolved Cd ($CdCl_2$), at 20 μg/mL for dissolved Te ($NaTeO_3$) ($p < 0.05$), and at 4 μg/mL for the Cd-Te mixture ($CdCl_2$ and $NaTeO_3$) (Table II). This shows again that Cd toxicity is increased (potentiated) by the presence of $NaTeO_3$ salts. The IC_{50} of macrophage viability was, in decreasing order of toxicity, dissolved Cd-Te mixture (5.3) > dissolved Cd (10) > dissolved Te (>18) QDs (>952) (Table IVA). Correlation analysis for mice revealed that cell viability changes were correlated between QDs and Cd-Te mixture ($r = 0.77$; $p < 0.05$). Human monocyte viability was significantly decreased at 14 μg/mL of Cd in the QDs, at 14 μg/mL for dissolved Cd ($CdCl_2$), and at 18 μg/mL for dissolved Te ($NaTeO_3$) (Table II). For the Cd-Te mix ($CdCl_2$ and $NaTeO_3$), a significant decrease was observed compared to the control at 4 μg/mL, revealing a synergistic effect of Cd and Te. Correlation analysis for humans revealed that cell viability changes were correlated between the QDs and the dissolved Cd ($r = 0.83$; $p < 0.05$), and between QDs and Te ($r = 0.97$; $p < 0.05$) (Table III). The reported IC_{50} shows a decreasing order of toxicity: dissolved Te (18) > dissolved Cd (19) > Cd-Te mixture (31) > QDs (217) (Table IVA).

In the attempt to determine whether each species responded similarly to QDs as well as Cd and Te-dissolved components, correlation analysis was performed. The analysis revealed that cell viability for QD exposure was correlated with each species, suggesting that each species could act as a surrogate with each other (Table V). The strongest correlations were found for cell viability between human cells and mussels ($r = 0.93$; $p < 0.05$), trout ($r = 0.92$; $p < 0.05$), and mice ($r = 0.91$; $p < 0.05$).

Phagocytosis

For mussels, immunoactivity and immunoefficiency were significantly decreased at 55 μg/mL of Cd contained in the QDs (Fig. 2). No significant decrease of phagocytosis was observed for dissolved Cd ($CdCl_2$), dissolved Te ($NaTeO_3$) [Fig. 2(A–D)] but for dissolved Cd-Te mixture a significant decrease was observed at 144 μg/mL for the two parameters [Figure 2(E,F)]. Correlation analysis for mussels revealed that cell immunoactivity changes were correlated between QDs and dissolved Cd ($r = 0.97$) and Cd-Te mixture ($r = 0.79$); the cell immunoefficiency changes were correlated between QDs and dissolved Cd ($r = 0.95$) and Cd-Te mixture ($r = 0.86$) (Table III).

In trout, phagocytosis activity was not significantly affected by QDs and dissolved Cd (Fig. 3). A significant increase in phagocytosis activity and efficiency was observed at 5 μg/mL for dissolved Te. No significant difference was found for Cd-Te mixture compared to the control. No correlations were observed between the QDs and the dissolved metals for immunoactivity and immunoefficiency (Table III).

In mice, the immunoactivity and immunoefficiency showed similar trends with trout, that is, no significant variation was observed with QDs and dissolved Cd (Table III). Immunoactivity was significantly decreased compared to the

TABLE IV. Concentration at 50% inhibition (IC_{50}) for different immune parameters. Immune cells (macrophages, monocytes, and hemocytes) were tested for (A) viability, (B) immunoactivity (phagocytosis of one bead or more), (C) immunoefficiency (phagocytosis of three beads or more), and (D) lymphoblastic transformation*

(A) Viability	QDs	$CdCl_2$	$NaTeO_3$	Mixed
Human	216.62	19.29	18.32	31.14
Mouse	>952.4	10.21	>18.25	5.30
Trout	715.39	>109.5	>18.25	>127
Mussel	581.92	>109.5	>18.25	>127
(B) Immunoactivity (phagocytosis 1 bead or more)				
Human	>952.4	17.92	30.45	15.15
Mouse	>952.4	>109.5	>18.25	>127
Trout	>952.4	>109.5	>18.25	>127
Mussel	598.51	>109.5	>18.25	>127
(C) *Immunoefficiency (phagocytosis three beads or more)*				
Human	424.65	9.10	22.53	11.36
Mouse	>952.4	>109.5	>18.25	>127
Trout	>952.4	>109.5	>18.25	>127
Mussel	435.22	42.95	>18.25	>127
(D) Lymphoblastic transformation				
Human	28.70	3.29	5.29	28.70
Mouse	4.38	2.86	0.78	<*M*1
Trout	20.04	9.81	1.44	3.03

*Note that *M*1 states for the first $CdCl_2$, $NaTeO_3$ mixture concentration. The IC values superior to the maximum concentration exposure are measured according a large dose effect exposure experiment.

TABLE V. Correlation between model species ($p < 0.05$) for viability, immunoactivity (*M*1), immunoefficiency (*M*2), and lymphoblastic transformation

Viability	Mussel	Trout	Mouse	Human
Mussel	1			
Trout	0.89*	1		
Mouse	0.79*	0.85*	1	
Human	0.93*	0.92*	0.91*	1

		Mussel		Trout		Mouse		Human	
Phagocytosis		*M*1	*M*2	*M*1	*M*2	*M*1	*M*2	*M*1	*M*2
Mussel	*M*1	1							
	*M*2	0.99*	1						
Trout	*M*1	−0.40	−0.48	1					
	*M*2	−0.22	−0.31	0.98*	1				
Mouse	*M*1	−0.83*	−0.86*	0.59	0.45	1			
	*M*2	0.38	0.44	−0.61	−0.56	−0.02	1		
Human	*M*1	0.87*	0.88*	−0.12	0.07	−0.63*	0.28	1	
	*M*2	0.86*	0.82*	0.03	0.19	0.92*	0.27	0.92*	1

Lymphoblastic transformation	Trout	Mouse	Human
Trout	1		
Mouse	0.88*	1	
Human	0.85*	0.73*	1

*The significant correlations.

control at 4 μg/mL for dissolved Te (Fig. 4). Immunoactivity and immunoefficiency were significantly decreased at 0.5 μg/ml of Cd-Te mixture ($p < 0.05$). Correlation analysis for mice revealed that immunoactivity changes were correlated between the QDs and the dissolved Te ($r = -0.78$), and between QDs and Cd-Te mixture ($r = -0.79$) (Table III).

