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# Aqueous suspensions of Fuller's earth potentiate the adsorption capacities of Paraoxon and improve skin decontamination properties

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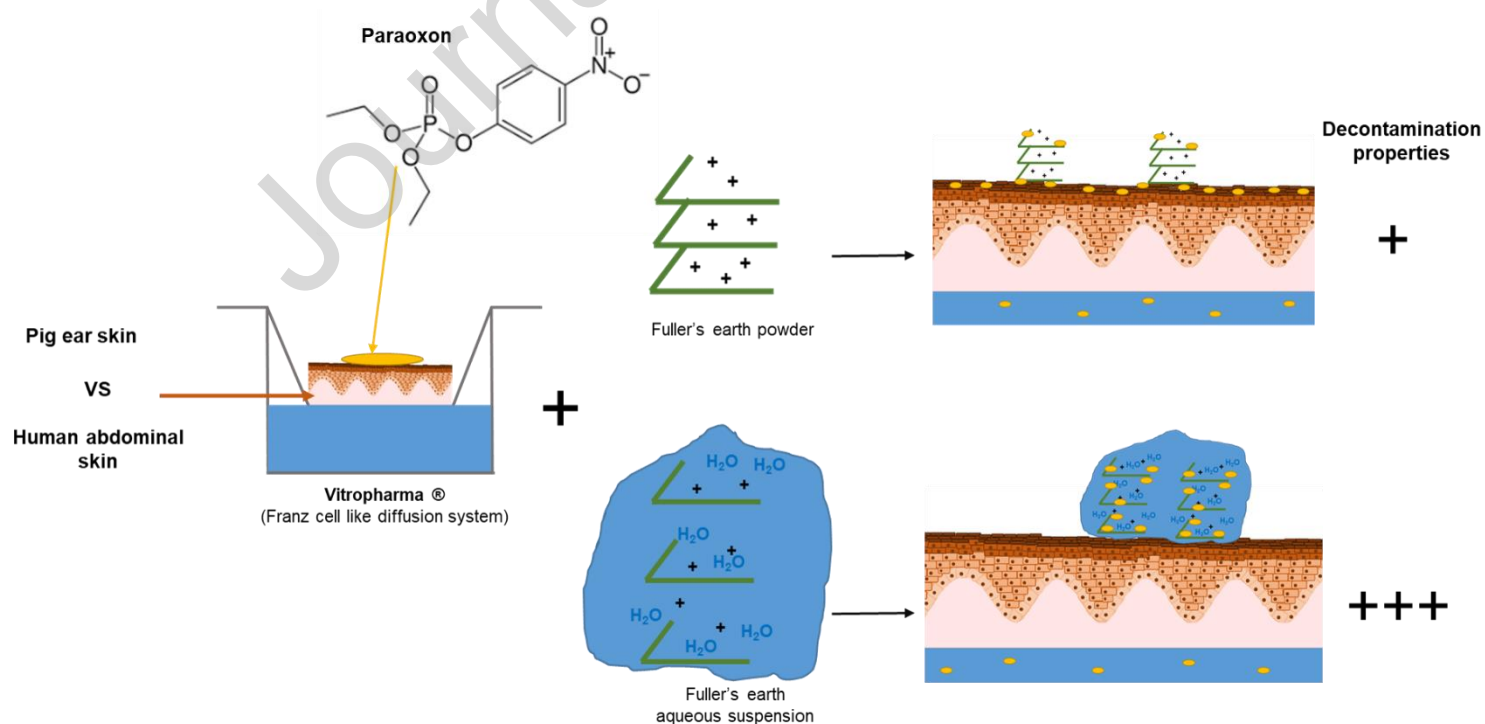
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**Abstract:** Fuller's earth (FE) is a phyllosilicate used as a powder for household or skin decontamination due to its adsorbent properties. Recent studies have shown that water suspensions exhibit similar adsorbent capacities. FE is heterogeneous due to its composition of elementary clay aggregates and heavy metal particles. Here, FE toxicity was assessed *in vitro* on skin cells and *in vivo* on *Danio rerio* embryos. Among the suspensions tested (5%, 9.1% and 15% w/w), only the highest one shows weak toxicity. Suspensions were tested for *ex vivo* dermal decontamination into pig ear skin and human abdominal skin using diffusion cells and paraoxon as organophosphorus contaminant. After 24 hours of diffusion, no difference was observed in the paraoxon concentration in the receptor compartment whether the decontamination was carried out with FE in powder or in suspension form. In presence of FE suspensions, we observed the disappearance of paraoxon from the *stratum corneum*, the reservoir compartment, independently of the suspensions' concentration. We suggest that water potentiates the absorbing capacities of FE powder by intercalating between clay lamellas leading to the appearance of new adsorption zones and swelling. These data support the use of FE aqueous suspensions as a safe tool for organophosphorus skin decontamination.

**Keywords:** smectite; paraoxon; toxicity; skin penetration; decontamination efficiency

## Graphical abstract :



## 1. Introduction

Fuller's earth (FE) is a clay that has been used for hundreds of years for woolen fulling in order to clean wool to eliminate oils, dirt and impurities (Kherdekar & Adivarekar, 2017). Due to its adsorptive capacities, modern uses of FE have been extensively developed, ranging from absorbent for cat litter to carrier for fertilizers or active ingredient in cosmetic products (Rehan et al., 2019). Interestingly, one of its most intriguing use concerns dry skin decontamination in the military field. Indeed, chemical warfare agent (CWA) still represents a threat, both for civilian (Ciottone, 2018; Haines & Fox, 2014; Pitschmann, 2014) and military populations. Organophosphorus compounds (OPC) are among the most detected chemical compounds in the environment due to their use as pesticides (such as paraoxon (POX)) and nerve agents (tabun, sarin, soman, VX) (Naughton & Terry, 2018). Because most OPCs have a low volatility, their absorption through the dermal route remains predominant in comparison with the respiratory one (John et al., 2015; Thors et al., 2016), meaning that any system acting by adsorption and displacement of the toxic agent from the skin surface could be an efficient decontaminant product. Thus, FE, which is unexpansive, easy to obtain, and composed of layers with high ion exchange capacities (Taylor et al., 2006) favoring interaction with OPCs, has largely been used for OPCs decontamination. As illustrated by previous *ex vivo* and *in vivo* studies, this phyllosilicate has been shown to be the reference agent applied for skin decontamination following CWA exposure (Bjarnason et al., 2008; Chilcott et al., 2001; Taysse et al., 2011).

FE is a dusty agent that easily induces aerosols, favoring the propagation of contaminated dust and particles which could be inhaled by surrounding people and thus represents a new threat by cross-contamination (Denet et al., 2020). One way to bypass this problem could be the use of water suspension of FE as recently described (Roul et al., 2017). Even if most of the OPC materials have low water-solubility (for example POX is soluble at 2.4g/L), an aqueous suspension of FE could offer a strong added value for decontamination. In their paper, Roul and collaborators (Roul et al., 2017) have shown that aqueous suspension of FE (10g into 100 mL) limit skin penetration of 4-cyanophenol. Thus, suspension's decontamination efficacy of this compound was proved to be better than FE powder or water alones, meaning that the contaminant is better entrapped.

