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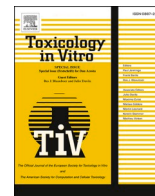
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## Gaining insight into genotoxicity with the comet assay in inhomogeneous exposure scenarios: The effects of tritiated steel and cement particles on human lung cells in an inhalation perspective

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### ABSTRACT

The comet assay was recently applied for the first time to test the genotoxicity of micrometric stainless steel and cement particles, representative of those produced in the dismantling of nuclear power plants. A large dataset was obtained from in vitro exposure of BEAS-2B lung cells to different concentrations of hydrogenated (non-radiative control) and tritiated particles, to assess the impact of accidental inhalation. Starting from the distributions of the number of nuclei scored at different extent of DNA damage (% tail DNA values), we propose a new comet data treatment designed to consider the inhomogeneity of the action of such particles. Indeed, due to particle behavior in biological media and concentration, a large fraction of cells remains undamaged, and standard averaging of genotoxicity indicators leads to a misinterpretation of experimental results. The analysis we propose reaches the following goals: genotoxicity in human lung cells is assessed for stainless steel and cement microparticles; the role of radiative damage due to tritium is disentangled from particulate stress; the fraction of damaged cells and their average level of DNA damage are assessed separately, which is essential for carcinogenesis implications and sets the basis for a better-informed risk management for human exposure to radioactive particles.

### 1. Introduction

The single cell gel electrophoresis assay, commonly referred to as comet assay, is widely used in genetic toxicology studies to identify substances causing DNA damage, as well as to provide a quantification of their genotoxic potential. In the assay, after exposing cells to the potentially genotoxic agent, the migration of DNA in a thin agarose layer due to the electrical field is used to obtain indirect information about exposure-induced DNA breaks. Measured breaks are resulting from direct interactions of the substance with DNA (as well as from indirect action on DNA, mediated by reactive oxygen species), alkali labile sites, or as a consequence of DNA excision repair. After electrophoresis, DNA is visualized with an appropriate stain and images are captured. The extent of DNA that has migrated depends on the frequency of DNA

breaks and their ability to relax the supercoiling of DNA loops, thus reflecting the level of the damage: the corresponding quantitative indicator is the percentage of total DNA that is found in the tail of the comet-shape *nucleoids* (also referred to as *cells*, for the sake of simplicity), calculated with respect to the total DNA content. This is referred to as “% tail DNA”, and such parameter can be extracted from image analysis as fluorescence intensity in the tail normalized to the total cell intensity. The migration distance reflects the spatial distribution of breaks, thus also being correlated to the size of fragments, and the corresponding tail length (from the trailing edge of the comet to its end), can be directly measured from the images. The product of % tail DNA and tail length expressed in  $\mu\text{m}$  gives the so-called tail moment, combining the two pieces of information in a single variable. OECD guidelines (OECD Test No, 2014) for in vivo comet assay recommend the

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use of % tail DNA as primary indicator to evaluate and interpret genotoxicity results. When applied in vitro on cultured cells, the comet assay proves to be a sensitive tool, also for low concentrations of the genotoxic agent (Bajpayee et al., 2019; Cordelli et al., 2021; Langie et al., 2015; Kumarave et al., 2009; Brendler-Schwaab et al., 2005) and in the specific case of genotoxic agents of particulate nature (Uboldi et al., 2016; Uboldi et al., 2019). The assay has been and is largely adopted also in radiation biology studies to investigate radiation-induced DNA damage and the associated repair kinetics (Olive, 2009). Dose-response relationships, also with the purpose of using comet for biodosimetry, have been studied for different quantitative indicators that can be extracted from image analysis (Bushmanov et al., 2022; Wang et al., 2013), but the % tail DNA largely remains the most frequently reported. Given its wide applications, and the fact that many features of the assay contribute to variability (see e.g. (Zainol et al., 2009)), collection and standardization of comet assay protocols to understand and reduce inter-laboratory differences, controlling for confounding in the assay, as well as recommendations for describing comet assay procedures and results, are issues of current interest (Brunborg et al., 2023; Collins et al., 2018; Azqueta et al., 2020; Møller et al., 2020).

Most applications of the comet assay however share a common limitation: once the quantitative indicator of genotoxicity is chosen, a single value derived from its full distribution in the analyzed cell population is usually proposed to condense the experimental information. This is useful for practical reasons, e.g. when comparing the result for the exposed population to the control condition, investigating the response at different levels of exposure to the same agent or comparing the genotoxic potential of agents of different nature. However, it is legitimate to use a single parameter, as the mean % tail DNA, as well as to apply statistical parametric tests to highlight differences between datasets, only when the underlying distribution of the variable is normal (Møller and Loft, 2014), which is not necessarily the case. Relevant scenarios can be identified, where considering the full distribution of % tail DNA, instead of its average value only, becomes very important, for instance: i) cells exposed to any agent that, for its specific nature (e.g., insolubility) and low concentration, is expected not to affect the whole population; ii) cells exposed to low and inhomogeneously distributed ionizing radiation dose. These scenarios share as a common aspect the inhomogeneity of the action of the potentially genotoxic agent, leaving a large fraction of unexposed cells, as well as the possibility of different sub-groups of cells being subject to highly varying exposure levels. It is therefore expected that the distribution of the number of nuclei scored with different amounts of DNA in the tail has a prominent peak towards low % tail DNA values. A distribution peaked at low % tail DNA values can also be found in the control sample, normally attributed to DNA damage because of the assay protocol and of replicative stress. In case of an inhomogeneous exposure, any average % tail DNA value in the exposed population could therefore be shifted towards lower values just because of the presence of unexposed cells, not giving correct information on the genotoxic potential of the agent. When analyzing % tail DNA distributions, OECD guidelines recommend the use of the median, which gives more weight to the presence of unexposed cells with respect to the mean value. However, the mean and median values from comet data are expected to have a high statistical correlation (Møller and Loft, 2014). When comparing two genotoxic agents sharing the feature of an inhomogeneous action, a different median or mean value for the % tail DNA distribution can result from a different balance between unexposed and exposed cells, and not necessarily from a different average damage when only exposed cells are considered. In principle, the full data distribution can also show a multi-peak structure, allowing to discriminate cells based on their different exposure level.

All the above considerations apply to a recent investigation of the genotoxic potential of tritiated stainless steel and cement particles acting on human lung cells by Lamartiniere et al. (Lamartiniere et al., 2022). Particles of these kinds are produced in the  $\mu\text{m}$ -size range following mechanical operations in the dismantling of old nuclear power

facilities. Their dispersion in the environment can lead to human exposure via inhalation, with a particular concern for workers. They have therefore been identified as worthy of attention, and their possible impact on human health has started being addressed with dedicated projects including experimental in vitro studies (Liger et al., 2018). In (Lamartiniere et al., 2022), both hydrogenated (as a non-radiative control) and tritiated SS316L stainless steel and cement particles have been administered to BEAS-2B human lung cells for 2 or 24 h, with concentrations in the range 0 to 200  $\mu\text{g}/\text{ml}$ , leading to exposures up to 100 kBq/ml for tritiated particles. Cell exposure to tritiated water (HTO) at activity levels reached by the highest tested concentrations of tritiated steel and cement particles have also been included in the study. Among other endpoints, the genotoxic potential of particles and HTO has been assessed with the comet assay, and the average % tail DNA was reported for cells in the different experimental conditions. Hydrogenated and tritiated steel and cement particles were found to lead to genotoxicity depending on their concentration. A net effect due to the radiative nature of tritiated particles in inducing additional DNA damage with respect to their corresponding hydrogenated control could not be determined nor ruled out.

