Bioanalytical evidence that chemicals in tattoo ink can induce adaptive stress responses

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Abstract

Tattooing is becoming increasingly popular, particularly amongst young people. However, tattoo inks contain a complex mixture of chemical impurities that may pose a long-term risk for human health. As a first step towards the risk assessment of these complex mixtures we propose to assess the toxicological hazard potential of tattoo ink chemicals with cell-based bioassays. Targeted modes of toxic action and cellular endpoints included cytotoxicity, genotoxicity and adaptive stress response pathways. The studied tattoo inks, which were extracted with hexane as a proxy for the bioavailable fraction, caused effects in all bioassays, with the red and yellow tattoo inks having the greatest response, particularly inducing genotoxicity and oxidative stress response endpoints. Chemical analysis revealed the presence of polycyclic aromatic hydrocarbons in the tested black tattoo ink at concentrations twice the recommended level. The detected polycyclic aromatic hydrocarbons only explained 0.06% of the oxidative stress response of the black tattoo ink, thus the majority of the effect was caused by unidentified components. The study indicates that currently available tattoo inks contain components that induce adaptive stress response pathways, but to evaluate the risk to human health further work is required to understand the toxicokinetics of tattoo ink chemicals in the body.

Keywords: tattoo ink; bioanalytical tools; polycyclic aromatic hydrocarbon; genotoxicity; oxidative stress; cytotoxicity

1. Introduction

Tattooing is becoming increasingly popular in many countries. For example, the number of adult Australians with one or more tattoos increased from 10.1% to 14.5% over a seven-year period [1, 2]. Further, young people tend to have a higher incidence of tattooing, with one recent study of US university students finding that 29.6% of participants were tattooed [3]. In addition to concerns regarding hygiene and disease transmission, there is growing interest in the chemical composition of tattoo ink. Tattoo inks are typically composed of negligibly soluble or insoluble pigments, dispersants in which the pigments are suspended and other additives for preservation or to alter the viscosity of the ink [4]. While coloured tattoo inks traditionally contained metals, modern coloured inks contain organic pigments, such as azo dyes for red and yellows inks or phthalocyanines for blue and green inks [5]. Tattoo inks are typically manufactured in industrial processes, for applications including paints and printing, and can contain up to 10% impurities [6]. Black ink often contains carbon black, which is formed from the incomplete combustion of hydrocarbons, and unsurprisingly, recent studies have found polycyclic aromatic hydrocarbons (PAHs) in black tattoo ink [7, 8]. Within the literature a number of adverse health effects from tattoos ranging from skin irritation to tumour formation have been reported [9], but the association between tattooing and cancer remains coincidental to date [10].

Given the complex mixture of chemicals that is likely to be present in tattoo ink, targeted chemical analysis alone is not sufficient to assess the potential health hazards associated with tattoo ink chemicals. *In vitro* bioanalytical tools can be applied complementary to chemical analysis. Bioanalytical tools or bioassays have the advantage that they can detect the mixture effects of all chemicals that act by the same mode of action and they are risk-scaled, so a more potent chemical will have a greater contribution to the effect than the same concentration of a less potent chemical [11]. These tools have been applied extensively for water quality assessment in recent years [e.g. 12], but they can also be applied to other matrices of interest, such as extracts from animal tissue samples [e.g. 13] or sediments [e.g. 14]. They can also be used for the assessment of chemical

products including tattoo ink. For example, Falconi et al. [15] found a significant reduction in cell viability of human fibroblast cells after exposure to red tattoo ink, but not black ink, while Regensburger et al. [8] assessed mitochondrial activity of human dermal keratinocytes in the presence of black tattoo ink extracts and UV light and found reduced activity for some black inks, but not others.

Knowledge of the mode of toxic action of tattoo ink chemicals is essential for understanding the potential impacts on human health. Modes of toxic action include non-specific (e.g. chemical partitioning to cell membrane), specific (e.g. chemical binding to an enzyme) and reactive (e.g. chemical reaction with biological molecules) and these can be targeted by applying different bioassays [11]. Also of relevance for human health are assays that focus on adaptive stress response pathways. These pathways, which are well conserved in all metazoan cells, are activated to restore the cell to homeostasis after damage to the cell structure [16].