In order to further investigate these observations, we decided to analyze the decontaminant properties of different concentration of FE suspension to highlight a potential dose effect decontamination efficacy. As skin is the first barrier to protect the body from external assaults, especially with OPC, we have explored the penetration of an OPC through this semi-permeable barrier to analyze if FE suspensions could diminish it. *Ex vivo* diffusion assays were done with specific derived Franz-cells (Salmon et al., 2015) (**see figure S1 in supplementary data**) allowing multiple runs at the same time and ensuring strong reproducibility. These devices consist of a donor and receiver compartments separated by a membrane make it possible to mimic passive cutaneous diffusion (Misik et al., 2011). Pig skin is commonly used as predictive model for human skin permeability because of the histological and pathophysiological similarities between the two species (Godin & Touitou, 2007; Matar et al., 2014). Although expensive and difficult to obtain, human skin remains the best model for *ex vivo* penetration study seeing as no interspecies extrapolation is needed (Moore et al., 2014) (Hui et al., 2012).

The primary aim of this study was to investigate the toxicity of different FE aqueous suspensions. This analysis is essential because the water in the suspension could favor the release of toxic compounds, such as heavy metals entrapped into Fuller's earth aggregates, jeopardizing the use of such decontamination system. The evaluation has been done *in vitro* on skin cells and *in vivo* on *Danio rerio* embryos. After toxicological validation of the suspensions, their effectiveness as a decontamination tool has been evaluated *ex vivo* with skin diffusion assays. POX was used as simulant agent for VX (Bartelt-Hunt et al., 2008; Sellik et al., 2017) and chose for its lower toxicity and its intrinsic properties (**see table S1 in supplementary data**). Furthermore, both pig ear and human abdominal skins have been used as model in order to determine if pig ear skin can be considered as a good predictive model of human skin for this contaminant.

## 2. Materials and Methods

### 2.1. Material

Fuller's earth (FE) was purchased as a free powder with adsorptive properties available for fire-fighter decontamination process (NBC-Sys, France).

## 2.2. Toxicity

### 2.2.1. *In vitro* cell toxicity

Normal Human Dermal Fibroblasts (NHDF), passages 9 to 12, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin (5000 U/mL). Human primary keratinocytes (HPK) extracted from foreskin, passage 1 to 4, were cultured in keratinocytes-SFM medium supplemented with bovine pituitary extract, 1% Penicillin-Streptomycin (5000 U/mL), Epidermal Growth Factor and  $\text{CaCl}_2$ . Both cell lineages were maintained in a 37 °C incubator (Heracell 150i, Thermo Scientific USA), under 5%  $\text{CO}_2$  and 95% humidity. All the compounds for the cell media were purchased from Gibco (Thermo Fisher Scientific, USA). Three FE suspensions were prepared, respectively 5%, 9.1% and 15% w/w, in PBS 1X (Gibco, USA) and then filtered on 0.22  $\mu\text{m}$  single-use polycarbonate filters (Merck Millipore, USA). The filtered supernatants were left at room temperature until they were added into cells for 30 minutes of incubation before washing with cell media and cell viability evaluation by the MTT technique. Cytotoxicity assays were done 40 hours after the coating of 96 wells plate with a density of 10 000 or 15 000 cells per well for respectively NHDF and HPK, with MTT cytotoxicity assays reagent from Sigma Aldrich (USA) as described by the protocol.

The values for the culture media and tested ones (i.e. PBS 1X, SD 5% and the three supernatants from the three FE suspensions) were compared and statistically analyzed using a two-way ANOVA coupled with Dunnett multiple comparison *post hoc* analysis.

### 2.2.2. *In vivo* *Danio rerio* toxicity

Fish embryo toxicity test (FET) on zebrafish (*Danio rerio*) was made in agreement with OECD 236 guideline (see **figure S2 in supplementary data**). Briefly, zebrafish were obtained from Institut de Génétique Fonctionnelle de Lyon (IGFL) where they were maintained in closed flow-through system with a temperature of  $26^\circ\text{C} \pm 1^\circ\text{C}$  and a 16h light / 8h dark cycle. After fertilization, newly fertilized eggs were collected and selected under binocular microscope: embryos with normal development staging from the zygote stage to the gastrula stage were distributed on a 24-well plate (Thermo Fisher Scientific, USA), one embryo per well. One condition per plate was tested: 3,4-dichloroaniline (Sigma Aldrich, France) at 8  $\mu\text{g}/\text{mL}$  used as negative control, aquarium water (E3) used as positive control, and FE suspensions in water aquarium at 5%, 9.1% and 15% w/w. Every well was filled with 2 mL of solution, and embryos viability was assessed every 24h using microscope and toxicological endpoints as criterions (coagulated embryo, absence of heartbeat, non-detachment of the tail, absence of somitis formation) for 4 days. Alive fishes were analyzed to determine if they had any morphological abnormalities before observing their mobility with a Zebrafish box equipped with Zebrafish software (Viewpoint Behavior Technology, France) by 5 minutes' light cycle exposition before 3 minutes' dark cycle.

The values for the aquarium media (E3) and tested ones (i.e. 3,4-dichloroaniline and the three FE aqueous suspensions) were compared and statistically analyzed using a two-way ANOVA coupled with Dunnett multiple comparison *post hoc* analysis.

## 2.3. *Ex vivo* cell diffusion assays

VitroPharma® (patent n° WO2013057401) was used as cell diffusion system (see **figure S1 in supplementary data**). Dermatomed skin at 500 $\mu\text{m}$ , either from pig ear or human abdomen, with a diffusion-available area of 0.71  $\text{cm}^2$  was mounted in the system with the dermis facing the receptor compartment filled with 5 mL of PBS 1X as receptor media. Heated magnetic stirrer was used to heat receptor fluid to  $37^\circ\text{C} \pm 1^\circ\text{C}$ , which allowed the skin to be at  $32^\circ\text{C}$  (i.e. the physiological average temperature of human skin surface), and to homogenize it with a magnetic rod according to the OECD 428 guideline.

### 2.3.1. Skin explants

Frozen pig ears (Proviskin, France) obtained from a slaughterhouse were thawed, shaved and dermatomed (Aesculap GA 630 model) at 500  $\mu\text{m}$ . Individual disks of 2  $\text{cm}^2$  were cut off to fit into VitroPharma system.

Abdominal human skin (Proviskin, France) was obtained after approval from women who have had abdominal plastic surgery and stored as frozen 2cm<sup>2</sup> disks dermatomed at 500 µm. Before the experiment, each skin explant was controlled with a caliper in order to have a homogeneous thickness of 500 µm ± 50 µm, and skin integrity was visually assessed by microscopy.

### 2.3.2. Decontamination protocols

Contaminated skins were obtained after the deposit of 10 µL /cm<sup>2</sup> of Paraoxon-diethyl (Sigma Aldrich, France) (paraoxon or POX) as simulant of VX nerve agent. In accordance with the standard NF X52-122, decontamination was realized after 30 minutes to evaluate decontamination efficiency for delayed decontamination. For dry decontamination, 0.16 g/cm<sup>2</sup> of Fuller's earth (NBC-Sys, France) were applied. Wet decontamination occurs through the use of water (Otec®, Aguettant, France) or FE aqueous suspensions prepared at 5%, 9.1% and 15% w/w using Otec® water (Aguettant, France). In each case 102 µL/cm<sup>2</sup> of liquid were deposited. For both decontamination protocols, the decontaminant was left on the skin for 2 minutes before being removed and then the skin was dry by patting with a pad.