There are several features of the in vitro exposures investigated in (Lamartiniere et al., 2022) that call for a careful consideration of the inhomogeneity in the action of steel and cement micrometric particles on human lung cells, among which: i) particles are either insoluble (steel) or only partially soluble (cement) in the cell culture medium, and in the investigated concentration range they are not expected to reach and be able to directly damage all cells, leaving a large fraction of the population as “unexposed”; ii) when radioactive particles are considered, though a fraction of the activity is released to the medium, tritium  $\beta$  electrons have a very short range (on average, 0.5  $\mu\text{m}$  in water), which means that only cells in close contact with a particle (“hit” by the particle) can receive radiation dose, and thus possibly be subject to radiation-induced DNA damage (Mentana et al., 2023); iii) particles might not be homogeneously distributed and create aggregates, which also adds to a final inhomogeneity in their action. Overall, in the light of the above discussion, when applying the comet assay to investigate the genotoxic potential of radioactive particles, a better insight could be gained considering the full data distribution, in terms of number of cells scored with different % tail DNA values in the exposed population.

In this study, we therefore present a novel analysis of the full dataset collected in (Lamartiniere et al., 2022), applying the single cell gel electrophoresis technique to human lung cells exposed to hydrogenated or tritiated stainless steel and cement particles. By means of this analysis, we are able to highlight the role of radiation-induced DNA lesions due to tritium, disentangled from the particulate stress due to steel and cement contamination. At the same time, we can discriminate the information on the amount of damaged cells and on the average level of DNA damage, which is essential in terms of implication for carcinogenesis. Results obtained with this data treatment thus contribute to a better definition of the hazard that could be useful to efficiently manage the occupational exposure to different kinds of radioactive particles.

## 2. Materials and methods

Full information on the experimental protocol for application of the comet assay on BEAS-2B cells is given in (Lamartiniere et al., 2022). Cells were stained with a 0.1 mg/ml propidium iodide solution. The slides were examined with fluorescent microscopes (Carl ZEISS Axio Imager A2, Bayern, Germany or Olympus BX40; Rungis, France) at 400 $\times$  final magnification. The digital pictures were analyzed using a CCD ANDOR Zyla camera (Andor Bioimaging, Nottingham, UK) and the determination of the % tail DNA was performed using the Komet Software 6.0 or 7.0 (Andor Bioimaging, Nottingham, UK). All the details related to the data processing using the Komet Software are given in the provider webpage at <https://andor.oxinst.com/>. More detailed information, following recommendations to report on the comet assay as

given in (Møller et al., 2020), is included as **Supplementary Materials (Table 1S)**.

The following exposure conditions were considered: exposure to hydrogenated or tritiated stainless steel or cement particles, for either 2 or 24 h, with concentrations: 1, 2.5, 5, 10, 25, 50, 100 and 200 (cement only)  $\mu\text{g}/\text{ml}$ . Tritiated particles were obtained with a specific activity of 1  $\text{kBq}/\mu\text{g}$  for steel and 0.4  $\text{kBq}/\mu\text{g}$  for cement. Exposures were in the range 0–100  $\text{kBq}/\text{ml}$  and 0–8  $\text{kBq}/\text{ml}$ , for steel and cement particles, respectively. Cells were also exposed to tritiated water (HTO), for either 2 or 24 h, at the two maximal activity levels reached for steel and cement particles (100 and 8  $\text{kBq}/\text{ml}$ ). Exposure conditions and corresponding comet images are illustrated in Fig. 1.

Each independent experiment consisted in a series of measurements following administration of the same particle type at different concentrations and of HTO at the corresponding maximal activity level, and included the analysis of an unexposed cell population as a control condition. 3 to 6 independent experiments were performed, depending on the particle type. In each experiment, for each exposure condition, the % tail DNA was scored for a total number of 100 nucleoids. In (Lamartiniere et al., 2022) results were presented as average % tail DNA  $\pm$  SEM (mean value  $\pm$  standard error of the mean) among all experiments for each exposure condition.

The data treatment presented in this work is explained in all its steps in Fig. 2 with a subset of conditions chosen as examples. The starting point is the full distribution of the number of nucleoids scored with different % tail DNA values for each exposure condition. Histograms with a 5% tail DNA bin width are obtained from such distributions. Cells are considered as undamaged if the corresponding % tail DNA value is found below 10%. The choice of this threshold value is critically discussed later. Once binned histograms (# cells vs. % tail DNA) are created, the analysis proceeds as follows:

1. The histograms for a chosen exposure condition (*test*) and the corresponding control (*ctrl*) are both normalized setting to 1 the number of nucleoids with % tail DNA < 10%. For each histogram, this number is obtained summing the contents of the first two bins (see Fig. 2a and b for representative histograms);
2. Starting from the two normalized histograms, the quantity  $\delta$  (arbitrary units, proportional to a number of cells) is calculated for each

bin, subtracting the number of cells in the control population from the number of damaged cells scored in the exposure condition under consideration:

$$\delta(\text{test}) = \# \text{cells}(\text{test}) - \# \text{cells}(\text{ctrl}) \quad (1)$$

The full distribution  $\delta(\text{test})$  vs. % tail DNA is obtained. By definition,  $\delta = 0$  for all tested exposure conditions when % tail DNA < 10% (see Fig. 2c and d for representative  $\delta$  distributions);

3.  $\delta$  values obtained in independent experiments for the same exposure condition can be averaged, leading to a single  $(\delta \pm \text{SEM})(\text{test})$  vs. % tail DNA distribution;
4. From each  $\delta(\text{test})$  vs. % tail DNA distribution, the quantity  $\Delta$  (arbitrary units, proportional to a number of cells) can be calculated summing up all  $\delta$  values over all % tail DNA bins, namely:

$$\Delta(\text{test}) = \sum_{\% \text{tail DNA}} \delta(\text{test}), \quad (2)$$

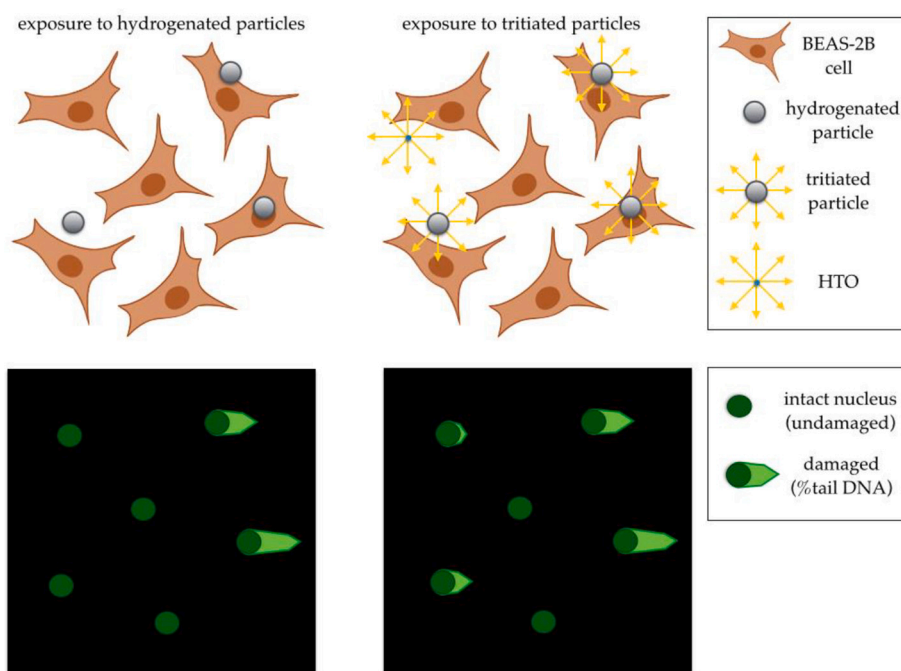
Examples of  $\Delta$  values are reported in Fig. 2c and d insets for the corresponding  $\delta$  distributions. Averaging  $\Delta$  values obtained in independent experiments a  $(\Delta \pm \text{SEM})(\text{test})$  indicator can be obtained for each exposure condition;

5. From each  $\delta(\text{test})$  vs. % tail DNA distribution, the average (first moment) of the distribution can be obtained