The aim of this study was to evaluate the toxicity potential of chemicals present in commercially available tattoo inks with cell-based bioassays indicative of cytotoxicity and adaptive stress response pathways. The studied assays included two measures of cytotoxicity, the bioluminescence inhibition test with bacteria *Vibrio fischeri* and the resazurin cell viability test with human colon carcinoma cells (HCT-116). While not directly relevant to human health, the bioluminescence inhibition test was selected as it is highly sensitive to organic compounds [17] and typically more sensitive than cytotoxicity assays with mammalian cells. In addition the cellular response to genotoxicity, p53 response and oxidative stress response were assessed. The bacterial umuC assay quantifies the SOS response after DNA damage [18] and the mammalian p53 assay is a measure of adaptive stress response regulation that responds to DNA damage, either inducing DNA repair and/or apoptosis [19]. The activation of p53 in mammalian cells has also been used as an indicator for genotoxic carcinogens [20]. The oxidative stress response, which is induced by reactive oxygen species or electrophilic chemicals [21] and mediated by Nrf2 and the antioxidant response element, was quantified with the AREc32 assay [22]. Such a comprehensive bioanalytical

assessment of tattoo inks has not been attempted previously. The SOS response and p53 protein induction are relevant for the tattoo ink cancer concerns as DNA damage can result in mutations, which can lead to cancer [11]. Further, Hutton Carlsen and Serup [23] hypothesised that skin complaints associated with tattooing may be related to the formation of reactive oxygen species (ROS), which is captured with the oxidative stress response endpoint [16]. Various coloured tattoo inks were tested in all bioassays. The bioanalytical assessment of black tattoo ink was complemented by chemical analysis to assess the contribution of known chemicals to the biological effect.

2. Materials and Methods

2.1. Chemicals

All chemicals were of analytical grade. Naphthalene, acenaphthylene, fluoranthene, phenanthrene and pyrene were purchased from Sigma Aldrich (Castle Hill, Australia). The liquid tattoo inks were purchased online from eBay and included Dragonhawk (tribal black, crimson red, country blue, true green, golden yellow, lavender, brite white) and Intenze (banana yellow) tattoo inks.

2.2. Sample treatment

1 g of liquid ink was extracted in 3 mL of hexane by sonicating for 60 minutes, then centrifuging for 10 minutes at $2500 \times g$. The supernatant was removed and the extraction process was repeated another two times. The combined solvent extract was blown down to 0.5 mL, enriching the sample 18 times and giving a final concentration of $2000 \text{ g}_{\text{ink}}/\text{L}_{\text{extract}}$.

2.3. Chemical analysis

The black ink hexane extract was blown to dryness and resuspended in 0.5 mL ethyl acetate. The extract was analysed for PAHs using GC-MS by a National Association of Testing Authorities (NATA) accredited analytical laboratory (Queensland Health Forensic and Scientific Service

(QHFSS), Coopers Plains, QLD, Australia). The analysed PAHs included naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo(b+k)fluoranthene, perylene, benzo(a)pyrene, benzo(e)pyrene, indeno(1,2,3-cd)pyrene, dibenz(a,h)anthracene, benzo(ghi)perylene, biphenyl, 2-ethyl naphthalene, 2,6-dimethyl naphthalene, 1,7-dimethyl naphthalene, 2,2-dimethyl biphenyl, 1,4-dimethyl naphthalene, 2-methoxy naphthalene, 1,2-dimethyl naphthalene, 1,8-dimethyl naphthalene, 3,3-dimethyl biphenyl, 4,4-dimethyl biphenyl, 1-methyl fluorene, 2-methyl anthracene and 9-methyl anthracene. Details of the analysis method can be found in Bi et al. [24]. The concentration was expressed as micrograms per gram of ink ($\mu\text{g/g}_{\text{ink}}$).

2.4. *Bioanalytical tools*

Prior to bioanalysis the hexane extracts were blown to dryness and resolubilized in the appropriate assay medium to prevent solvent effects in the assay, while the PAH stocks were prepared in methanol with low volumes added to the assay (not exceeding 1.04% methanol in final assay volume for the AREc32 and p53 assays and not exceeding 2.8% methanol in final assay volume for the bioluminescence inhibition assay). All methanol extracts were blown to dryness in the umuC assay due to the larger volume requirements. Solvent controls were included in all assays to ensure no effect from the methanol.

The bacterial bioluminescence inhibition assay is based on ISO11348-3 [25], but was performed in a 96-well format [26]. Lab-grown *V. fischeri* were prepared according to Tang et al. [17]. Briefly, the blown down extracts were resuspended in test buffer and serially diluted, with 100 μL added to 50 μL of bacteria. Inhibition of bioluminescence was measured using a Fluostar Omega (BMG Labtech, Ortenberg, Germany) and used to determine the concentration causing 10% effect (EC_{10}) in units of $\text{g}_{\text{ink}}/\text{L}$. Phenol was used as the positive control in all experiments. EC_{10} was derived from a log-logistic concentration-effect curve.