### 2.3.3. Collection and treatment of samples

To determine POX penetration kinetics, 500 µL of fluid receptor were collected over time (1, 2, 4, 6, 8, 10 and 24 hours of diffusion) and replaced by 500 µL of fresh media. After the set exposure period of 24 hours, VitroPharma® diffusion cells were dismantled and the surface of skin sample was wiped with an absorbent paper in order to recover the fraction of POX remaining on the skin. Skin layers were separated using the cyanoacrylate glue method (Salerno et al., 2017) for *stratum corneum* (SC), and immersion into PBS 1X heated at 60 °C for epidermis and dermis. For each sample, POX was extracted for 24 hours into HPLC grade ethanol before HPLC-UV quantification.

### 2.3.4. Quantification by HPLC-UV

Concentration of POX was determined by liquid chromatography using a quaternary pump (Agilent Technologies 1260 Infinity), vial auto sampler (Agilent Technologies 1260 Infinity II) and photodiode array detector (Agilent Technologies 1200 Series). Mobile phase was composed by 52.25% of methanol (HPLC grade, Carlo Erba, Italy); 42.75% of milliQ water (ultrapure analytical water with  $r=18$  mΩ/cm, Millipore, USA) and 5% of acetic acid glacial (HPLC grade, Carlo Erba, Italy), the whole at a flow rate of 0.7 mL/min. 10 µL of sample were brought to the C18 Cliepus column (5 µm, 100x40 mm) heated at 40°C. POX was identified at 269 nm UV wavelength with a retention time of 5.9 minutes (Salerno et al., 2017). The linearity of the method was assessed for POX concentration in the range 1.95-2000 ng/mL ( $R^2=0.999$ ) with a limit of detection (LOD) = 0.31 ng/mL and a limit of quantification (LOQ) = 1.95 ng/mL.

The value for the kinetic profiles and for the quantification into cell diffusion compartments and skin layers were compared between them and statistically analyzed using a one-way ANOVA coupled either with Dunnett or Tuckey multiple comparison *post hoc* analysis.

## 2.4. Ex vivo evaluation of a skin decontaminant

Skin decontamination methods need to be evaluated and compared to each other in order to determine the most effective one, based on skin penetration data. *Ex vivo* decontamination efficiency have been calculated as follows to compare skin decontamination methods:

$$DE (\%) = \frac{(Q3T + Q4T) - (Q3 + Q4)}{Q3T + Q4T} \times 100$$

Where Q3 = the amount of POX present into skin layers (SC + epidermis + dermis) at the end of the experiment; Q4 = the amount of POX in the receptor (meaning that it passed through skin) at the end of the experiment; T is for control samples



According to the standard NF X52-122, skin decontamination has an impact on functional prognosis because of its ability to capture the contaminant on the skin's surface. The improvement of the functional prognosis for each decontamination method have been calculated as follows:

$$FP = \frac{Q2T + Q3T + Q4T}{Q2 + Q3 + Q4}$$

Where Q2 = the amount of POX present on the skin after decontamination; Q3 = the amount of POX present into skin layers (SC + epidermis + dermis) at the end of the experiment; Q4 = the amount of POX in the receptor (meaning that it passed through skin) at the end of the experiment; T is for control samples

## 2.5. Statistical

## analysis

Data was statistically analyzed using GraphPad Prism Version 7.0 software (San Diego, CA, USA). All of the data are presented as the mean  $\pm$  SD, where n=4 for *in vitro* cell toxicity experiments, n=3 for *in vivo* *Danio rerio* toxicity experiments and n=6 for *ex vivo* skin decontamination assays. The statistical difference between groups was analyzed using either unpaired t-test, one-way ANOVA or two-way ANOVA tests followed by specific multiple comparison *post-hoc* tests as mentioned with each observed parameter individually.

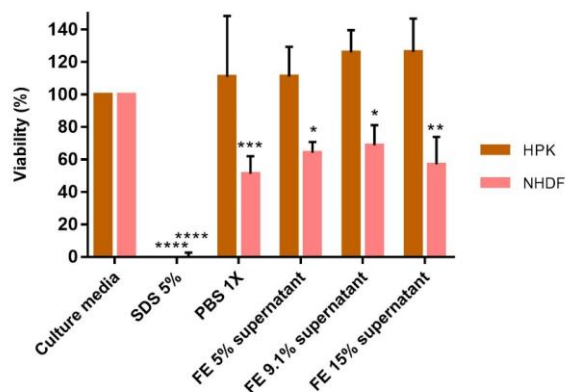
## 3. Results

### 3.1. Toxicity of Fuller's earth suspensions

Fuller's earth is a heterogeneous smectite composed of particles sizing from nanometers to millimeters, corresponding to heavy metals particles and elementary clay aggregates. In order to assess its toxicity, it is necessary to take into account the presence of these different populations and to identify appropriate toxicity tests. First, *in vitro* experiments were performed using 0.22 $\mu$ m filtered FE suspensions, on two different skin cells to identify ultra-filterable toxicity, such as heavy metal or nanoparticles. In parallel, toxicity of the whole suspensions was assessed *in vivo* on zebra fish embryos model to determine the potential side effects of clay aggregates.

#### 3.1.1. FE suspensions' supernatant cytotoxicity

Toxicity of particles with a size < 0.22 $\mu$ m was evaluated *in vitro* on two types of skin cells: Human Primary Keratinocytes (HPK) and Normal Human Dermal Fibroblasts (NHDF) after 30 minutes of contact (see materials and methods section 2.2.1.). As keratinocytes compose the top skin layer, which is epidermis, they are the first ones in contact with the product. Regardless of the concentration of the suspensions, no toxic effect on cell viability of FE supernatant was observed, in comparison with the two negative controls: cell media and PBS 1X (Figure1). NHDF, on the other hand, are founded in the dermis under the epidermis: they are in contact with an external element only in the case of a skin lesion. Interestingly, PBS 1x in which the suspensions were made also exhibits a significant toxic effect on NHDF, which indicates that the suspensions are not more toxic than PBS (Figure 1).