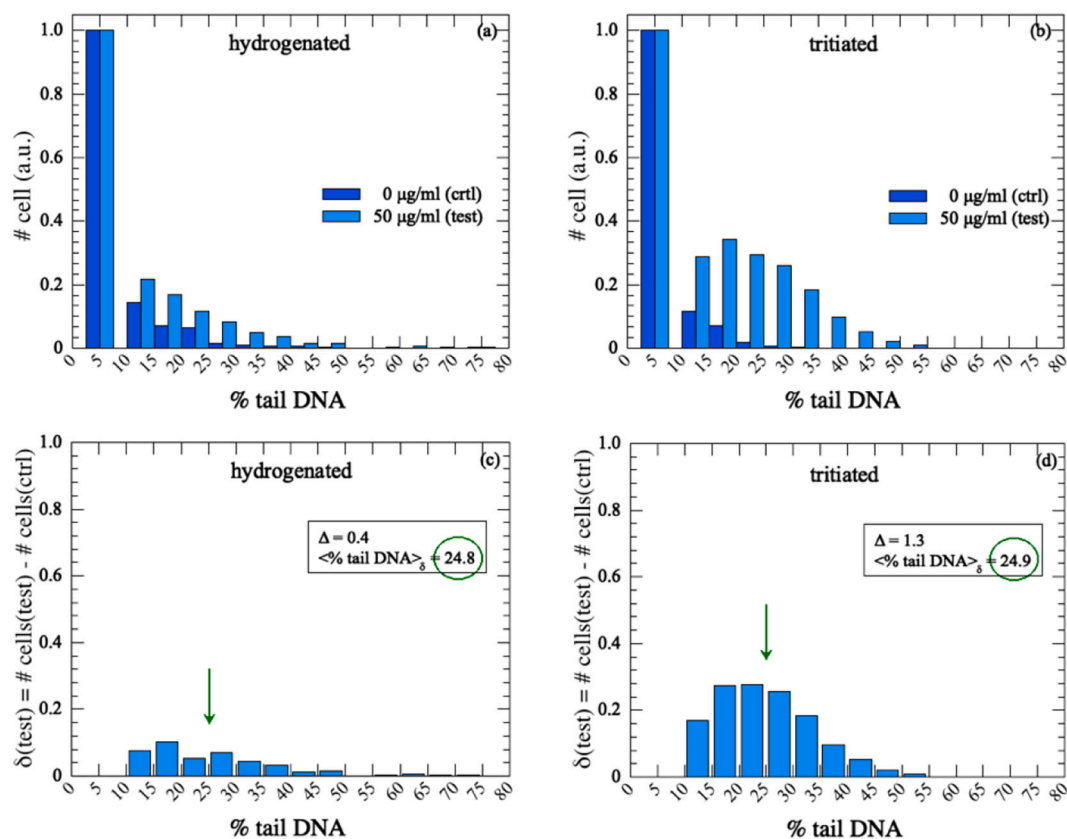
$$\langle \% \text{tail DNA} \rangle_{\delta}(\text{test}) = \frac{1}{\Delta} \sum_{\% \text{tail DNA}} \delta \cdot (\% \text{tail DNA}), \quad (3)$$

Examples of  $\langle \% \text{tail DNA} \rangle_{\delta}$  values are reported in Fig. 2c and d insets for the corresponding  $\delta$  distributions, and indicated by arrows. An average  $(\langle \% \text{tail DNA} \rangle_{\delta} \pm \text{SEM})(\text{test})$  indicator can be obtained for each exposure condition.

Statistical significance for differences in the  $\Delta$  and  $\langle \% \text{tail DNA} \rangle_{\delta}$  quantities was evaluated with the two-tailed *t*-test, always comparing exposures to the same concentration of tritiated vs. hydrogenated particles.



**Fig. 1. Illustrations of the exposure conditions of BEAS-2B cells to hydrogenated and tritiated particles at the same concentration, and of the corresponding comet images for quantification of DNA damage.** Tritiated particles are source of  $\beta$  decays (yellow arrows represent decay electrons), which causes a radiative stress, in addition to the particulate (steel or cement) stress. A fraction of tritiated particle activity is also lost to the cell medium, presumably in form of tritiated water (HTO). Because of the low tested particle concentrations and of the inhomogeneity in their action, a large fraction of cells remains undamaged. Comet images are acquired to score the number of cells at different DNA damage level, quantified with the % of DNA in the tail. Comparing exposed populations to the control conditions (0  $\mu\text{g}/\text{ml}$  particle concentration), the data treatment discussed in this work gives information on extra damaged cells when hydrogenated or tritiated particles are administered, and on the average DNA damage level these cells suffer. Illustration partly realized with BioRender. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2. Representative comet data distributions for cells exposed to SS316L stainless steel particles and examples of the data treatment.** For single experiments, histograms in the upper panels report the number of cells (# cells) scored with different % tail DNA levels (5% tail DNA bin width), after setting to 1 the number of cells with % tail DNA < 10%. Both panels include the distributions measured in the control population (ctrl, 0 µg/ml) and in the cell population after 24 h administration of 50 µg/ml (test) of: (a) hydrogenated particles; (b) tritiated particles (1 kBq/µg). (c) and (d): corresponding distributions of the  $\delta$  quantity (see Eq. 1), obtained for each bin as # cell (test) - #cell (ctrl). Insets report:  $\Delta$  values (Eq. 2), obtained summing  $\delta$  over all % tail DNA bins;  $\langle \% \text{ tail DNA} \rangle_{\delta}$  values (Eq. 3), obtained as average values of the  $\delta$  distributions, also indicated by arrows.

### 3. Results

#### 3.1. Analysis of the full distribution in comet data

The full dataset from the comet assay applied to BEAS-2B cells in different exposure conditions in (Lamartiniere et al., 2022) was made available for the analysis. Experiments were performed for cells exposed for 2 and 24 h to: hydrogenated or tritiated (1 kBq/µg) SS316L stainless steel particles, at different concentrations in the range 0 (control) - 100 µg/ml, and HTO at the activity level of 100 kBq/ml; hydrogenated or tritiated (0.4 kBq/µg) cement particles, at different concentrations in the range 0 (control) - 200 µg/ml, and HTO at the activity level of 8 kBq/ml.

Fig. 2 shows an example of representative comet data distributions from two different single experiments: histograms report the number of cells (# cells) scored with different % tail DNA values, comparing results for the control conditions (0 µg/ml particle concentration) to those for cells after 24 h administration of 50 µg/ml of hydrogenated (Fig. 2a) and tritiated (Fig. 2b) stainless steel particles. In both panels, all distributions are normalized, setting to 1 the number of cells with % tail DNA < 10%, referred to as “undamaged” (see later section in the Discussion, §3).

The actual percentages of undamaged cells, over the whole analyzed cell population, are reported in Fig. 1S, **Supplementary Materials**, for control conditions and all tested exposures. Data visualization as proposed in Fig. 2a and b helps appreciating that, at equal number of undamaged cells, both hydrogenated and tritiated steel particles induce genotoxicity with respect to the control, and tritiated particles seem to have a higher effect with respect to their non-radiative counterparts.