The mammalian AREc32 assay, which assesses induction of the Nrf2 pathway using a luciferase report gene assay [22], was performed according to Escher et al. [27]. The blown down tattoo ink extracts were resuspended in DMEM medium, which contained 10% fetal bovine serum (FBS), serially diluted and 100 μ L was added to cells with a density of 1.2×10^5 cells/mL in white, clear bottom 96 well plate. After incubating for 24 h at 37°C, luciferase production was measured using a Fluostar Omega to determine induction. The results were presented as effect concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$) in units of g_{ink}/L . *tert*-Butylhydroquinone served as the positive control. To prevent interference from cytotoxicity, only the linear portion of the concentration-effect curve was evaluated, with induction ratios above 5 excluded from the linear regression [28]. Previous studies have shown that cytotoxicity in the AREc32 often occurs when the induction ratios level off or drop [27], so that a parallel cytotoxicity test is not necessary. The full concentration-effect curves are shown in Figure S1 in the Supplementary Material.

The CellSensor™ p53RE-*bla* HCT-116 assay (Life Technology, Mulgrave, Australia) was used to assess the activation of p53 in human colon HCT-116 cells. The blown down tattoo ink extracts were resuspended in Opti-MEM reduced serum medium, which contained 0.5% dialysed FBS, and serially diluted. 8 μ L of serially diluted extract was added to 32 μ L of cells with a density of 9.4×10^5 cells/mL in black, clear bottom 384 well plates. The plates were incubated for 48 h at 37°C, then 8 μ L of LiveBLAzer™ substrate mix was added with a final resazurin concentration of 50 μ M and incubated for a further 2.5 h in darkness at room temperature. The addition of resazurin made it possible to simultaneously measure cell viability via cellular mitochondrial activity. Induction of p53 was assessed by measuring fluorescence using a Fluostar Omega, with results presented as $EC_{IR1.5}$ values, while cytotoxicity results were presented as EC_{10} values. Benzo(a)pyrene was used as the positive control. Further details on the method can be found in Yeh et al. [29].

The bacterial umuC assay, which is indicative of the SOS response pathway, was run according to the protocol described in Macova et al. [30]. To account for compounds that may only

be genotoxic after metabolic activation, the assay was performed with and without 3% Sprague Dawley rat liver supernatant fraction (S9) (Molecular Toxicology Inc., Boone, US). Briefly, the blown down extracts were resuspended in TGA medium, serially diluted and the bacterial culture was added. After incubating for 2 h, 30 μ L was added to fresh TGA medium and incubated for a further 2 h. Bacterial density and β -galactosidase activity was measured using the Fluostar Optima (BMG Labtech, Ortenberg, Germany) to determine $EC_{IR1.5}$. 2-aminoanthracene and 4-nitroquinoline-oxide were used as positive controls with and without S9, respectively.

The tattoo ink extracts were also tested in the AhR-CAFLUX assay [31], which can detect compounds that activate the aryl hydrocarbon receptor; however, the extracts were cytotoxic in the assay at high concentrations and did not induce the aryl hydrocarbon receptor at lower concentrations.

2.5. Data analysis

The bioassay responses of the tattoo ink extracts were expressed as bioanalytical equivalent concentrations (BEQ_{bio}). BEQ_{bio} was calculated from the EC_{10} or $EC_{IR1.5}$ of the respective reference compound and the EC_{10} or $EC_{IR1.5}$ of the tattoo ink extract (Equation 1). Further details about the BEQ (formerly called TEQ) concept can be found in Escher et al. [26].

$$BEQ_{bio} = \frac{EC_{10} \text{ or } EC_{IR1.5} (\text{reference compound})}{EC_{10} \text{ or } EC_{IR1.5} (\text{tattoo ink extract})} \quad (1)$$

Fluoranthene served as the reference compound in the present study, thus the BEQs were expressed as fluoranthene-EQ (Equation 2). The fluoranthene EC values were different for the different assays and are provided in the Section 3. While it was necessary to use mole units for the calculation, the fluoranthene-EQ values were all presented in mass units.