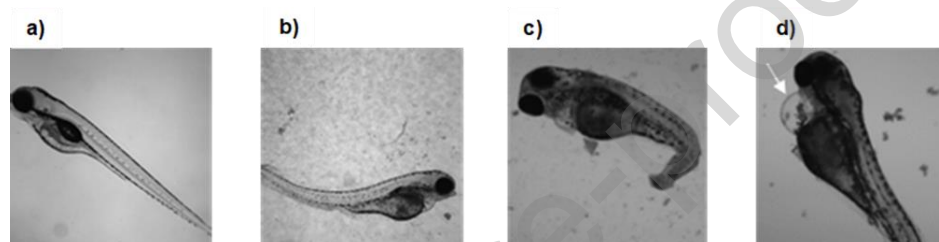
**Figure 1.** *In vitro* cell toxicity of FE supernatants filtered (size <0.22  $\mu$ m) from FE PBS 1X suspensions at different concentrations, on Human Primary Keratinocytes (HPK) and Normal Human Dermal Fibroblasts (NHDF) (n=4 for each). Results were statistically analyzed using two-

way ANOVA followed by Dunnett's multiple comparison tests using culture media as control treatment. Values are presented as mean  $\pm$  SD. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.005$ ; \*\*\*\*:  $p < 0.0001$

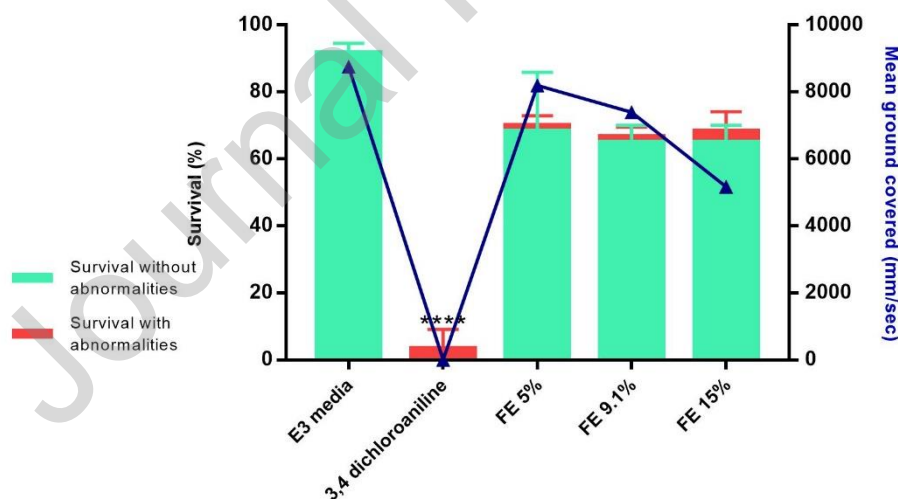
### 3.1.2. In vivo toxicity of FE suspensions

To address a potential toxicity due to the FE suspension concentration, *Danio rerio* fish embryos have been immersed for 4 days into different amount of FE suspensions, respectively 5, 9.1 and 15% w/w. Their viability was assessed as described in section 2.2.2 using optical microscopy. Observations have been done to differentiate embryos without and with morphological abnormalities such as development default, curved tail or cardiac edema (**Figure 2**). The presented figure is a picture of the viability after 4 days of immersion, meaning that fish embryos that have died on previous days are also counted.

No significant difference was observed on global survival and the occurrence of anomalies depending on the suspensions' concentration (**Figure 3**). When analyzing the mean ground covered for 3 minutes of light after 4 days of immersion, the 15% suspension impact fish mobility but not significantly. However, as 15% of FE implies a strong increase in water viscosity, we could not exclude that these observations are due to physical impairment of fish mobility.



**Figure 2:** Morphological observation of 4 days old *Danio rerio*. a) Normal development; b) Global development default; c) Curved tail; d) Cardiac edema.



**Figure 3:** Survival rate of *Danio rerio* with and without morphological abnormalities and mean ground covered after 4 days on immersion in aquarium media (E3), negative control (3.4-dichloroaniline) and three different concentrations of FE aqueous suspensions into E3 media ( $n=3$  for each). Results were statistically analyzed using two-way ANOVA followed by Dunnett's multiple comparison tests using E3 medium as control treatment. Values are presented as mean  $\pm$  SD. \*\*\*\*:  $p < 0.0001$

### 3.2. Ex vivo decontamination assays

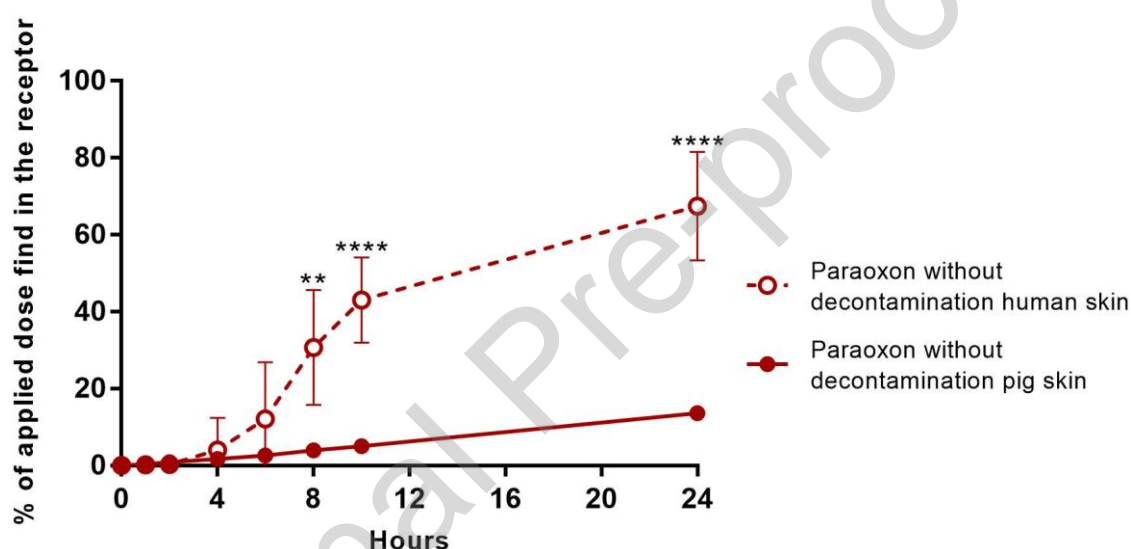
Most of the *ex vivo* skin diffusion experiments are done on pig skin (Godin & Touitou, 2007), which is also true for skin decontamination studies (Matar et al., 2014; Taysse et al., 2007). This model is widely used as human skin penetration model due to the respect of the physiopathology in relation to human skin (In et al., 2019). Nevertheless, it is still necessary to compare penetration profile for a given molecule on both skin origins as



they differ slightly, leading to misleading extrapolations on human decontamination efficiency (Jung & Maibach, 2015; Netzlaff et al., 2005).

### 3.2.1. Differences in paraoxon penetration kinetics through pig ear skin and human abdominal skin

*Ex vivo* POX penetration kinetics through pig ear skin is linear over time for 24 hours, reaching the value of  $13.59 \pm 0.78\%$  of the applied dose found in the receptor compartment (**Figure 4**). At the opposite, *ex vivo* skin diffusion on human abdominal skin follows a more complex kinetics. Indeed, the penetration seems to take place in several stages: it follows the same pattern as the one for pig ear skin during 4 hours before a strong increase in the amount of POX in the receptor. From 8 hours of POX diffusion, significant differences are observed between pig ear skin and human abdominal skin profiles. These observations are supported by the quantification of POX into each compartment that makes up the diffusion cell, i.e. the skin surface, the skin and the receptor, and each skin layers, i.e. *stratum corneum* (SC), epidermis and dermis (**Table 1**). Stronger significant differences between both skin origins are found in the receptor and in the skin surface, whereas pig ear epidermis contains significantly lower quantity of contaminant than the human abdominal epidermis.