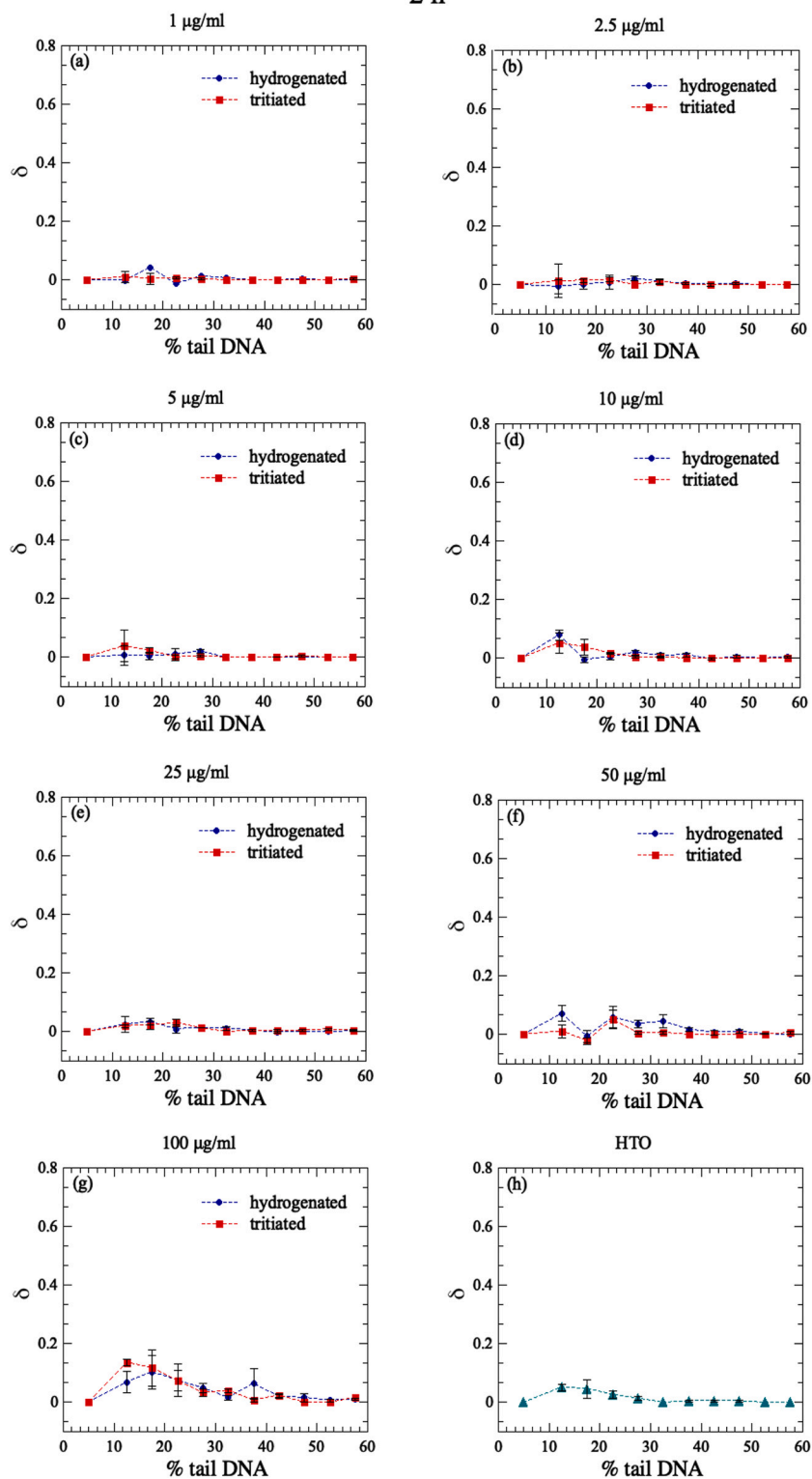
#### 3.2. Evaluation of the net genotoxic effect of hydrogenated and tritiated particles

From normalized histograms as those shown in Fig. 2a and b, we calculated the full distributions of the quantity  $\delta$  (Eq. 1): for each % tail DNA histogram bin,  $\delta$  is obtained subtracting the number of cells in the control population from the number of damaged cells scored in the exposure condition under consideration. The value of  $\delta$  (arbitrary units) is therefore proportional to the number of extra cells that can be found at a given damage level when the population is exposed to the potentially genotoxic agent. By construction,  $\delta = 0$  when % tail DNA < 10%. Representative  $\delta$  distributions are shown in Fig. 2c and d, obtained respectively from the histograms shown in Fig. 2a and b, and corresponding to 24 h exposures to 50 µg/ml of hydrogenated and tritiated stainless steel particles.

$\delta$  distributions for a given particle concentration are averaged over all experiments and shown for hydrogenated and tritiated steel particles administered for 2 and 24 h, respectively in Figs. 3 and 4. At both time points, the  $\delta$  distribution after administration of 100 kBq/ml HTO is also obtained, and results are included in the figures. From Fig. 3, it can be seen that, after 2 h exposure, all  $\delta$  values are always close to 0 for particle concentrations below 50 µg/ml, indicating the absence of a net genotoxic effect for both hydrogenated and tritiated particles.

At 50 and 100 µg/ml,  $\delta$  values below ~25–30% tail DNA seem to show an increasing trend. No biologically significant difference is observed between tritiated and hydrogenated particles, with a possible exception at 100 µg/ml for  $10 \leq \% \text{ tail DNA} < 15$ . HTO seems to induce a net genotoxic effect in the same range of % tail DNA values, with  $\delta$

2 h



**Fig. 3.** Distributions of extra damaged cells at different % tail DNA levels with respect to the control (ctrl) population, after 2 h exposure to SS316L stainless steel particles or HTO. For each % tail DNA bin and tested condition,  $\delta$  (a.u.) is calculated as  $\#cells (test) - \#cells (ctrl)$  (Eq. 1), starting from full comet distributions with  $\#cells$  set to 1 for % tail DNA < 10%. Distributions are averaged over all experiments ( $n = 3$  to 6) and data are given as  $\delta \pm SEM$ . Panels show data for both hydrogenated (blue) and tritiated (red) particles at concentrations of: (a) 1  $\mu\text{g/ml}$ ; (b) 2.5  $\mu\text{g/ml}$ ; (c) 5  $\mu\text{g/ml}$ ; (d) 10  $\mu\text{g/ml}$ ; (e) 25  $\mu\text{g/ml}$ ; (f) 50  $\mu\text{g/ml}$ ; (g) 100  $\mu\text{g/ml}$ . (h) Exposure to 100 kBq/ml HTO. Lines are a guide to the eye. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

values close but generally lower than those for 100  $\mu\text{g/ml}$  tritiated particles. After a 24 h exposure (data in Fig. 4),  $\delta$  values start to be higher than 0 at lower concentrations ( $\sim 10 \mu\text{g/ml}$ ), and a net difference between the effect of hydrogenated and tritiated particles becomes visible for concentrations greater or equal to 25  $\mu\text{g/ml}$ . The range of % tail DNA for which  $\delta$  is different than 0 also extends towards higher values, up to 50% for the highest concentration.  $\delta$  values for the HTO case after 24 h seem to be higher with respect to the 2 h exposure, but

with large fluctuations that do not allow a net effect to be measured, as later discussed.

Average  $\delta$  distributions for different concentrations of hydrogenated and tritiated cement particles and after administration of 8 kBq/ml HTO, for exposures of 2 and 24 h, are included as **Supplementary Materials** (Fig. 2S and 3S).

For a given particle concentration, the information carried by the full  $\delta$  distribution can be condensed in two quantities:  $\Delta$  (Eq. 2), obtained