In humans, immunoefficiency and immunoactivity of monocytes were significantly decreased at a threshold concentration of 109 μg/mL of Cd contained in QDs [Fig. 5(A,B)] of 18 μg/mL of Te contained in QDs [Fig. 5(C,D)], and of 128 μg/mL of Cd-Te mixture contained in QDs [Fig. 5(E,F)]. For dissolved Cd ($CdCl_2$), immunoactivity and immunoefficiency of phagocytosis were decreased significantly at 15 μg/mL. For the dissolved Cd-Te mixture, a significant decrease at 70 μg/mL ($p < 0.05$) for immunoactivity, and at 17 μg/mL for immunoefficiency, was observed ($p < 0.05$) (Fig. 5). Correlation analysis for humans revealed that immunoactivity changes were correlated between the QDs and the dissolved Te ($r = 0.95$; $p < 0.05$) (Table III). The toxicity based on the IC_{50} for human macrophage immunoactivity was Cd-Te mixture (15) ∼ dissolved Cd (16) > dissolved Te (30) > QDs (>952); and for immunoefficiency was dissolved Cd (9) ∼ dissolved Cd-Te mixture (11) > dissolved Te (22) > QDs (>425) (Table IVB and C).

In an attempt to determine key species for NPs immunotoxicity assessment, correlation analysis revealed that immunoactivity was correlated between mussels and mice ($r = -0.83$), mussels and humans ($r = 0.87$), and mice and humans ($r = -0.63$) (Table V). For immunoefficiency, correlations were found between mussels and humans only ($r = 0.82$) (Table V). Hence, the results suggest that, for immunoactivity, the fish species should be added to either the mussel, mouse or human models, in a test battery for environmental health issues. However, for immunoefficiency, the mice and either humans or mussels should be considered.

Lymphoblastic Transformation

In rainbow trout, lymphocyte transformation was significantly decreased at 2 μg/mL of Cd contained in QDs after 72 h of exposure. For dissolved Cd ($CdCl_2$), the lymphoblastic transformation was significantly decreased at 7 μg/mL (Fig. 6). A decrease was observed at the lowest concentration of dissolved Te ($NaTeO_3$) concentration (0.6 μg/mL) (Fig. 6). Lymphocyte proliferation was significantly decreased at the first mixed Cd-Te concentration. Correlation analysis for trout revealed that lymphocyte viability changes were correlated between the QDs and the dissolved Cd ($r = 0.99$; $p < 0.05$) (Table III). The toxicity (IC_{50}) for trout lymphocytes was dissolved Te (1.4) > dissolved Cd-Te mixture (3) > dissolved Cd (10) > QDs (20) (Table IVD).

In mice, lymphoblastic transformation of lymphocytic T cells exposed to QDs was significantly decreased at 4 μg/mL