**Figure 4:** *Ex vivo* POX penetration assessment through pig ear skin and human abdominal skin without decontamination: kinetic of diffusion of applied POX dose through skin for 24 hours (n=6 for each time). Results were statistically analyzed using unpaired t-test. Values are presented as mean  $\pm$  SD. \*\*: p<0.01, \*\*\*\*: p<0.0001

**Table 1:** Percentage of applied dose of POX found at the skin surface, trapped into skin layers and passed through the skin after 24 hours of diffusion without decontamination method into pig ear skin and human abdominal skin (n=6 for each). Results were statistically analyzed using unpaired t-test. Values are presented as mean. \*\*\*\*: p<0.0001

Skin model	Skin surface	Stratum corneum	Epidermis	Dermis	Receptor
Pig ear skin	69.84%****	5.55%	0.64%*	2.21%	13.59%****
Human abdominal skin	3.82%	3.13%	1.57%	3.99%	67.46%

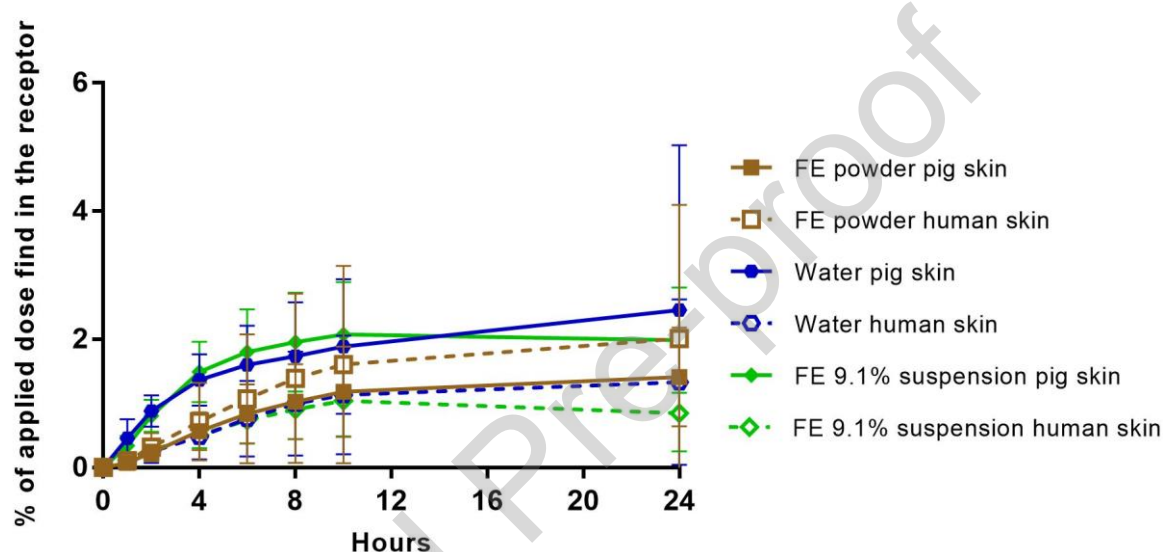
### 3.2.2. Efficacy of FE suspension compared to FE powder and water

After assessing the POX penetration profiles on both skin origins explants, decontamination efficiencies of FE powder, water and FE 9.1% suspension were evaluated into both pig and human skins. This suspension' concentration was chosen as it was the one developed by Roul et al., 2017, that has proven to have a better decontamination efficiency than FE powder or water alones for 4-cyanophenol skin decontamination.

The same decontamination protocol was applied for each decontamination method and the kinetics of POX penetration through skin were evaluated for 24 hours (**Figure 5**). The profiles of the contaminant penetration curves differ according to the type of decontaminant used but not according to the nature of the skin. After 8

to 10 hours of diffusion, a plateau is observable when the decontamination was done with the 9.1% suspension, whereas for FE powder and water decontamination there is only an inflection point in the curve. Despite these observations, there is not significant difference in the quantity of POX found in the receptor after 24 hours of diffusion, depending on the decontamination methods or skin origins.

At the end of the experiment, POX quantification in each skin layers highlighted differences between decontaminants and skin origins (**Table 2**). Indeed, after FE powder decontamination POX significantly accumulates in the SC regardless of the skin' nature. Furthermore, for this condition, when contaminant content in human abdominal skin is compared with the one in pig ear skin a significant difference showing an accumulation in favor of the first one is observed for skin surface, epidermis and dermis. Tested decontamination formulations are effective on both pig ear and human abdominal skins, with an increase efficiency between FE powder, water and FE 9.1% suspension.



**Figure 5:** *Ex vivo* POX penetration assessment through pig ear skin and human abdominal skin with 3 decontamination methods following the same protocol (FE powder, water and FE 9.1% aqueous suspension): kinetic of diffusion of applied POX dose through skin for 24 hours (n=6 for each time). Results were statistically analyzed using two-way ANOVA followed by Tukey's multiple comparison tests. Values are presented as mean  $\pm$  SD.

**Table 2:** Percentage of applied dose of POX found at the skin surface, trapped into skin layers and passed through the skin after 24 hours of diffusion with 3 different decontamination methods (FE powder, water, FE 9.1% suspension) into pig ear skin and human abdominal skin (n=6 for each). Results were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison tests between each skin origins and decontamination methods. Values are presented as mean. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*\*:  $p < 0.0001$

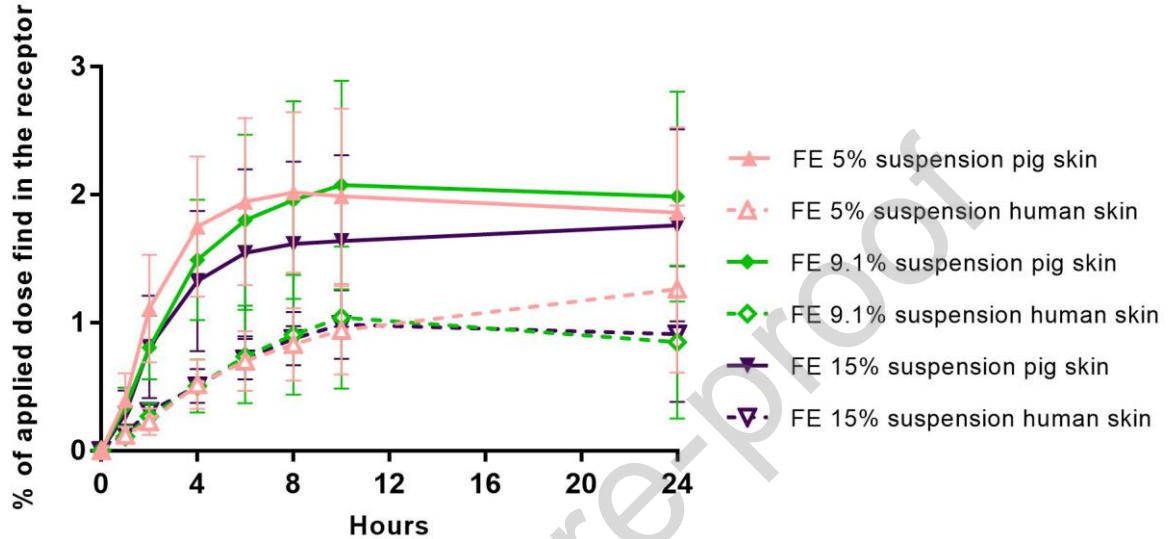
Decontamination method	Skin model	Skin surface	Stratum corneum	Epidermis	Dermis	Receptor
FE powder	Pig ear skin	0.89%	3.18%*	0.30%	0.36%	1.43%
	Human abdominal skin	5.28%***	3.98%**	1.71%**	3.48%**	2.01%
Water	Pig ear skin	0.78%	0.21%	0.05%	0.09%	2.45%
	Human abdominal skin	0.56%	0.59%	0.24%	0.46%	1.33%
FE 9.1% suspension	Pig ear skin	0.06%	0%	0.01%	0.02%	1.99%
	Human abdominal skin	0.48%	0.87%	0.29%	0.29%	0.85%