24 h

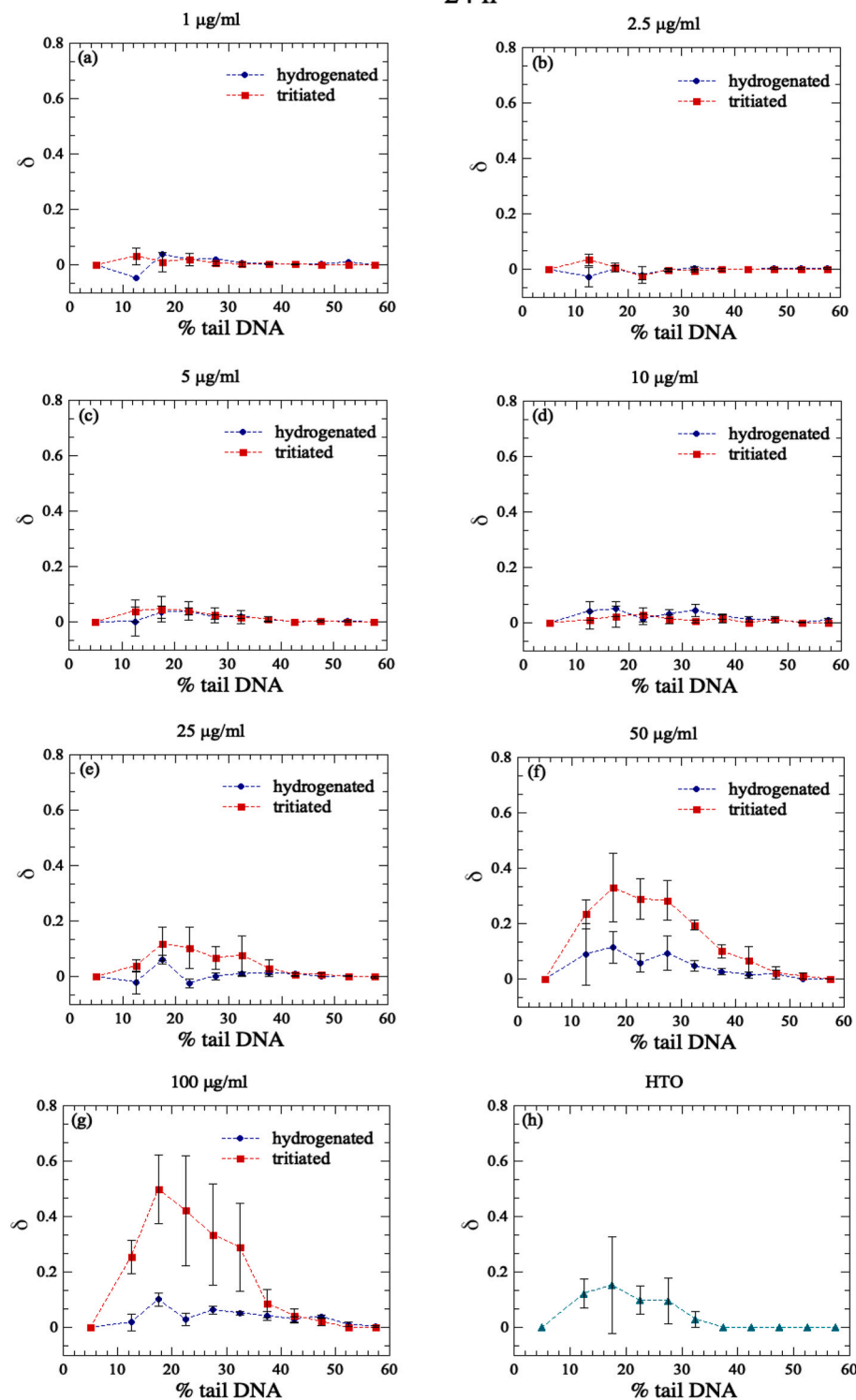
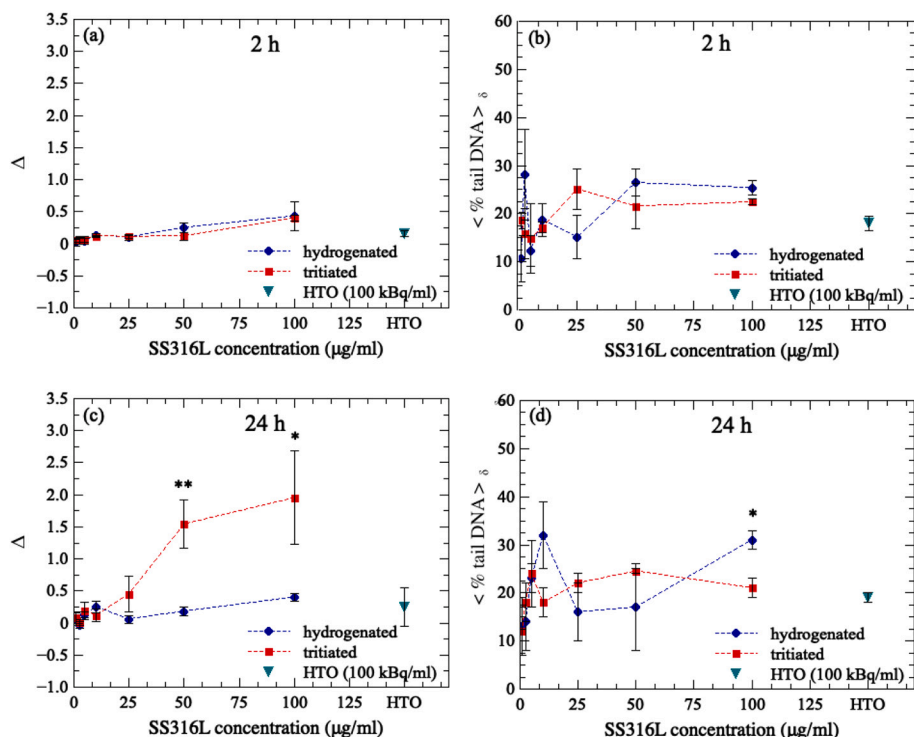


Fig. 4. Distributions of extra damaged cells at different % tail DNA levels with respect to the control (ctrl) population, after 24 h exposure to SS316L stainless steel particles or HTO. For each % tail DNA bin and test condition,  $\delta$  (a.u.) is calculated as  $\#cells (test) - \#cells (ctrl)$  (Eq. 1), starting from full comet distributions with  $\#cells$  set to 1 for % tail DNA < 10%. Distributions are averaged over all experiments ( $n = 3$  to 6) and data are given as  $\delta \pm SEM$ . Panels show data for both hydrogenated (blue) and tritiated (red) particles at concentrations of: (a) 1  $\mu\text{g/ml}$ ; (b) 2.5  $\mu\text{g/ml}$ ; (c) 5  $\mu\text{g/ml}$ ; (d) 10  $\mu\text{g/ml}$ ; (e) 25  $\mu\text{g/ml}$ ; (f) 50  $\mu\text{g/ml}$ ; (g) 100  $\mu\text{g/ml}$ . (h) Exposure to 100 kBq/ml HTO. Lines are a guide to the eye. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

summing up all  $\delta$  values over all % tail DNA bins; and its average  $\langle \% \text{ tail DNA} \rangle_{\delta}$  (Eq. 3). The value of  $\Delta$  (arbitrary units) is proportional to the number of extra damaged cells (independently of the damage level) with respect to the control condition that can be attributed to the potentially genotoxic agent. The average  $\langle \% \text{ tail DNA} \rangle_{\delta}$  value gives an indication on the average level of damage that is measured in these cells (hence considering the comparison to the control and excluding undamaged cells from the analysis). As an example of the calculation procedure,  $\Delta$  and  $\langle \% \text{ tail DNA} \rangle_{\delta}$  values corresponding to the

representative  $\delta$  distributions shown in Fig. 2c and d are given in the figure insets, and the average values of the distributions are also indicated by arrows.

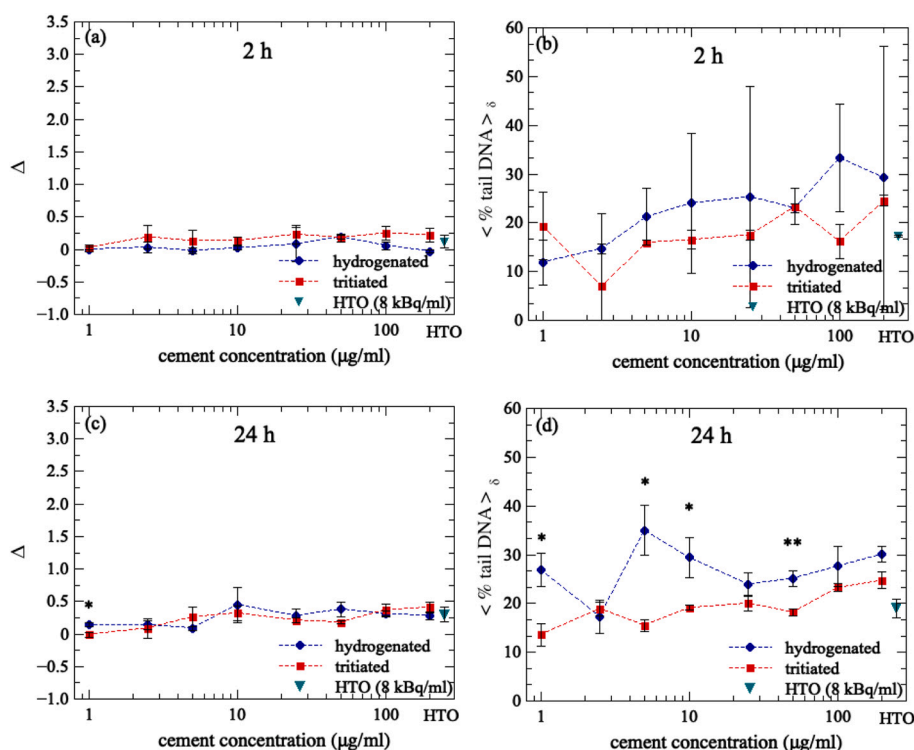
In Fig. 5 we show  $\Delta$  and  $\langle \% \text{ tail DNA} \rangle_{\delta}$  values averaged over all experiments as a function of particle concentration, both for hydrogenated and tritiated SS316L steel particles, after administration for 2 (Fig. 5a and b) and 24 h (Fig. 5c and d). At both time points, results after administration of 100 kBq/ml HTO are also included. After 2 h,  $\Delta$  starts being higher than 0 at 50  $\mu\text{g/ml}$ , and an increasing trend for increasing