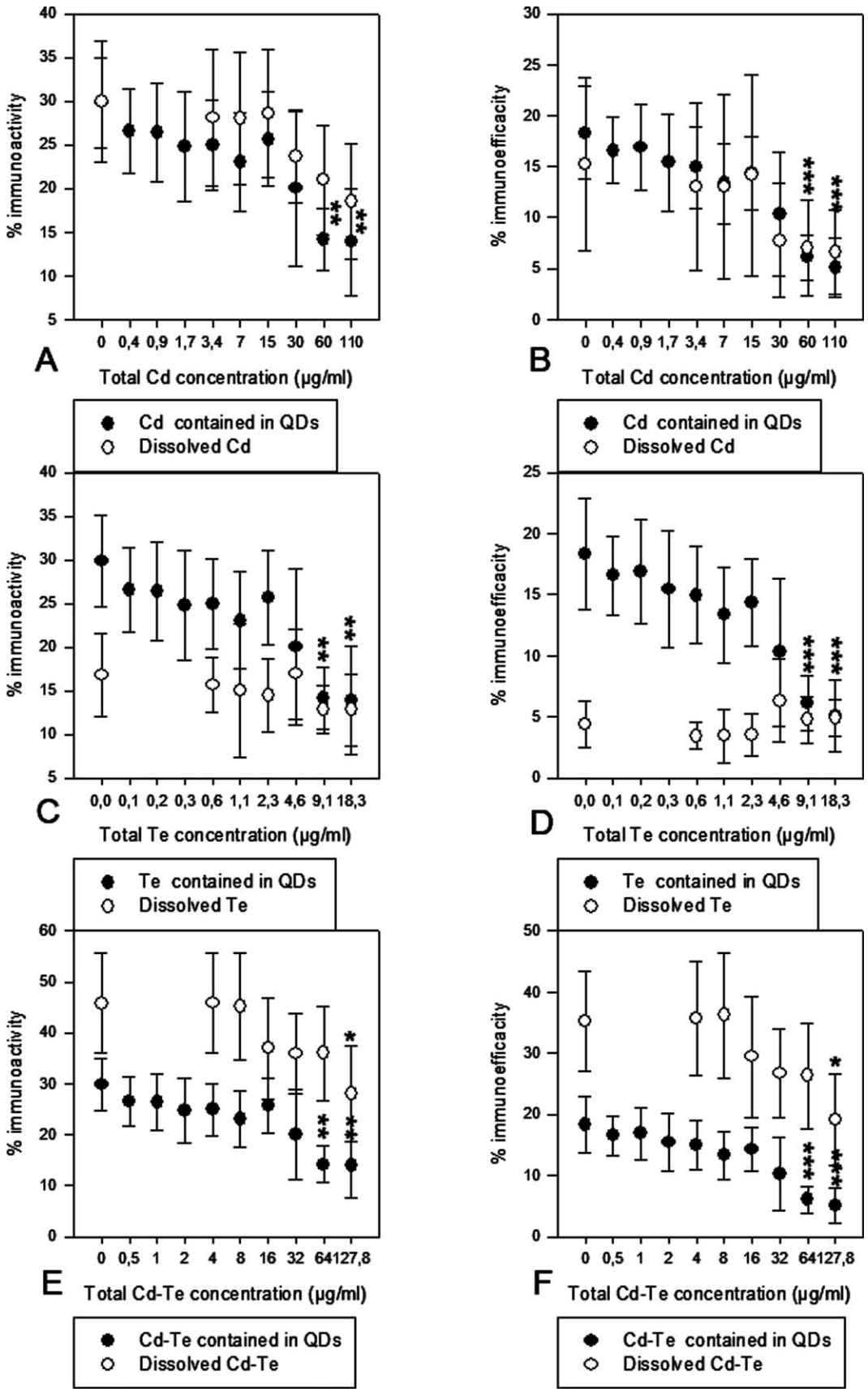

Fig. 2. Immunoactivity and immunoefficiency of mussel hemocytes exposed for 21 h to QDs and their metallic components. (A and B) QDs ($N = 9$) versus dissolved Cd ($N = 6$); (C and D) QDs ($N = 9$) versus dissolved Te ($N = 6$); (E and F) QDs ($N = 9$) versus dissolved Cd/Te ($N = 6$). $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$. The error bars correspond to the standard deviations.

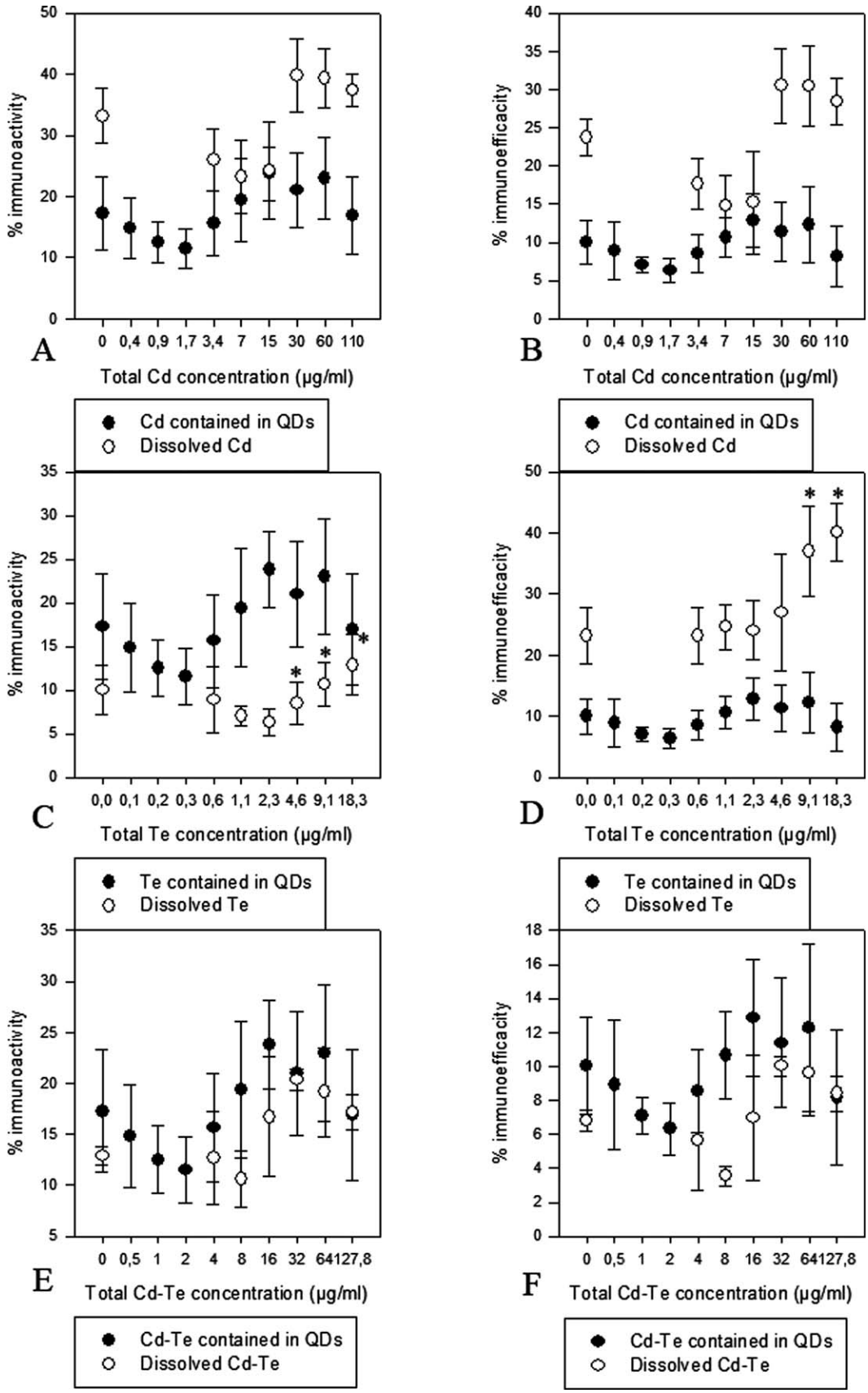

Fig. 3. Immunoactivity and immunoefficiency of trout macrophages exposed for 21 h to QDs and their metallic components. (A and B) QDs ($N = 11$) versus dissolved Cd ($N = 3$); (C and D) QDs ($N = 11$) versus dissolved Te ($N = 3$); (E and F) QDs ($N = 11$) versus dissolved Cd/Te ($N = 3$). $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$. The error bars correspond to the standard deviations.