### 3.2.3. Relationship between FE suspensions' concentration and decontamination efficiency

The added value of the FE 9.1% suspension compared to FE powder in terms of POX accumulation in the SC has been illustrated by the **table 2**. Then, a dose effect of FE concentration has been further investigated using 5% and 15% suspensions.

For each concentration, a plateau effect in the kinetic profile is observed from 8 hours of POX diffusion onwards, as presented in **Figure 6**. No significant differences are observed in the amount of contaminant in the receptor depending on the FE concentration for each suspension and on the skin origin. The contaminant

quantification in skin layers after 24 hours of diffusion highlights some differences mostly depending on the skin nature (**Table 3**). Indeed, higher POX accumulation into skin surface, epidermis and dermis for the human skin decontamination with FE 5% suspensions is mainly due to an outlier in the dataset. For the SC compartment, there is a significant accumulation of the contaminant in the human abdominal skin in comparison with the pig ear skin. This can lead to a prolonged release even if the penetration kinetic of the POX shows a plateau from 8 hours in human skin, leading to same percentage of applied dose find in the receptor in the two skins for a given decontamination method.



**Figure 6:** *Ex vivo* POX penetration assessment through pig ear skin and human abdominal skin with FE aqueous suspensions as decontaminant at 3 different concentrations (5%, 9.1% and 15%): kinetic of diffusion through skin for 24 hours (n=6 for each time). Results were statistically analyzed using two-way ANOVA followed by Tukey's multiple comparison tests. Values are presented as mean  $\pm$  SD.

**Table 3:** Percentage of applied dose of POX found at the skin surface, trapped into skin layers and passed through the skin after 24 hours of diffusion with FE aqueous suspensions as decontaminant at 3 different concentrations (5%, 9.1%, 15%) into pig ear skin and human abdominal skin (n=6 for each). Results were statistically analyzed using one-way ANOVA followed by Tukey's multiple comparison tests: comparison were made between all the values for given compartment and significance is by comparison with the letter (i.e., for skin surface, values are only significantly different to the one for human skin abdominal skin decontaminated with FE 5% suspension). Values are presented as mean. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.005$ .

Decontamination method	Skin model	Skin surface	Stratum corneum	Epidermis	Dermis	Receptor
FE 5% suspension	Pig ear skin	0.06% <sup>*(a)</sup>	0.02% <sup>***(b)</sup>	0% <sup>*(e)</sup>	0.07% <sup>*(f)</sup>	1.86%
	Human abdominal skin	0.83% <sup>(a)</sup>	0.81% <sup>(b)</sup>	0.28%	0.40% <sup>(f)</sup>	1.26%
FE 9.1% suspension	Pig ear skin	0.06% <sup>*(a)</sup>	0% <sup>*** (c)</sup>	0.01% <sup>*(e)</sup>	0.02% <sup>*(f)</sup>	1.99%
	Human abdominal skin	0.48%	0.87% <sup>(c)</sup>	0.29% <sup>(e)</sup>	0.29%	0.85%
FE 15% suspension	Pig ear skin	0.12% <sup>*(a)</sup>	0.16% <sup>*(d)</sup>	0% <sup>*(e)</sup>	0.04% <sup>*(f)</sup>	1.76%
	Human abdominal skin	0.53%	0.78% <sup>(d)</sup>	0.18%	0.13%	0.91%

### 3.3. Comparison of skin origins and of decontamination methods

Decontamination methods were evaluated in comparison with POX skin contamination alone for both pig and human skins (**Table 4**). The quantity of contaminant absorbed by the skin, meaning find in the *stratum corneum*, the epidermis, the dermis and the receptor compartment, is significantly lower when decontamination methods are applied, no matter which type. Furthermore, for the same applied dose, the quantity of POX absorbed by human abdominal skin is significantly higher than the one for pig ear skin.

For both skin models, *in vitro* decontamination efficiency of FE powder is the lowest. Whereas there are not significant differences for the *in vitro* decontamination efficiencies depending on the decontamination method applied on pig ear skin, wet decontamination is significantly more effective than dry decontamination into

human abdominal skin. The use of FE suspensions into pig ear skin leads to significant lower decontamination efficiencies values than the ones on human abdominal skin with the same aqueous decontaminants. Thus, it appears that pig ear skin is a good predictive model for the assessment of the decontamination efficiency because there is no risk of overestimation when extrapolated to human skin.

The functional prognosis improvement factor defined as the ability of each decontaminant to capture the contaminant on the skin surface (see materials and methods section 2.4.) is not impacted by the type of decontamination method used for a given skin origin. Although FE powder decontamination presents the lowest value in both skins, there are not significant differences with wet decontamination. However, the use of FE 5% suspension induces an increase of the functional prognosis improvement factor significantly higher in the pig ear skin than in the human abdominal skin: these result can be explained by an exceptional value in the human skin dataset, increasing the global percentage of contaminant find on the skin surface. This factor can't be used to significantly classified decontamination methods according to their efficiency.

The more pronounced effect of the suspensions on pig ear skin is explained by histological differences with human skin, that will be discussed in the next session.

**Table 4:** Evaluation of skin decontamination methods following the quantity of POX absorbed by the skin (i.e. the percentage of applied dose find in the *stratum corneum*, the epidermis, the dermis and the receptor), the *in vitro* decontamination efficiency and the functional prognosis improvement depending on skin origin (n=6 for each). Results were statistically analyzed using unpaired t-test for the comparison of each values of pig ear skin and human abdominal skin; and one-way ANOVA followed by Tuckey's multiple comparison test was used to compare values between them for a given skin origin. Values are presented as mean. \*: p<0.05; \*\*: p<0.01; \*\*\*\*: p<0.0001.