**Fig. 5.** Evaluation of extra damaged cells and of their average level of DNA damage after exposure to steel particles with respect to the control (ctrl) population. Cells exposed to hydrogenated (blue) and tritiated (red) SS316L particles at different concentrations and to 100 kBq/ml HTO (light blue). (a) and (c):  $\Delta$  (sum of  $\delta$  values over all % tail DNA bins, Eq. 2) after 2 h and 24 h exposures; (b) and (d):  $\langle \% \text{ tail DNA} \rangle_{\delta}$  (average of % tail DNA over the  $\delta$  distribution, Eq. 3) after 2 h and 24 h exposures. Results are given as average  $\pm$  SEM over  $n = 3$  to 6 experiments. Lines are a guide to the eye. Statistical significance was evaluated by the two-tailed  $t$ -test for the comparison of tritiated vs. hydrogenated conditions at the same particle concentration: \*  $p < 0.05$ , \*\*  $p < 0.01$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

particle concentration can be guessed from the data. No difference is observed between hydrogenated and tritiated particles. The value of  $\Delta$  for the HTO condition is lower than that for the highest particle concentration. The  $\langle \% \text{ tail DNA} \rangle_{\delta}$  value does not vary significantly with particle concentration, being always close to  $\sim 20\%$ . A slightly lower average damage is found in cells exposed to HTO. The situation is different after a 24-h exposure:  $\Delta$  deviates from 0 and increases for concentrations higher than  $\sim 10 \mu\text{g/ml}$ , both for hydrogenated and

tritiated particles. A net effect due to radiation can be observed for concentrations greater or equal to  $25 \mu\text{g/ml}$ , with  $\Delta$  for tritiated particles being significantly higher than for hydrogenated particles (comparison with statistical significance). A saturating trend of  $\Delta$  for tritiated particles can be guessed above  $50 \mu\text{g/ml}$ , while this is not observed for their non-radiative counterparts. The average level of damage seems instead not strongly affected by the length of the exposure, with  $\langle \% \text{ tail DNA} \rangle_{\delta}$  values remaining around  $\sim 20\%$  and not varying with particle



**Fig. 6.** Evaluation of extra damaged cells and of their average level of DNA damage after exposure to cement particles with respect to the control (ctrl) population. Cells exposed to hydrogenated (blue) and tritiated (red) cement particles at different concentrations and to 8 kBq/ml HTO (light blue). (a) and (c):  $\Delta$  (sum of  $\delta$  values over all % tail DNA bins, Eq. 2) after 2 h and 24 h exposures; (b) and (d):  $\langle \% \text{ tail DNA} \rangle_{\delta}$  (average of % tail DNA over the  $\delta$  distribution, Eq. 3) after 2 h and 24 h exposures. Results are given as average  $\pm$  SEM over  $n = 3$  to 6 experiments. Lines are a guide to the eye. Statistical significance was evaluated by the two-tailed  $t$ -test for the comparison of tritiated vs. hydrogenated conditions at the same particle concentration: \*  $p < 0.05$ , \*\*  $p < 0.01$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



concentration. However, the average damage following exposure to 10  $\mu\text{g/ml}$  and to the highest concentration of tritiated steel particles seems lower than that observed for hydrogenated particles. Neither the total amount of damaged cells nor their average damage level seem to be affected by the length of the exposure to HTO.

In Fig. 6 we show the corresponding set of results obtained after administration of different concentrations of hydrogenated and tritiated cement particles and 8 kBq/ml HTO. For cement particles,  $\Delta$  remains closer to 0 after a 2 h exposure for hydrogenated particles, with the exception of the 50  $\mu\text{g/ml}$  concentration, while more cells seem to be affected in the case of tritiated particles starting from relatively low concentrations. This difference seems to be washed out after 24 h, where higher  $\Delta$  values are reached for both kinds of exposures and for HTO.  $\langle \% \text{ tail DNA} \rangle_{\delta}$  values show unclear trends and high statistical fluctuations at 2 h. At 24 h data are less sparse, and a general higher damage level seems to be observed when cement particles are non-radiative (with statistically significant differences at several concentrations), as well as a slight increasing trend with increasing particle concentration above  $\sim 25 \mu\text{g/ml}$ . Averaging the  $\langle \% \text{ tail DNA} \rangle_{\delta}$  value over concentrations we obtain  $\sim 30\%$  for hydrogenated particles and  $\sim 20\%$  for tritiated ones. The average damage following exposures to 8 kBq/ml HTO seems lower than that found at the maximal concentration of tritiated particles, with a similar percentage of damaged cells. HTO data seem not affected by the length of the exposure, neither by the difference in activity when comparing Fig. 6 to Fig. 5.

### 3.3. Comparison to standard comet data treatment

Fig. 7 shows the direct comparison of  $\% \text{ tail DNA}$  values that can be obtained averaging the full comet data distribution, as done in (Lamartiniere et al., 2022) using mean values, or when considering the comparison to the control population and excluding undamaged cells ( $\langle \% \text{ tail DNA} \rangle_{\delta}$  values, as introduced in this work). The comparison is conducted only for 24 h exposures, and results are given as a function of particle concentration for all exposures and for the two tested activity

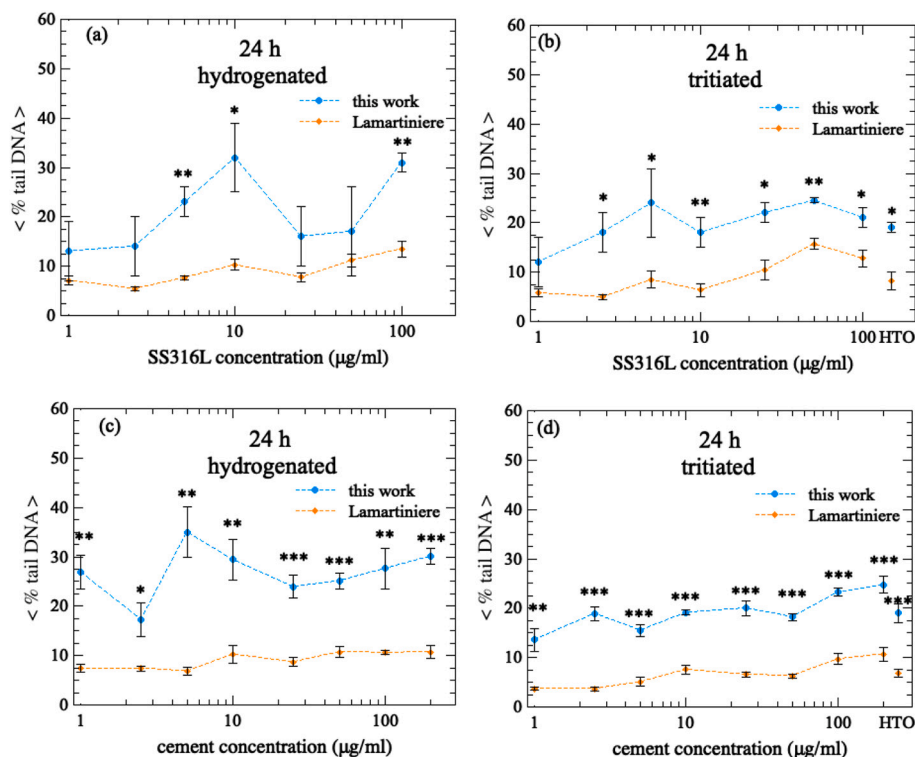


Fig. 7. Comparison of  $\% \text{ tail DNA}$  values obtained with standard averaging applied to full comet data distributions (Lamartiniere et al., 2022) and  $\langle \% \text{ tail DNA} \rangle_{\delta}$  values (Eq. 3) as defined in this work. Cells exposed for 24 h to different concentrations of: hydrogenated (a) and tritiated steel particles, and 100 kBq/ml HTO (b); hydrogenated (c) and tritiated cement particles and 8 kBq/ml HTO (d). Results are given as average  $\pm$  SEM over  $n = 3$  to 6 experiments. Lines are a guide to the eye. Statistical significance was evaluated by the two-tailed t-test for the comparison of results obtained with the two data treatment procedures in the same exposure conditions: \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