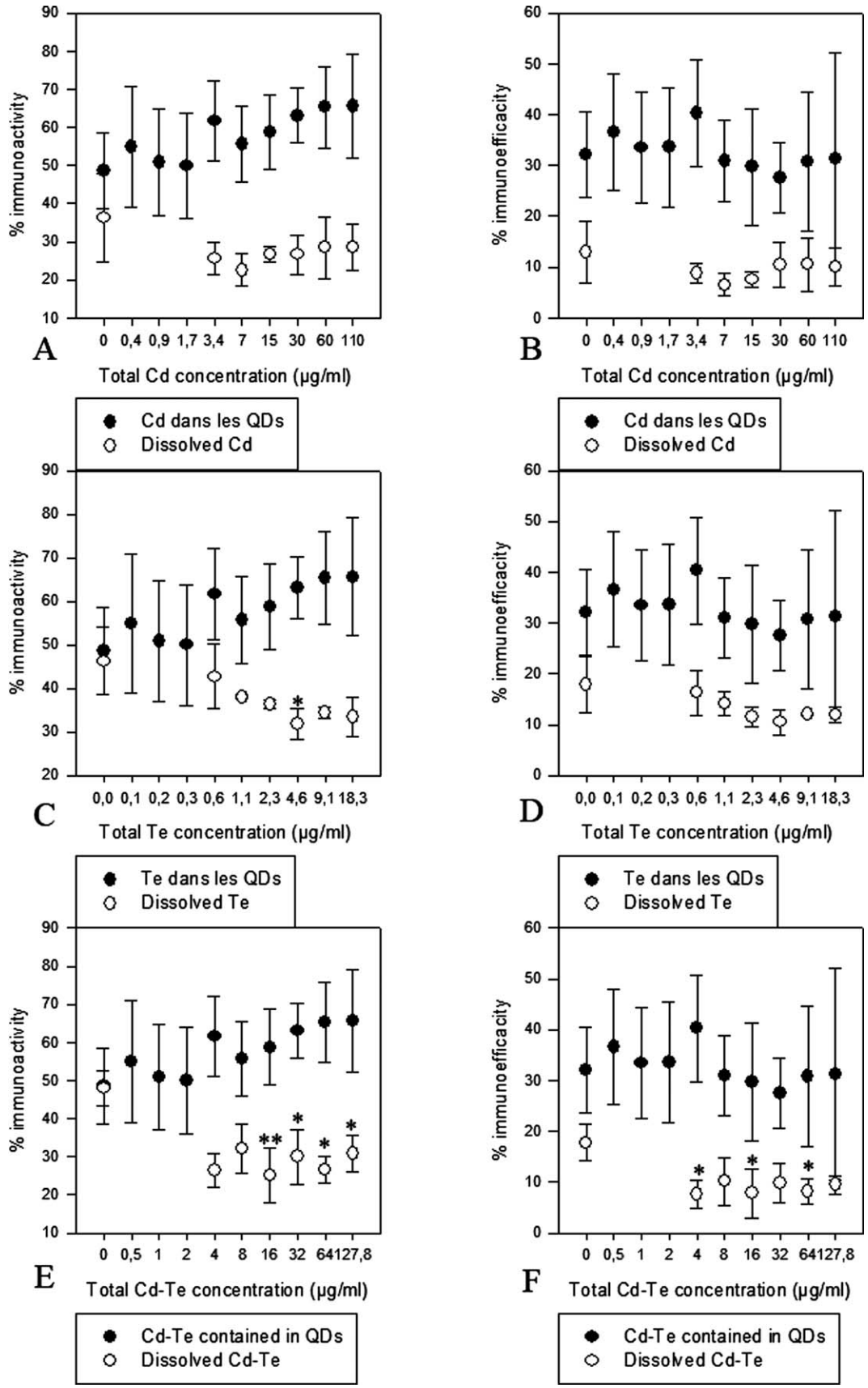

Fig. 4. Immunoactivity and immunoefficiency of mouse macrophages exposed for 21 h to QDs and their metallic components. (A and B) QDs ($N = 10$) versus dissolved Cd ($N = 3$); (C and D) QDs ($N = 10$) versus dissolved Te ($N = 3$); (E and F) QDs ($N = 10$) versus dissolved Cd/Te ($N = 3$). $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$. The error bars correspond to the standard deviations.