$$\begin{aligned}
\text{Fluoranthene-}EQ_{bio} \left(\frac{\mu g_{fluoranthene}}{g_{ink}} \right) &= \frac{EC_{fluoranthene} \left(\frac{mol_{fluoranthene}}{L} \right)}{EC_{ink} \left(\frac{g_{ink}}{L_{extract}} \right)} \\
&\cdot MW_{fluoranthene} \left(\frac{\mu g_{fluoranthene}}{mol_{fluoranthene}} \right)
\end{aligned}
\tag{2}$$

Bioanalytical equivalent concentrations can also be derived from the chemical analysis if the relative effect potency (REP_i) of each detected chemical i is known. The REP_i of the detected PAHs (i) in black tattoo ink were calculated using Equation 3, as the ratio of the EC_{10} or $EC_{IR1.5}$ of the reference compound fluoranthene to the EC_{10} or $EC_{IR1.5}$ of the detected PAH.

$$REP_i = \frac{EC_{10} \text{ or } EC_{IR1.5} (\text{reference compound})}{EC_{10} \text{ or } EC_{IR1.5} (i)}
\tag{3}$$

The bioanalytical equivalent concentration from chemical analysis (BEQ_{chem}) was determined from the REP_i and the measured concentrations C_i (in mol/g_{ink}) of the detected PAHs (Equation 4).

$$BEQ_{chem} = \sum_{i=1}^n REP_i \cdot C_i
\tag{4}$$

Specifically, for this study fluoranthene served as the reference compound and Equation 4 can be rewritten as Equation 5.

$$\begin{aligned}
\text{Fluoranthene-EQ}_{chem} \left(\frac{\mu g_{fluoranthene}}{g_{ink}} \right) &= MW_{fluoranthene} \left(\frac{\mu g_{fluoranthene}}{mol_{fluoranthene}} \right) \\
&\cdot \sum_{i=1}^n REP_i \left(\frac{mol_{fluoranthene}}{mol_i} \right) \cdot C_i \left(\frac{mol_i}{g_{ink}} \right)
\end{aligned}
\tag{5}$$

A comparison between fluoranthene-EQ_{bio} and fluoranthene-EQ_{chem} will allow an estimation of the contribution of the detected PAHs to the overall observed effects.

To express the bioanalytical results in a form that they can be compared with other PAH exposure routes, a tattoo bioanalytical equivalent mass (fluoranthene-EQ_{tattoo} in units of $\mu g_{fluoranthene}$ per average tattoo) was established (Equation 6). Fluoranthene-EQ_{tattoo} was calculated from the fluoranthene-EQ_{bio} values for the different tattoo inks (in units of mol/g_{ink}), the average mass of ink injected per cm^2 of skin (C_{ink} , in units of g_{ink}/cm^2) and the average tattooed skin area (A_{skin} , in units of cm^2 per average tattoo).

$$\begin{aligned}
\text{Fluoranthene-EQ}_{tattoo} (\mu g_{fluoranthene}) &= \text{fluoranthene-EQ}_{bio} \left(\frac{mol_{fluoranthene}}{g_{ink}} \right) \\
&\cdot MW_{fluoranthene} \left(\frac{\mu g_{fluoranthene}}{mol_{fluoranthene}} \right) \cdot C_{ink} \left(\frac{g_{ink}}{cm^2} \right) \cdot A_{skin} (cm^2)
\end{aligned}
\tag{6}$$

To compare fluoranthene-EQ_{tattoo} with other sources of fluoranthene exposure, the annual exposure of fluoranthene from food (Exposure, in units of $\mu g_{fluoranthene}/year$) was estimated using the average fluoranthene concentration in different food groups (C_{food} , in units of $\mu g_{fluoranthene}/kg_{food}$) and median annual consumption of different food groups per person (Consumption, in units of $kg_{food}/year$) (Equation 7).

$$Exposure\left(\frac{\mu g_{fluorant\ hene}}{year}\right)=C_{food}\left(\frac{\mu g_{fluorant\ hene}}{kg_{food}}\right)\cdot Consumption\left(\frac{kg_{food}}{year}\right)$$

(7)

3. Results and Discussion

3.1. Soluble fraction of tattoo ink

The main components of tattoo inks are water-insoluble dyes, as well as inorganic particles [5]. They are considered not bioavailable and the particles may disturb the cell-based bioassays. Therefore, the tattoo inks were not dosed directly but mild solvent extraction with hexane was applied to extract the readily desorbable compounds to mimic the fraction of chemicals in the tattoo ink that would be bioavailable in the body [32]. The tattoo inks were purchased pre-dispersed in an unidentified solution and the mass of extracted chemicals was unknown and likely varied for the different inks, hence all bioanalytical and chemical analysis results were normalised to the initial weight of the extracted ink (g_{ink}/L). Hexane was selected over other solvents as it has been previously shown to be suitable for extracting PAHs, which are known to be present in black tattoo ink, from soil samples [33]. While mild solvent extraction is relatively simple and fast, it does have some limitations, as chemical extraction can be influenced by the selected solvent. An alternative approach would be to apply passive sampling techniques to determine the bioavailable fraction of a wide range of chemicals in tattoo ink. While this has not been previously applied to tattoo ink, passive sampling techniques using polymer disks have been applied to extract bioavailable chemicals from sediment and biological material [13, 14].