levels of HTO. As expected, the data treatment discussed in this work reveals a generally higher genotoxicity level with respect to what can be concluded with standard averaging. Of note, information on a higher genotoxicity has to be interpreted in light of the data obtained on the fraction of damaged cells, as later discussed. For a further term of comparison, the analysis performed in (Lamartiniere et al., 2022) has been repeated calculating median instead of mean values, following OECD guidelines. Results are shown in Supplementary Materials (Fig. 4S). The mean and the median of each distribution have a high statistical correlation (Møller and Loft, 2014), and their trends as a function of particle concentration are the same. Using median values leads to an even higher difference with results presented in this work.

## 4. Discussion

The comet assay is a sensitive and powerful tool to assess chemically- or radiation-induced genotoxicity. In most studies, also following OECD guidelines (OECD Test No, 2014) for its in vivo applications, the  $\% \text{ tail DNA}$  is chosen as indicator of DNA damage, and a single value (the median, per recommendations) over the analyzed cell population is reported.

Such assay was recently applied for the first time to an in vitro lung cell model (BEAS-2B cells) to address the impact of accidental inhalation of tritiated stainless steel and cement particles representative of those produced in the dismantling of a nuclear power plant (Lamartiniere et al., 2022). Based on results available in the literature on the exposure to stainless steel and cement in the working environment, neither of these materials is expected to exert a significant cytotoxicity on humans (Santonen et al., 2010; Hedberg et al., 2010; Stockmann-Juvala et al., 2013; Van Berlo et al., 2009; Kain et al., 2012; Bauer et al., 2012). However, DNA appeared to be a possible target of their action in previous studies (Sellappa et al., 2010; Krishna et al., 2020). The question naturally arises whether the combination of particulate stress due to steel or cement contamination with a radiative stress can significantly concur to the genotoxicity level that can be measured in vitro with the comet assay.

The experimental characterization of steel and cement particles and of their behavior in biological media (the cell culture medium, in this case) for in vitro studies, together with the short-range nature of tritium as a  $\beta$ -emitter, call however for a careful consideration of the inhomogeneity in their genotoxic action: in the concentration range investigated in (Lamartiniere et al., 2022), a significant fraction of cells in the population is expected to remain “unexposed”, at least in terms of absence of a direct contact with particles. This leads to a non-Gaussian distribution of the number of cells scored with different % tail DNA values, and results given only in terms of average values do not allow to reach a full understanding of the consequence of the exposure. Previous experimental studies have also exploited the potential of full data distributions in single cell gel electrophoresis measurements: as an example, the distribution of the tail moment (% tail DNA multiplied by tail length in  $\mu\text{m}$ ), measured in different mammalian cells after UV irradiation, was reported to follow a  $\chi^2$ -distribution (Bauer et al., 1998). The degree of freedom  $n$  in the  $\chi^2$ -probability density function fitted to reproduce experimental data was proposed as a quantitative indicator of genotoxicity, taking account at the same time of the amount and complexity of DNA damage. The factors in the product tail moment however were found to be normally distributed, which is reasonable for the rather uniform exposure resulting from UV irradiation. A full comet data analysis based on these findings was also applied to evaluate the genotoxic activity of chemotherapeutic agents (Digue et al., 1999).

To our knowledge, this study is proposing a new way to exploit full comet data distributions, which is particularly suited and applied for the first time in case of inhomogeneous exposures. The data treatment we present aims at assessing, for all exposure conditions, both the extra damaged cells with respect to the control population (at different % tail DNA levels, and in total), as well as the average damage level affecting these cells. In the unexposed population, the distribution of the number of cells vs. % tail DNA is expected to be sharply peaked at 0 (intact nuclei). However, cells with damaged DNA can also be found in the control, either as a consequence of replicative stress or of the assay protocol itself. For the analysis presented in this work, only cells with % tail DNA  $\geq 10\%$  have been considered as having an appreciable level of DNA damage. This threshold should be adapted to the cell population under study: in our dataset, obtained with BEAS-2B cells, it leads on average to a  $\sim 80\%$  of cells being undamaged in the control condition, which is deemed reasonable for a healthy and proliferating cell population. A lower value could also be used: however, if the threshold for an appreciable level of DNA damage is set to 5% in our data, only  $\sim 60\%$  of BEAS-2B cells are undamaged in the control condition, which seems too low. Importantly however, this choice does not affect the conclusions of the analysis that we now discuss (see **Supplementary Materials**, Fig. 5S).

When exposing cells to micrometric steel and cement particles, the percentage of undamaged cells is expected to be lower than in the control condition and presumably dependent on particle concentration. Data included as **Supplementary Materials** (Fig. 1S) indicate that: after administration of the highest concentration of stainless steel particles (100  $\mu\text{g}/\text{ml}$ ), 60% of cells remain undamaged if particles are hydrogenated, while this fraction further decreases to 40% if particles are tritiated; after administration of the highest concentration of cement particles (200  $\mu\text{g}/\text{ml}$ ), undamaged cells are on average the 70% of the population, with no difference between hydrogenated and tritiated particles.

Concerning the role of tritium radiation in addition to the particulate stress due to stainless steel and cement, the following considerations are important: first, the total activity levels for the two highest particle concentrations is different for the stainless steel and cement cases. Indeed, 100  $\mu\text{g}/\text{ml}$  of stainless steel particles result in a total activity of 100 kBq/ml, while a maximal activity of 8 kBq/ml is measured for 200  $\mu\text{g}/\text{ml}$  of cement particles. Also, for both kinds of particles, a  $\sim 60\%$  (steel) and  $\sim 70\%$  (cement) of activity loss to the cell culture medium (presumably in form of HTO) occurs quickly (in  $\sim 2$  h for steel and  $<30$

min for cement particles) (Lamartiniere et al., 2022). Of note, exposures to HTO at the two maximal activity levels reached by particles at their maximal concentrations result in an almost constant  $\sim 70\%$  of undamaged cells, which seems also not dependent on the length of the exposure (2 h vs. 24 h), though variability among replicates increases with exposure time.