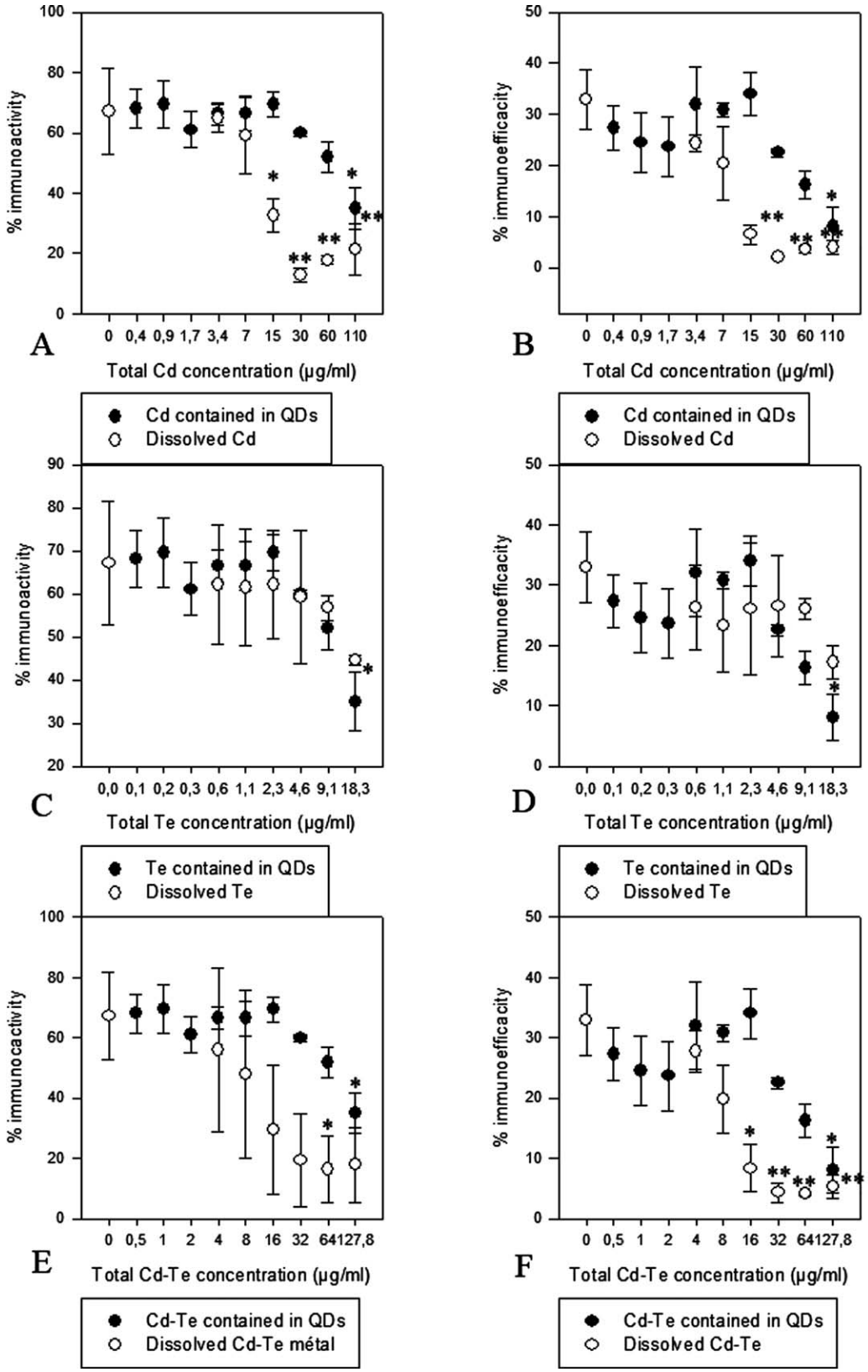

Fig. 5. Immunoactivity and immunoefficiency of human immune cells exposed for 21 h to QDs and their metallic components. (A and B) QDs ($N = 3$) versus dissolved Cd ($N = 3$); (C and D) QDs ($N = 3$) versus dissolved Te ($N = 3$); (E and F) QDs ($N = 3$) versus dissolved Cd/Te ($N = 3$). $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$. The error bars correspond to the standard deviations.

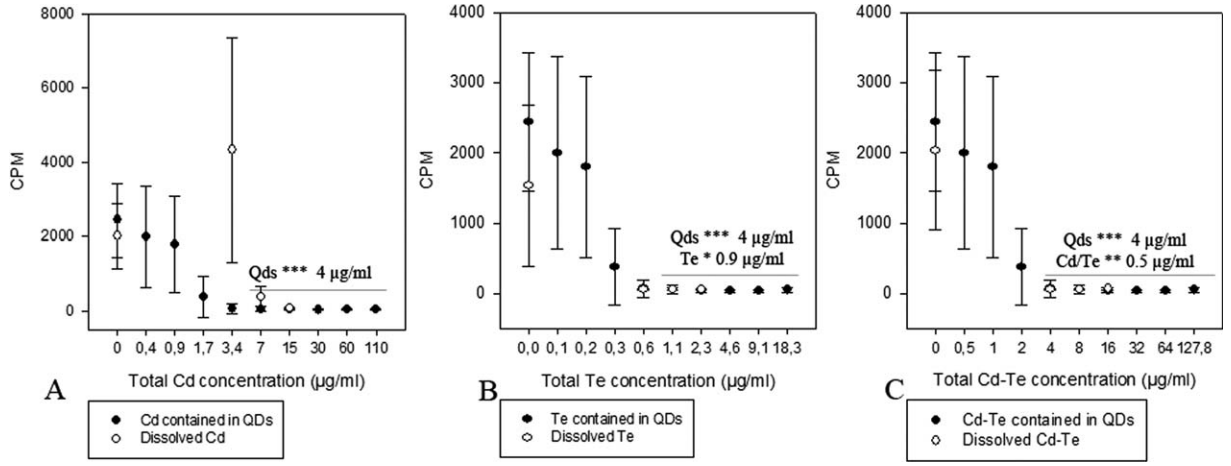

Fig. 6. Lymphoblastic transformation of trout lymphocytes exposed to QDs and their metallic components (Cd, Te, and Cd-Te mixture). Cells were exposed for 21 h at 15°C. $N = 9$ for QDs, and for Cd, Te, Cd-Te, $N = 3$. $^{*}p < 0.05$, $^{**}p < 0.001$. The error bars correspond to the standard deviations.

of Cd contained in the QDs. Lymphoblastic transformation was significantly decreased at the lowest Cd-Te concentration (at 3.6 µg/mL) (Fig. 7). Correlation analysis for mice revealed that cell viability changes were highly correlated between the QDs and the dissolved Cd ($r = 0.99$), and between dissolved Te ($r = 0.91$) and dissolved Cd-Te mixture ($r = 0.99$) (Table III). The toxicity (IC_{50}) for mouse lymphocytes was dissolved Cd-Te mixture (>0.5) > dissolved Te (0.8) > dissolved Cd (3) > QDs (4.4) (Table IVD). In humans, lymphoblastic transformation was significantly decreased at 2 µg/mL of QDs (Fig. 8). Significant decreases were also observed for dissolved Cd at 4 µg/mL, dissolved Te at 1.2 µg/mL, and dissolved Cd-Te mixture at the lowest exposure concentration (Fig. 8). Correlation analysis for humans revealed that cell viability changes were correlated between the QDs and the dissolved Cd ($r = 0.93$; $p < 0.05$) (Table III). The toxicity based on the IC_{50} for human lymphocytes was dissolved Cd (3.3) > dissolved Te (5.3) > dissolved Cd-Te mixture (29) ≥ QDs (29) (Table IVD). Correlation analysis revealed that lymphoblastic transformation was correlated between trout and mice ($r = 0.88$), trout and humans ($r = 0.85$), and mice and humans ($p = 0.73$) (Table V). This suggests that either one of the test species (trout, humans, or mice) could be used as a test system for lymphoblastic proliferation, because they were related to each other.

Discriminant Function Analysis

DA revealed that blue mussels discriminated the toxicity of QDs, Cd, Te, and Cd-Te mixture (Partial Wilk's $\lambda = 0.021$ and $p < 0.0001$, Fig. 9(A)). In rainbow trout, the immunotoxic effects of QDs were similar to those obtained with the