3.2. Bioanalytical assessment of tattoo ink

The concentration-effect curves for all studied assays are depicted in Figure 1 and the thereof derived effect concentrations are summarised in Table 1. The EC values for the different inks were derived from either log-logistic (bacterial cytotoxicity) or linear (mammalian p53 cytotoxicity,

oxidative stress response, p53 response and SOS response for genotoxicity) concentration-effect curves and are compared in Figure 2A. The lower the EC value the higher the effect, so in Figure 2A the scale has been reversed so the extracts with the highest effect are at the top of the graph.

Figure 2A indicates that the mammalian assays, particularly the AREc32 assay, were generally more sensitive than the bacterial assays for all tattoo ink extracts. The AREc32 assay is indicative of the oxidative stress response pathway, and adaptive stress response pathways tends to activate at much lower chemical concentrations than concentrations that cause actual damage, such as cytotoxicity [16]. However, the tattoo ink extracts caused cytotoxicity in the p53 adaptive stress response assay at slightly lower concentrations than induction of the p53 response (Table 1). Yeh et al. [29] also found that cytotoxicity often masked induction of the p53 response for haloacetic acids, thus this is likely to be a feature of this assay. As cytotoxicity and induction occurred within the same order of magnitude in the present study, the p53 induction data were still used to calculate fluoranthene-EQ values.

Red and yellow inks generally had the greatest effect in the assays. In contrast, the white tattoo ink was below the limit of detection in the studied assays, with the exception of the oxidative stress response where it had the lowest effect. The difference in effect can be explained by the likely components of the inks. White ink predominately contains inorganic titanium dioxide, which is known to have low toxicity to human cells [34, 35]. Further, the applied extraction method in this study targets organic contaminants, thus titanium dioxide should not be present in the final extract. Red and yellow inks are expected to contain azo pigments, and previous studies have shown that certain azo pigments can release hazardous compounds after exposure to natural and UV light [36, 37]. Further, probable human carcinogen 3,3-dichlorobenzidine is used as an intermediate in the manufacture of some azo dyes and has been identified in tattoo inks in Europe [4]. The presence of such impurities in the studied red and yellow inks could explain the increased effect.

Only the red and yellow (Intenze) inks had an effect in the umuC assay, with the rest of the inks not exceeding the effect threshold in both repeats. The red ink was around 8 times more toxic

after metabolic activation. The identity of metabolically active chemicals in the red ink in the current study is unknown; however, chemical analysis of other red inks have shown that they can contain aromatic amines, including o-anisidine and 4-aminobiphenyl [4], and many aromatic amines exhibit genotoxicity after metabolic activation [38]. Red ink is one of the more popular tattoo ink colours [39] and a recent review of adverse health effects associated with tattoos found examples of skin irritation and tumours associated with red parts of tattoos [9]. Bioanalytical assessment indicated that the impurities in one brand of red tattoo ink can induce the oxidative stress response, p53 response and SOS response for genotoxicity but the causal link between the chemical identity of constituents and biological effects requires further research.

3.3. Chemical analysis of black tattoo ink

Six PAHs were detected in the black tattoo ink including the possible human carcinogen naphthalene (International Agency for Research on Cancer Class 2B) (Table 2). Pyrene and fluoranthene were found at the highest concentrations in ink, with the total PAH concentration, 1.1 $\mu\text{g/g}_{\text{ink}}$, over twice as high as the maximum allowable PAH concentration in tattoo ink (0.5 ppm or $\mu\text{g/g}_{\text{ink}}$) advised by the Council of Europe [40]. The PAH content of black ink can vary significantly with different brands, with Regensburger et al. [8] finding PAH concentrations ranging from 0.14 to 201 $\mu\text{g/g}_{\text{ink}}$ in commercially available black inks.