The analysis presented in this work gives striking evidence of tritium effects in inducing extra damage with respect to non-radiative particles only for stainless steel particles at concentrations greater or equal to 25  $\mu\text{g}/\text{ml}$ . The comet data treatment we propose allows not only to put in evidence such effects, but also to gain a better insight into the underlying mechanisms: indeed, comparing the  $\Delta$  quantity and the  $< \%$  tail DNA  $>_{\delta}$  values for tritiated and hydrogenated steel particles, it becomes clear that a larger fraction of cells in the population is damaged when particles are radioactive, but that the average damage that these cells suffer is not strongly affected by radioactivity. This seems coherent with the fact that steel particles are insoluble (Report on production of steel particles, 2019), spread over the population of cells at administration and not internalized by cells (Lamartiniere et al., 2022). If cells are damaged only when “hit” by a particle, the fraction of hit cells is expected to increase with particle concentration. If particles are also radioactive, they deliver dose to the nucleus even if not in direct contact with it, but still only if they fall within a given distance: if they are too far from the nucleus, since tritium is a short-range emitter, they cannot induce radiative damage. The radioactivity of particles could be therefore described as an increase in their “effective radius”, as introduced in (Mentana et al., 2023), and this naturally leads to an increase in the fraction of affected cells. However, tritium emits radiation with a relatively low linear energy transfer (LET), and the associated DNA damage is not expected to be complex, leading to a relatively low fraction of damaged DNA as measured in the comet assay. Interestingly, extra damaged cells (hence the  $\Delta$  quantity) seem to show a tendency towards saturation when increasing the concentration of tritiated particles above 50  $\mu\text{g}/\text{ml}$ . This could at least be partially attributed to bystander signaling phenomena (Klammer et al., 2015; Kadhim et al., 2013): at concentrations around 50  $\mu\text{g}/\text{ml}$  and below, a larger fraction of cells with respect to those being directly hit by particles suffers from DNA damage, hence  $\Delta$  increases and deviates from a linear trend. As characteristic of bystander phenomena (Prise and O’Sullivan, 2009), this plays a less important role at further increasing concentrations, resulting in higher radiation dose to the cell population. The extreme case of all cells being hit will therefore be approached with a smaller slope in the  $\Delta$  vs. concentration trend. As a possible mechanisms at the basis of bystander signaling, all tested conditions were found not to induce an oxidative stress able to disturb the GSH/GSSG ratio in (Lamartiniere et al., 2022), but neither reactive oxygen species induction (that, if moderate, could not alter the GSH/GSSG balance) nor proteins involved in cell-to-cell communications were measured. Also, as an additional hypothesis, steel particle aggregates will be more easily formed at higher concentrations, which has the same effect in shaping the  $\Delta$  vs. concentration trend.

The situation is completely different for cement particles, which are found to be partially soluble with a  $\sim 60\%$  release of Ca in aqueous medium very quickly (Report on production of cement particles and characterization on steel and cement suspension, 2019). As a consequence of this behavior, the fraction of cells hit by particles is not expected to strongly depend on particle concentration, coherently with experimental observations. Intriguingly, the lower average DNA damage level measured when cement particles are tritiated (and, though only for few concentration values, when steel particles are tritiated) could suggest an adaptive response mechanism (Guéguen et al., 2019), with the low radiation dose to the population rendering cells more effective in repairing particle-induced DNA damage. The computational approach for the reconstruction of the average radiation dose to the cell population discussed in (Mentana et al., 2023) leads indeed to very low radiation levels, of the order of few cGy’s for the longest 24 h exposure to the highest concentration of tritiated steel particles, and lower doses are

expected in the cement case.

On top of all these considerations on the inhomogeneity of the action of the genotoxic agent, it is also important to recall that cells might vary in sensitivity. This would probably become more relevant for heterogeneous cell populations, like primary or cancer cells. In principle, different sensitivity levels could be highlighted with this comet data treatment applied to heterogeneous cells uniformly exposed to a genotoxic agent as e.g. X rays.

For the specific features of the exposures under study in this work, the analysis of the full distribution of comet data instead of average values only turns out to be important. Average % tail DNA values reported in (Lamartiniere et al., 2022) as the mean of the distributions are necessarily lower than the ones found considering both the comparison to the control and excluding undamaged cells from the analysis. As examples, for the longest exposure to the highest particle concentrations we have on average: for steel, % tail DNA  $\sim 10\%$  in (Lamartiniere et al., 2022) vs.  $< \% \text{ tail DNA } >_{\delta} \sim 20\%$  (tritiated) and  $\sim 30\%$  (hydrogenated) in this work; for cement, % tail DNA  $\sim 10\%$  in (Lamartiniere et al., 2022) vs.  $< \% \text{ tail DNA } >_{\delta} \sim 25\%$  (tritiated) and  $\sim 30\%$  (hydrogenated) in this work. Averaging seems to lead to an underestimation of the effect, if the mean percentage of DNA in the tail is chosen as indicator of genotoxicity. The use of median values, according the OECD guidelines, leads to the same conclusions, as a high correlation is found between the mean and the median of each distribution (Supplementary Materials, Fig. 4S).

However, the inhomogeneity in particle action cannot be the only cause of the difference in the results, as a similar underestimation is found also for the HTO case, and HTO is expected to be distributed and act homogeneously on the cell population. Of interest, data obtained after exposures to HTO seem also to indicate that the percentage of damaged/undamaged cells remains constant in the population, irrespective of the length of the exposure. It is important to keep in mind that DNA damage levels measured at the end of 24 h exposures are necessarily the result of the balance between the continuous induction of damage by the stressor and the ongoing DNA repair processes. This finding could therefore be interpreted assuming that the tested HTO activity levels are too low to significantly impair DNA repair mechanisms: if the rate of damage induction is counterbalanced by a constant rate of DNA repair, neither the amount of damaged cells nor their average damage level are expected to vary for a longer exposure. The combination of radiation and the particulate stress due to steel or cement, as well as the inhomogeneity features of the exposures to particles, could instead significantly alter the response of the DNA damage repair machinery.

More generally, the comet data treatment proposed in this work seems to be more sensitive than standard analysis procedures, and might give more correct estimations of genotoxicity. Its robustness should be tested, applying the same analysis to different datasets. Of note, the common visual scoring of comets, i.e. sorting comets into classes with increasing tail intensities, also gives data that, in principle, can be analyzed with this approach, though a lower sensitivity is expected (because of fewer intensity classes with respect to the number of 5% tail DNA bins as here proposed).

We expect that, in spite of a higher difficulty of data treatment with respect to standard averaging, important insight on genotoxicity could be gained with this approach both for exposure to agents with an inhomogeneous action, including radioactive particles (also see (Baiocco et al., 2022) for a general discussion of the consequences of a non-uniform distribution of radiation dose), and to agents that are expected to act homogeneously on the cell population, but still leading to a low genotoxicity level (as the tested activities of HTO in this work). Also, if more genotoxicity mechanisms are at play, and experimental data are available, this data treatment has a better potential to disentangle their roles, as it is the case for particulate stress due to the chemical nature of particles and to radiation-induced stress due to their radioactivity.

As well known, both the level of damage suffered by the cells and the

amount of damaged cells play a crucial role in determining the possible outcome in terms of initiation of carcinogenesis in the cell population: highly damaged cells are less prone to survive, while lower damage levels might more easily result in misrepair leading to mutation, but preserving clonogenic potential. In this respect, in particular for tritiated steel particles, the finding of a higher fraction of cells affected by DNA damage, but with the same relatively low (or an even lower) damage level due to non-radiative particles, is an important information for risk assessment.

## 5. Conclusions

When applying single cell gel electrophoresis to obtain quantitative information on genotoxicity, great care should be given to the possible inhomogeneity in the action of the agent under study on the cell population. In fact, if a large fraction of cells remains undamaged (or presents a very low damage), or if different cells suffer a highly varying damage level, averaging the %tail DNA (to obtain a single genotoxicity indicator from the comet assay) over the whole cell population might lead to an underestimation of the damage and to misinterpretation of results when comparing the outcome of the exposure to different agents.

Instead of using only single values as the median or mean values, the comet data treatment proposed in this work is based on the analysis of the whole distributions of the number of nuclei scored at different extent of DNA damage (% tail DNA values) in the exposed and in control populations. These latter are used for a sort of “background” subtraction: the analysis therefore disentangles the information on the amount of extra damaged cells, when the potentially genotoxic agent is administered, and on the average DNA damage level these cells suffer. In this way, the quantitative result on the genotoxicity level is no more affected by the presence of undamaged cells. Both pieces of information are essential to understand the possible implications, e.g. for carcinogenesis.

When applied to comet data obtained from lung epithelial cells exposed for the first time to micrometric stainless steel and cement particles, this data treatment also allows to disentangle the role of radiation from particulate stress, when such particles are tritiated, as it might happen in dismantling operations of a nuclear power plant. Besides this specific case study, the methodology presented in this work could be applied to a variety of scenarios with potentially inhomogeneous exposures (e.g. involving contamination with radioactive particles), gaining a better insight into genotoxicity and allowing for a better definition of the hazard and consequent risk management.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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for production and tritiation of steel and cement particles and for the characterization of their behavior in biological media.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tiv.2023.105656>.

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