Decontamination method	Pig ear skin			Human abdominal skin		
	Quantity of POX absorbed by the skin	<i>In vitro</i> decontamination efficiency	Functional prognosis improvement factor	Quantity of POX absorbed by the skin	<i>In vitro</i> decontamination efficiency	Functional prognosis improvement factor
No decontamination	21.77% <sup>(a)</sup> ; ****	-	-	76.15% <sup>(b)</sup>	-	-
Fuller's earth powder	5.27% **** <sup>(a)</sup>	75.81%	23.18	11.18% <sup>(c)</sup> ; **** <sup>(b)</sup>	85.32% <sup>(d)</sup>	14.53
Water	2.81% **** <sup>(a)</sup>	87.11%	48.71	2.62% **** <sup>(b)(c)</sup>	96.55% **** <sup>(d)</sup>	31.81
FE 5% suspension	1.95% **** <sup>(a)</sup>	91.04% **	53.19 *	2.75% **** <sup>(b)(c)</sup>	96.39% **** <sup>(d)</sup>	23.51
FE 9.1% suspension	2.01% **** <sup>(a)</sup>	90.75% **	53.10	2.30% **** <sup>(b)(c)</sup>	96.98% **** <sup>(d)</sup>	36.14
FE 15% suspension	1.96% **** <sup>(a)</sup>	91.01% **	71.5	2.00% **** <sup>(b)(c)</sup>	97.38% **** <sup>(d)</sup>	34.21

#### 4. Discussion

Toxicity of clays in general is not very well documented, and even more surprising when Fuller's earth (FE) is concerned, in particular its potential unsafe use regarding skin contact. First of all, when analyzing the toxicity of ultrafiltrable compounds released by water suspension of FE, our *in vitro* skin cells toxicity tests on filtered supernatant illustrated two information. Keratinocytes, which are cells from the epidermis, are not impacted in their viability by the application of neither PBS 1X or various concentrated supernatants of filtered FE suspensions. Although the measured toxicity of PBS 1X on NHDF can be attributed to their structural differences with HPKs (due to a stronger resilience and tolerance to external aggressions), it allows to determine that FE supernatants do not induce a higher cytotoxicity than PBS 1X. Han et al., 2011, previously demonstrated that clays suspensions at 1000 µg/mL are not toxic on human skin fibroblasts (CCD986sk) whereas Sandri et al., 2014, shaded this purpose by the observation of a tendency of decrease in cell viability of NHDF incubated with montmorillonite nanocomposites from 150 to 300 µg/mL. However, *in vitro* toxicity assessment is highly dependent on the clay mineral and its structure in addition of the nature of the cell line (Maisanaba et al., 2015). Based on suspensions' effect of raw FE, the results on keratinocytes are in accordance with the ones of Janer et al., 2014, that recorded low toxicity of FE supernatants. To our knowledge, it is the first study that characterizes FE's intrinsic toxicity using *Danio rerio* model. Most of the time this type of

experiment allows to determine adsorption properties of clays in order to diminish environmental water contamination (Gupta et al., 2016; Kansara et al., 2019). Here, the classical fish embryo toxicity test was coupled with a mobility test: no suspension has an effect of fish viability, while the more concentrated one has an impact on mobility. These results are in accordance with the ones of Abdel-Wahhab et al., 1999 and Dwyer et al., 1997, who gave ingested clays to rats and chickens in order to detoxify them after toxin ingestion. Neither models showed toxicity leading to the conclusion that clays are safe to use. Taken together, *in vitro* and *in vivo* toxicity tests suggest that FE suspensions are safe to use on undamaged and healthy skin in a concentration range from 5 to 9.1% w/w.

Furthermore, pig ear skin was studied as a predictive model for *ex vivo* human skin decontamination studies of paraoxon as a model for VX organophosphorus compound. Indeed, although human excised skin is considered as the gold standard for *in vitro* skin penetration experiments (Barbero & Frasch, 2009), its use is complicated due to ethical and supply difficulties. The presence of the papillary body and the large content in elastic tissue of its dermis in addition to the highly differentiated epidermis make pig skin a good substitute for human skin to assess *ex vivo* skin penetration. Just like for human skin, pig skin presents differences in terms of permeability as a function of the age, sex or anatomical region. In et al., 2019, compared histological and functional anatomical regions of porcine skin, including the ear, with human abdominal skin. Several significant differences have been identified, in particular for the SC, the dermo-epidermal interface and the dermis thickness that are larger in the human model. These variations in layer thickness leads to a significant difference in the epidermal-dermal thickness ratio; 1:19 for human abdominal skin versus 1:14 for pig ear skin. It means that even if both explants are dermatomed at 500  $\mu\text{m}$ , the pig one has a larger dermal hydrophilic component. Despite these differences, skin from the pig ear is the more appropriate anatomical site in order to predict human skin penetration (Boudry et al., 2008; Gerstel et al., 2016; Schmook et al., 2001). Penetration abilities of various compounds are impacted by the skin nature which is mainly greater for animal skin than for human skin, leading to a possible overestimation of the human percutaneous absorption (Kraeling et al., 2018; Vallet et al., 2007).

The receptor medium could as well influence POX penetration behavior between human and pig skins. However, various studies have shown that receptor medium do not modify the differences of contaminant penetration between pig and human skin. For instance, a study by Boudry et al., 2008 showed higher parathion penetration in pig skin compared to human skin (7.5% against 20.7%), while using Eagle medium supplemented with BSA and Gentamycin as receptor medium. Similarly, Chilcott et al., 2001, showed that the absorption of Sulfur mustard through pig ear skin was 2.6 times higher than that of human skin using 50% aqueous ethanol in the receptor medium. Finally, other studies showed that using another receptor medium, such as ethanol and water (1:1) (Dalton et al., 2018; Thors et al., 2016) or HBSS solution (Bignon et al., 2017), does not impact the penetration of compounds through the skin. The use of different membranes origins at the interface of the diffusion device can lead to differences in the evaluation in skin decontaminants (Chilcott et al., 2001). Despite this, pig ear skin can be used as a cheap predictive model for human skin POX diffusion and its decontamination studies, whereas reconstructed skin could not be because of its weak barrier function (Schmook et al., 2001).

The improvement of decontamination efficiency for FE aqueous suspensions in comparison to FE powder or water alone can be explained by the molecular structure of the particles. Clays, more particularly smectites, are compounds of negatively charged organized units called lamellas, spaced by cations and water molecules, that are then organized as particles and further as aggregates (Salles et al., 2010). Generally speaking, the adsorption model for this type of material is based on pore diffusion through and inside aggregates before sorption by intern particles (van Beinum et al., 2005). This sorption mechanism was validated for pesticides: diffusion occurs through migration inside aggregates and their pores leading to the adsorption on the solid phase inside the aggregate before reaching a local equilibrium between the concentration in the pore solution and the locally absorbed concentration (Villaverde et al., 2009). Although the inter lamellar space is filled with cations, it remains the privileged area for chemical sorption with aggregates' surface (Shah et al., 2018). The nature of exchangeable cations and their degree of isomorphic substitution as well as the clay chemical composition are going to be key factors to understand clays' wettability properties with water (Iannuccelli et al., 2016). Indeed, when clays are added in aqueous media, aggregates become oriented and ordered (Salles et al., 2010): lamellas are separated by water molecules layers and an increase in micropore size is observed as the result of the increase of basal space leading to a bigger BET surface area (the Brunauer, Emmet and Teller



method permit to measure the surface areas) (Bertella & Pergher, 2017). Hydration sequence is well described for smectites clay: firstly, an external hydration occurs on mesopore with single layers of water before filling of inter lamellar spaces and osmotic swelling.