The detected PAHs, with the exception of benzo[ghi]perylene, were characterized in all bioassays. All studied PAHs caused bacterial cytotoxicity and induced the oxidative stress response, while only fluoranthene induced the p53 response (Figure 3A). Naphthalene and acenaphthylene induced cytotoxicity in p53 assay, but only at the highest concentration. None of the PAHs had an effect in the umuC assay, even after metabolic activation. This was also observed by Nakamura et al. [41] who found that naphthalene, phenanthrene, fluoranthene and pyrene did not induce genotoxicity in the umuC assay. Consistently, the black ink did not induce the SOS response. The PAH EC values are provided in Table 2.

Fluoranthene-EQ_{chem} values for bacterial cytotoxicity, oxidative stress response and p53 response were calculated for the black ink using Equation 5 (Figure 3B). Fluoranthene was selected as the reference compound in this study as it was detected at high concentrations in the black ink and had an effect in three of the studied assays. As fluoranthene did not have a response in the umuC assay it was not possible to determine the REP_i, meaning that fluoranthene-EQ_{chem} could not be calculated for this endpoint. The predicted effect based on chemical analysis varied for the different endpoints, with the highest fluoranthene-EQ_{chem} value for bacterial cytotoxicity and the lowest fluoranthene-EQ_{chem} value for p53 response (Figure 3B).

While the PAHs were dosed at the solubility limit for the bacterial bioluminescence inhibition assay and the umuC test, the EC_{IR1.5} values in the mammalian assays were above the solubility of the PAHs. A mass balance calculation of the fraction of PAHs sorbed to cells and FBS indicated that the majority of PAHs would be bound to these components [42], leaving the freely dissolved or bioavailable PAH concentration below the solubility limit. However, the bioavailable concentration was not used to determine the PAH EC_{IR1.5} values in the present study in order to allow comparison with the tattoo ink EC_{IR1.5} values, which could only be evaluated using the nominal concentration due to the unknown mixture of chemicals in the extract.

3.4. Fluoranthene equivalent concentration (fluoranthene-EQ_{bio})

For a better comparison between the bioassay data the tattoo ink EC values were converted to fluoranthene-EQ_{bio} values using the EC values of reference compound fluoranthene (Table 2). An advantage of the BEQ concept is that it can facilitate a quantitative comparison between the different tattoo inks. It must be noted that the reference compound fluoranthene only serves as a proxy to explore effects and its use as a reference compound does not imply that it is contributing substantially to the mixture effect. While the EC values give an indication of the sensitivity of the assays, the fluoranthene-EQ_{bio} values translates the effect into the mass of fluoranthene that would elicit the same effect as one gram of tattoo ink. However, fluoranthene-EQ_{bio} does not mean that

there would be fluoranthene in the inks. The fluoranthene-EQ_{bio} values can only be used for comparison. The fluoranthene-EQ_{bio} values for bacterial cytotoxicity, oxidative stress response and p53 response are compared in Figure 2B for the different inks (also shown in Table 1). The fluoranthene-EQ_{bio} values for the oxidative stress response, which were up to 1000 times higher than the corresponding values for bacterial cytotoxicity, ranged from 195 $\mu\text{g}_{\text{fluoranthene}}/\text{g}_{\text{ink}}$ for white ink to 13704 $\mu\text{g}_{\text{fluoranthene}}/\text{g}_{\text{ink}}$ for yellow ink (Intenze brand). As fluoranthene-EQ_{bio} values have not been derived previously for tattoo ink, it is not possible to compare with existing literature values. It was also not possible to determine fluoranthene-EQ_{bio} for the mammalian cytotoxicity or SOS response for genotoxicity as fluoranthene did not elicit an effect in these assays.