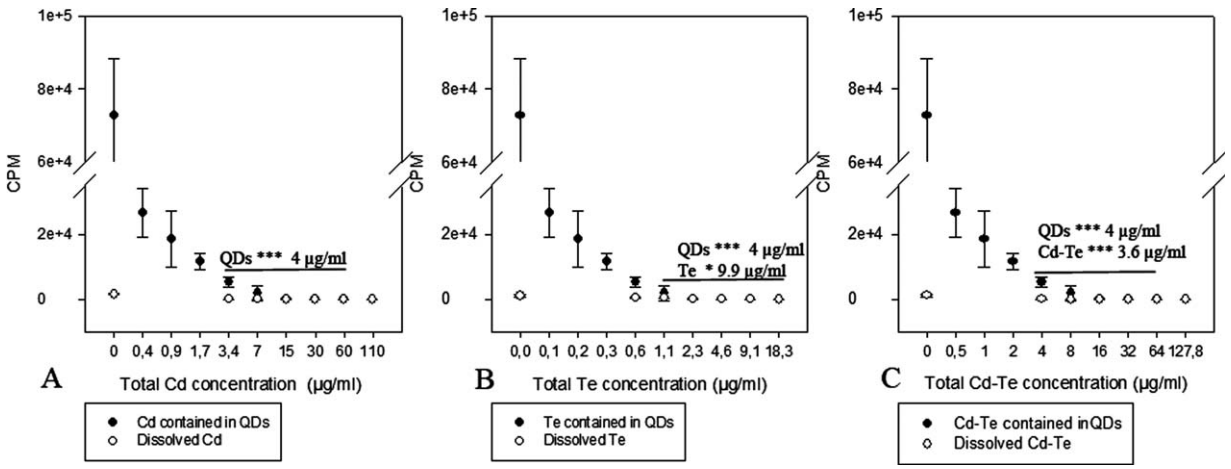

Fig. 7. Lymphoblastic transformation of mouse macrophages exposed to QDs and their metallic components (Cd, Te, and Cd-Te mixture). Cells were exposed for 48 h at 37°C. $N = 10$ for QDs, and for Cd, Te, Cd-Te, and $N = 6$. $^{*}p < 0.05$, $^{**}p < 0.001$. The error bars correspond to the standard deviations.

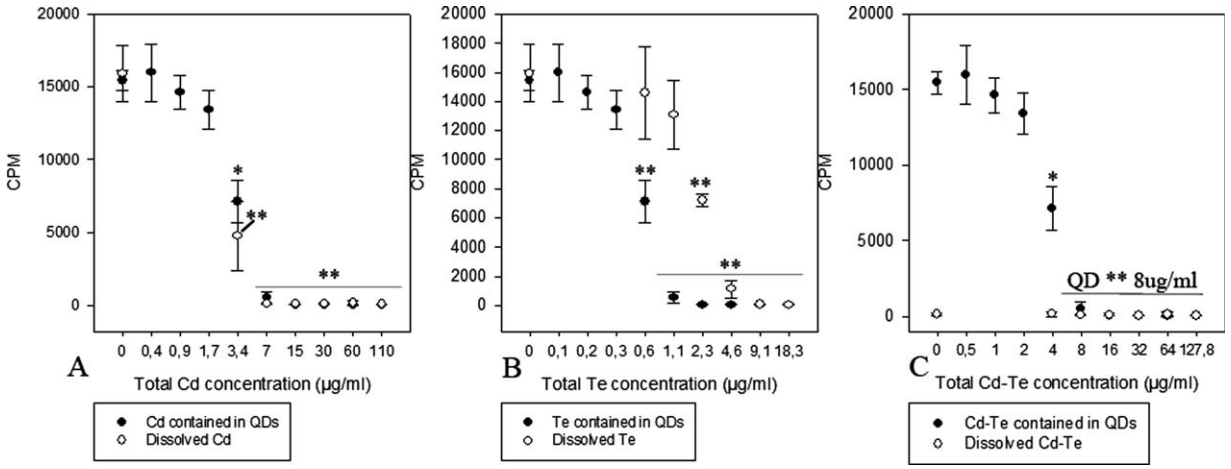

Fig. 8. Lymphoblastic transformation of human lymphocytes exposed to QDs and their metallic components (Cd, Te, and Cd-Te mixture). Cells were exposed for48 h at 37°C. $N = 3$ for QDs, Cd, Te, and Cd-Te. $^{*}p < 0.05$, $^{**}p < 0.001$. The error bars correspond to the standard deviations.

Cd-Te mixture, suggesting that QD-induced toxicity could be explained by the concomitant release of dissolved Cd and Te (Partial Wilk's $\lambda = 0.0019$ and $p < 0.0001$, Fig. 9(B)). For mice, the toxicity of QDs markedly differed from those observed with Cd, Te, and dissolved Cd-Te mixture, suggesting that other factors were at play (Partial Wilk's $\lambda =$