With the use of FE suspensions, new hidden and non-accessible adsorption territories are released (van Beinum et al., 2005) which explains the decontamination efficiency improvement of water suspensions in comparison with dry FE. Nevertheless, this phenomenon is limited because intercalation of molecules into inter lamellar spaces is controlled by two parameters: attractive forces and cations exchange capacity (Moraes et al., 2017). This restriction explains the absence of a relationship between the concentration of suspensions and decontamination efficiency or functional prognosis improvement factor. All decontamination methods tested are significantly effective but depending on the decontaminant, different mechanisms are involved: for dry decontamination with FE as a powder, there is contaminant adsorption and fixation site saturation leading to free contaminant available to pass through skin layers. The excess contaminant will accumulate in the SC because of its lipophilic properties, and create a tank reservoir that allows a delayed release and a later contamination which is represented by a low Functional Prognosis, in accordance with the one found by Salerno et al., 2017 for FE powder applied on pig ear skin. To the opposite, wet decontamination with water alone allows only physical displacement and no adsorption with FE is involved. Thus, aqueous suspensions of FE used as innovative wet decontamination potentiates FE powder decontamination ability with the emergence of new adsorption sites as a result of inter lamellar swelling induced by water molecules. As it was already detailed for pesticide sorption by wet soils, contaminant will migrate by a diffusion phenomenon into aggregates' internal pores filled with water (Villaverde et al., 2009).

## 5. Conclusion

Fuller's earth (FE) is a smectite clay well known for its adsorbent properties used for household or skin decontamination. This compound is very heterogeneous in terms of nature and particle size as it is mostly composed of elementary clay aggregates and few amount of minerals such as heavy metals. Our results clearly indicate that aqueous suspension of FE, regardless of its concentration, does not promote the release of toxic compounds from the clay. We have identified a minimal dose of 5% FE suspension in water as an optimal concentration for *ex vivo* dermal decontamination of paraoxon when loaded on porcine or human skin explants. Indeed, water suspension increases the decontaminant properties of FE, as noticed by the disappearance of a *stratum corneum* reservoir of paraoxon. All together these results are in favor of the use of water suspension of FE as a new tool to organophosphate decontamination.

## CRedit authorship contribution statement

**Alix Danoy:** Conceptualization; Methodology; Formal analysis; Investigation; Writing – Original Draft; Writing – Review & Editing. **Kardelen Durmaz:** Investigation. **Margaux Paoletti:** Investigation. **Laetitia Vachez:** Methodology; Investigation. **Annick Roul:** Conceptualization. **Jérôme Sohier:** Review & Editing, **Bernard Verrier:** Conceptualization; Resources; Writing – Review & Editing; Supervision; Funding acquisition.

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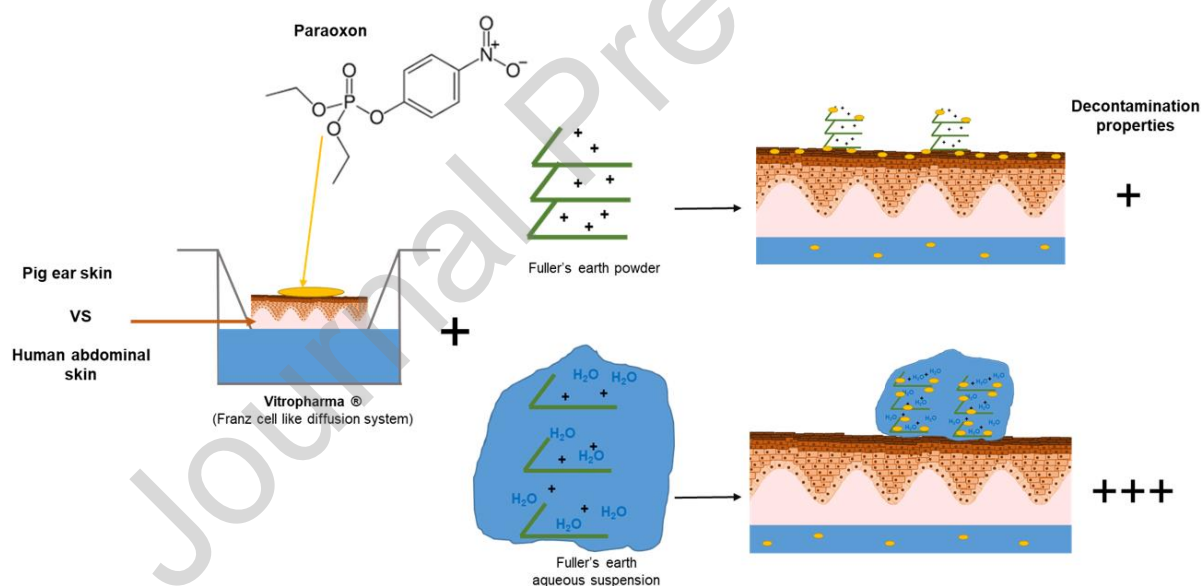
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#### Graphical abstract



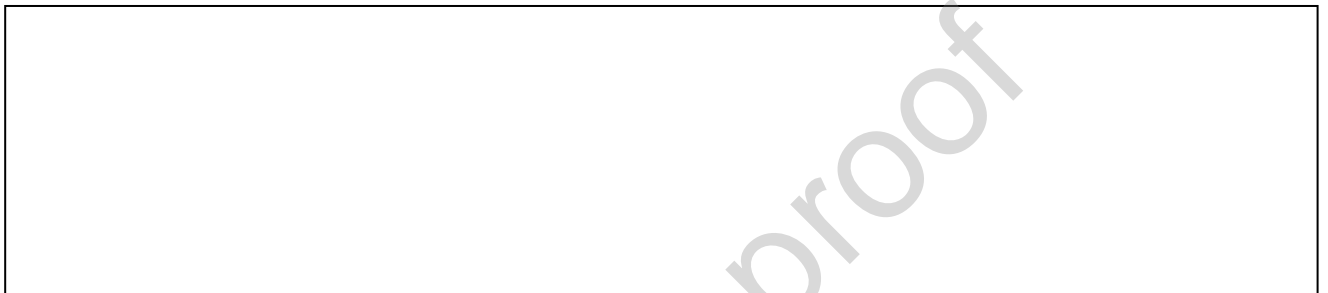
#### CRedit authorship contribution statement

**Alix Danoy:** Conceptualization; Methodology; Formal analysis; Investigation; Writing – Original Draft; Writing – Review & Editing. **Kardelen Durmaz:** Investigation. **Margaux Paoletti:** Investigation. **Laetitia Vachez:** Methodology; Investigation. **Annick Rouil:** Conceptualization. **Jérôme Sohier:** Review & Editing. **Bernard Verrier:** Conceptualization; Resources; Writing – Review & Editing; Supervision; Funding acquisition.

**Declaration of interests**

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

**Highlights:**

- Fuller's earth is non-toxic *in vitro* and *in vivo*
- Increase of the decontamination efficiency not related to the clay concentration
- Water drags the contaminant deeper into the pores of the Fuller's earth
- Pig ear skin as cheap predictive model for human skin contamination with paraoxon