3.5. Can the detected chemicals explain the effect of black tattoo ink?

By comparing the fluoranthene-EQ_{bio} and fluoranthene-EQ_{chem} of the black tattoo ink extract it is possible to determine how much of the effect in the different assays can be explained by the detected and quantified chemicals. Typically, target chemical analysis can explain less than 1% of effect in water samples for non-specific and adaptive stress response endpoints, such as bioluminescence inhibition and oxidative stress response, respectively [17, 28]. Similar to the water samples, the five PAHs could only explain 0.06% of the oxidative stress response (fluoranthene-EQ_{bio} 929 $\mu\text{g}_{\text{fluoranthene}}/\text{g}_{\text{ink}}$ compared to fluoranthene-EQ_{chem} $0.59 \pm 0.03 \mu\text{g}_{\text{fluoranthene}}/\text{g}_{\text{ink}}$) and 0.04% of the p53 response (fluoranthene-EQ_{bio} 465 $\mu\text{g}_{\text{fluoranthene}}/\text{g}_{\text{ink}}$ compared to fluoranthene-EQ_{chem} $0.17 \pm 0.04 \mu\text{g}_{\text{fluoranthene}}/\text{g}_{\text{ink}}$). However, 14.6% of non-specific toxicity could be explained by the PAHs in the bioluminescence inhibition assay (fluoranthene-EQ_{bio} 11.1 $\mu\text{g}_{\text{fluoranthene}}/\text{g}_{\text{ink}}$ compared to fluoranthene-EQ_{chem} $1.6 \pm 0.1 \mu\text{g}_{\text{fluoranthene}}/\text{g}_{\text{ink}}$). As discussed above, it was not possible to determine fluoranthene-EQ_{bio} and fluoranthene-EQ_{chem} for the SOS response for genotoxicity as fluoranthene did not have a response in this assay. The difference between fluoranthene-EQ_{bio} and fluoranthene-EQ_{chem} indicates that chemicals other than the detected PAHs are causing the majority of the effect, particularly for the studied adaptive stress response pathways. Høgsberg et al. [7] also found no

relationship between ROS production, which can induce oxidative stress, and PAH concentration. Hence, the bulk of the effect in the studied bioassays is due to unidentified components.

3.6. Benchmarking biological effects from tattoo ink with other exposure routes

The measure fluoranthene-EQ_{tattoo} was developed in this study to provide a more meaningful indication of chemical exposure per tattoo (Equation 6). Based on work by Engel et al. [43], the average mass of ink injected per cm² of skin is 2.53 mg, so this was used for C_{ink}, while 300 cm² was used for A_{skin}, as this is a common tattoo size [39]. Different C_{ink} and A_{skin} values can be applied to calculate fluoranthene-EQ_{tattoo}. The fluoranthene-EQ_{tattoo} values ranged from 5.4 ± 0.1 to 11.6 ± 3.2 µg_{fluoranthene} for bacterial cytotoxicity, 148 ± 42.9 to 10401 ± 2300 µg_{fluoranthene} for the oxidative stress response and 39.2 ± 18.1 to 2413 ± 1263 µg_{fluoranthene} for the p53 response (Figure 4). The highest fluoranthene-EQ_{tattoo} value was determined for the yellow (Intenze brand) ink in the oxidative stress response endpoint and this suggests that a yellow 300 cm² tattoo would elicit an oxidative stress response equivalent to 10 mg of fluoranthene.

To put the fluoranthene-EQ_{tattoo} values in context with other exposure pathways, estimated annual exposure to fluoranthene from food was determined using Equation 7. C_{food} values were collected from Martorell et al. [44] who measured average fluoranthene concentrations in a range of different food groups (C_{food} was 5.2 µg_{fluoranthene}/kg_{food} for meat and meat products and 0.4 µg_{fluoranthene}/kg_{food} for fish and seafood). Fluoranthene concentrations in other food types were below the limit of detection. Median annual consumption was extrapolated from the daily median consumption per person of different food groups from 16 European countries (48.2 kg_{food}/year for meat and meat products and 24.8 kg_{food}/year for fish and seafood) [45]. The estimated annual exposure from food ranged from 10.1 to 251 µg_{fluoranthene}/year (Figure 4). The fluoranthene-EQ_{tattoo} values for bacterial cytotoxicity were comparable to the estimated annual exposure to fluoranthene from fish and seafood; however, fluoranthene-EQ_{tattoo} values for oxidative stress response and p53

response were generally higher than the estimated annual exposure to fluoranthene from meat (up to 40 times higher).

This example has some obvious limitations, including differences in exposure pathway (e.g., ingestion vs. injection) and comparing only fluoranthene concentrations to mixture effects of PAHs, but the intent is to demonstrate a potential approach to compare the effect from tattoos to other sources of chemical exposure. More work is required to achieve this, including bioanalytical assessment of food.

4. Conclusions

This study demonstrated that tattoo ink contains a range of chemical impurities that can induce oxidative stress response, p53 response and cytotoxicity. In all endpoints, the effect was greatest for red and yellow inks, which are expected to contain azo pigments. Chemical analysis of black tattoo ink found PAHs at concentrations twice the recommended PAH level in tattoo ink. The detected PAHs could explain almost 15% of bacterial cytotoxicity by the black tattoo ink, but only 0.06% of the oxidative stress response. The significant contribution of undetected components to the biological effect emphasises the advantage of using bioanalytical tools complementary to chemical analysis to assess the hazard potential of tattoo inks. This study represents the most comprehensive bioanalytical assessment of tattoo inks to date; however, it focuses only on the extractable fraction of chemicals in tattoo ink, not necessarily the fraction that will be available in the body. Hence, further work on toxicokinetics of tattoo ink, including uptake and metabolism, is necessary for improved understanding of the health risks associated with tattoo ink chemicals.