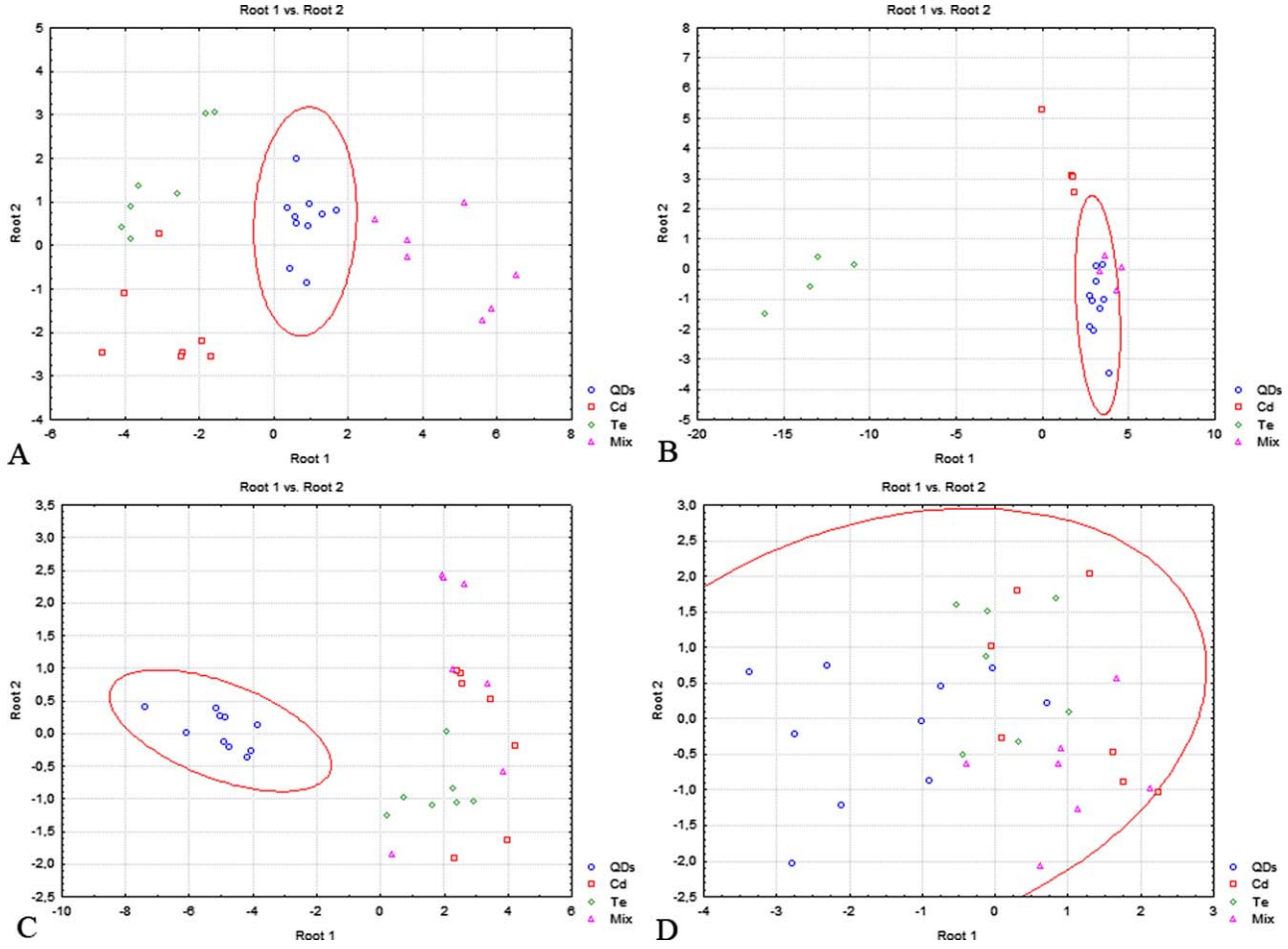

Fig. 9. DA for different animal models. (A) Mussel, (B) rainbow trout, (C) mouse, and (D) human. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

0.041 and $p < 0.0001$, Fig. 9(C)). For human cells, the immunotoxic effects of the QDs were similar to those observed with Cd, Te, and Cd-Te mixture, suggesting that QD-induced toxicity was closely associated with dissolved Cd and Te effects. Although the response pattern was similar to that observed in mice, the discrimination was less clear in human cells (Partial Wilk's $\lambda = 0.28$ and $p < 0.001$, Fig. 9(D)).

DISCUSSION

According to a previous study, the QDs exist in three different states in solution: aggregated, fragmented, and monomeric forms (Louis et al., 2010). The proportion of these three forms will depend on the density of surface charge of the NPs (ζ-potential), surface reactivity, and the presence of salts in the media. In this study, the total metallic content of NPs was conserved in the exposure media, as evidenced by the total Cd concentration in the exposure media being similar to the Cd concentration contained in the QDs (as noted by the manufacturer).

Several studies revealed that the initial phase of QDs' toxicity could be owing to the release of the metallic components of the NPs (Kirchner et al., 2005; Lovric et al., 2005).Based on a previous study with the same QDs, the proportion of labile Cd and Te in the dissolved fraction for the QDs in this study was estimated at 2 and 11% of the total nominal concentrations, respectively (Louis et al., 2010). For rainbow trout, which is good sentinel specie for contaminant risk assessment in fresh water, the effects observed for the QDs were similar to those observed with dissolved Cd-Te mixture, according to the DA results. For the lymphoblastic transformation, the lowest observed-effect concentration (LOEC) was 2 μg/mL of Cd contained in the QDs, which would correspond to 0.04 μg/mL of the labile fraction (2%) and 0.036 μg/mL for Te. For the dissolved Cd-Te mixture, the LOEC was <0.5 μg/mL. This suggests that the toxicity of QDs to rainbow trout could have been explained, in part at least, by the dissolved fraction of Cd-Te mixture of the QDs. However, the toxicity could not be explained by the Te concentration alone, because the LOEC of the dissolved Te was 0.6 μg/mL (and was superior to 11% of the labile fraction of Te from the QDs). This suggests that the QDs' toxicity may be determined by the synergistic action of the Cd and Te. Ours LOEC results were in agreement with those of Gagné et al. (2008). The authors demonstrated that viability of hepatocytes cells was significantly decreased at 4.5 μg/mL of Cd-Te. Moreover, Gagné et al. (2008) observed that toxic effects of NPs could be separated from their metallic content with biomarkers analyses where the biomarker response was not entirely explained by the release of Cd ions (confirmed by DA analysis). In this study, our results confirm that the toxicity could be explained partially by the coaction of Cd-Te mixture of the QD.

For *M. edulis,* which is good sentinel specie for contaminant risk assessment in sea water, the viability of hemocytes was more affected by the QDs than by their metallic components ($CdCl_2$ and $NaTeO_3$). Results for dissolved components indicate that mussel hemocyte viability was not affected even at high Cd and Te concentrations, which suggests that dissolved metals were less toxic to bivalves or less bioavailable in salt-rich environments. This was further supported by: (1) the lack of correlation of hemocyte viability responses with QDs and dissolved Cd, Te, and Cd-Te mixture; and (2) the global response pattern of the immunocompetence data differing between QDs and dissolved Cd, Te, or Cd-Te mixture groups. In mussels, it is noteworthy that the response pattern of the Cd-Te mixture readily differed from dissolved Cd and Te, which suggests a mixture interaction. This could be explained by the properties of the Te in the Cd-Te mixture.

Selenium (Se) is a metal that belongs to the same family as Te in the periodic table. It is well known for its antioxidant action on metals such as Cd (Zwolak and Zaporowska, 2011). Some studies report that Se has induced biochemical changes in animals exposed to heavy metals, and reduced the toxicity of the metals by a sequestration process (Talas et al., 2008). In our case, the presence of Te increased the toxicity of the QDs. Studies on the Te immunotoxicity are lacking at the present time. Te was recognized as affecting metabolic functions such as the maintenance of the adenosine-5′-triphosphate and glutathione (Ogra, 2009). Moreover, Te salts such as tellurate (TeO_4^{2-}) and tellurite (TeO_3^{2-}) are recognized as inducing the formation of free radicals, principally hydroxyl radicals. TeO_4^{2-} ions are highly toxic and induced oxidative stress (Nordberg et al., 2008; Chasteen et al., 2009). Our results were similar to those of Sandoval et al. (2010), who demonstrated a toxicity of Te on HeLa cells at a concentration of 18 μg/mL of TeO_3^{2-}. In our study, we could expect that the formation of oxidized forms of Te increased the toxicity of the QDs.

Moreover, the association of the Cd-Te mixture could explain the difference in toxicity between the complex and the individual metal. For mammals (mice and humans), Fthenakis et al. (1999) demonstrated that the mixture of Cd and Te is very toxic to mammalian species such as rats. An acute exposure (72 h) of dissolved Cd-Te (minimal dose, 12.5 mg/kg) caused an increased percentage of polymorphonuclears, lymphocytes, and alveolar macrophage cells (Morgan et al., 1995). In this study, we examined whether the dissolved mixture of Cd-Te could explain, in part at least, QDs' immunotoxicity. The results showed that this mixture was more toxic for most species (except mussels) with respect to QDs.

Our correlation results indicate that the mechanistic effects (such as phagocytosis) of the QDs were correlated with those of the dissolved Cd and the dissolved mixture, but that the QDs were more toxic for blue mussels than their metallic components. Given that QDs could aggregate in high-salinity

media, the aggregates of QDs should be more toxic than individual NPs for mussels. Previous studies have demonstrated that agglomerated NPs could be found in the cytosol of rainbow trout gill-W1 cells, and the cytotoxicity has been recently demonstrated irrespective of the exposure medium (Kühnel et al., 2009; Canesi et al., 2010). Indeed, NPs could be internalized in immune cells and degraded in phagosomes, and induce immunomodulation (Zolnik et al., 2010). Moreover, the positive relationship between the dissolved Cd and the QDs suggests liberation of Cd during the oxidative burst in the phagosomes. According to our results, these aggregates will likely damage macrophages or hemocytes.

Mussel hemocytes were resistant at dissolved Cd concentrations that could be lethal for other aquatic species (Suedel et al., 1997) or mammalian cells (ATSDR, 1999; Olabarrieta et al., 2001). This could be explained by the attenuation of Cd toxicity by the salinity of the sea water. However, a recent study by Canesi et al. (2010) confirmed that the behavior of the NPs (C_{60} fullerene, titanium dioxide [TiO_2], and silicon dioxide [SiO_2]) may be affected by sea water. The formation of stable agglomerates of nano-micrometric size was observed. Hence, the effects of QDs in marine organisms would differ from those of soft water or mammalian systems. A more recent study of Kadar et al. (2013) with zero-valent nano-iron reported a twofold decrease in fertilization success and a delay in the embryo development of one bivalve's species (*M. galloprovincialis*) (Kadar et al., 2013). The effect was more severe with the coated form of the NP (zero-valent nano-iron), possibly owing to its different behavior, mainly its stability, under marine condition.

For humans and mice, DA analyses were similar to the dissolved components (Cd, Te, and Cd-Te mixture), suggesting that, in lower salinity conditions, the toxicity of dissolved components of QDs becomes more apparent. The LOEC of humans for viability was 14 μg/mL of Cd contained in the QDs, which would correspond to 0.28 μg/mL of the labile fraction (2%) and 0.256 μg/mL for Te (11%). The effects of dissolved Cd and Te were above 2% of the Cd labile fraction (LOEC = 7 μg/mL) and 11% of the Te-labile fractions (20 μg/mL) of QDs. The same results were also observed for lymphoblastic transformation and for the mouse model. Hence, the toxic concentration of the QDs (labile fraction) was inferior to the toxic concentration of the dissolved Cd and Te, and the QDs' toxicity was more important; as well, the dissolved fractions could not entirely explain the QDs' toxicity. The estimated labile fractions of Cd and Te of the QDs were lower than the toxic concentration of dissolved Cd and Te, suggesting that QDs' toxicity was not mediated only by the release of Cd and Te in the short term. This hypothesis was in accordance with the recent studies on Cd Te core NPs on Hela cells (Chen et al., 2012). The authors demonstrated that the cytotoxicity of QDs was not only the results of Cd ions' release but the distribution of the particles incorporated in the cell and a size effect as well (Chen et al., 2012).

CONCLUSIONS

In summary, our results suggest that the cytotoxicity of the QDs could be partially owing to the presence of dissolved Cd^{2+} and TeO_3 ions in fish, mice, and humans, as shown by DA. Moreover, the labile fraction of the QDs (2% of Cd and 11% of Te) indicates that QDs' toxicity was not mediated only by the release of Cd and Te in the short term. In marine mussels, the toxicity of QDs was not explained by the presence of labile Cd or Te salts, and this organism was able to clearly discriminate the toxic fingerprint for all substances.

Moreover, we found that macrophages and monocytes were less sensitive to QDs and dissolved metals exposition than T-lymphocytes for humans, mice, and fish. This suggests that the adaptive immunity represents a more sensitive target than the innate immunity toward QDs' toxicity.

The correlation analysis between species revealed that the trout responses observed during QD exposure were significantly different from other model species' responses. Rainbow trout behaves differently during a QD exposition (phagocytosis results were not correlated with other model species). Blue mussels were the most resistant models in this study, during an exposition to dissolved metals, and were more affected by the QDs than by its metallic components.

Human cells were the most sensitive species tested in this study, both to QDs and to dissolved metals. Moreover, the human model associates the toxicity of the QDs with their components (which was not observed with mice). According to this observation, a set of species, not just one species, should be used as a model for the QD risk assessments. Considering the different immune response, the mussels and mice were most able to discriminate the effects of Cd-based NPs from the effects of dissolved Cd and Te at the immunocompetence level.

ACKNOWLEDGMENTS

The authors acknowledge the Centre Interinstitutionnel de Recherche en Écotoxicologie du Québec (CIRÉ) and the immunotoxicology network laboratory in the Institut national de la recherche scientifique (INRS) for the immune assessments for the four species in this study. The authors further acknowledge Dr. Stephane Masson for supplying blue mussels from the Aquarium of Quebec. This work was supported by funds from Health Canada's Chemical Management Plan.

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