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Table 1: Summary of effect concentrations of all inks in all bioassays and bioanalytical equivalents (fluoranthene-EQ_{bio}) for bacterial cytotoxicity, oxidative stress response and p53 response (EC values are the average of 2 to 3 repeats $\pm$ standard deviation (SD)).

	<i>Bacterial cytotoxicity</i>		<i>Mammalian p53 cytotoxicity</i>	<i>Oxidative stress response</i>		<i>p53 response</i>		<i>umuC (SOS response to genotoxicity)</i>	
Ink	EC₁₀ $\pm$ SD (g_{ink}/L)	Fluoranthene-EQ_{bio} $\pm$ SD (μg_{fluoranthene}/g_{ink})	EC₁₀ $\pm$ SD (g_{ink}/L)	EC_{IR1.5} $\pm$ SD (g_{ink}/L)	Fluoranthene-EQ_{bio} $\pm$ SD (μg_{fluoranthene}/g_{ink})	EC_{IR1.5} $\pm$ SD (g_{ink}/L)	Fluoranthene-EQ_{bio} $\pm$ SD (μg_{fluoranthene}/g_{ink})	-S9 EC_{IR1.5} $\pm$ SD (g_{ink}/L)	+S9 EC_{IR1.5} $\pm$ SD (g_{ink}/L)
Black	26.6 $\pm$ 2.5	11.1 $\pm$ 1.1	18.4 $\pm$ 17.6	15.1 $\pm$ 3.7	929 $\pm$ 234	34.6 $\pm$ 12.4	464 $\pm$ 182	>200	>200
Red	34.0 $\pm$ 1.6	8.7 $\pm$ 0.5	15.8 $\pm$ 6.3	2.5 $\pm$ 0.2	5570 $\pm$ 566	35.5 $\pm$ 3.4	453 $\pm$ 83	163 $\pm$ 23	19.9 $\pm$ 0.3
Blue	41.9 $\pm$ 0.6	7.1 $\pm$ 0.2	25.2 $\pm$ 4.7	22.8 $\pm$ 7.8	613 $\pm$ 210	37.1 $\pm$ 0.1	434 $\pm$ 68	>200	>200
Green	34.9 $\pm$ 1.0	8.5 $\pm$ 0.3	4.0 $\pm$ 0.1	6.5 $\pm$ 0.3	2150 $\pm$ 142	7.1 $\pm$ 1.3	2284 $\pm$ 556	>200	>200
Yellow (Dragonhawk)	27.5 $\pm$ 7.1	10.8 $\pm$ 2.8	2.2 $\pm$ 1.3	1.2 $\pm$ 0.6	12111 $\pm$ 6151	10.4 $\pm$ 7.3	1555 $\pm$ 1117	>200	>200
Yellow (Intenze)	19.5 $\pm$ 5.4	15.2 $\pm$ 4.3	3.7 $\pm$ 1.0	1.0 $\pm$ 0.2	13704 $\pm$ 3030	5.1 $\pm$ 2.5	3180 $\pm$ 1664	>200	166 $\pm$ 28
Lavender	30.5 $\pm$ 9.3	9.7 $\pm$ 3.0	133.9*	19.1 $\pm$ 1.9	731 $\pm$ 78	312 $\pm$ 136	52 $\pm$ 24	>200	>200
White	>400	-	>400	71.8 $\pm$ 20.6	195 $\pm$ 57	>400	-	>200	>200

Table 2: Solubility, concentration and effect concentrations of detected polycyclic aromatic hydrocarbons in black tattoo ink.

Compound	Molecular Weight (g/mol)	Solubility (mg/L)	Detected conc. ($\mu\text{g/g}_{\text{ink}}$)	Bacterial cytotoxicity $\text{EC}_{10} \pm \text{SD}$ (mg/L)	Oxidative stress response $\text{EC}_{\text{IR1.5}} \pm \text{SD}$ (mg/L)	p53 response $\text{EC}_{\text{IR1.5}} \pm \text{SD}$ (mg/L)
Naphthalene	128	25.6	0.07	2.9 ± 0.7	243 ± 49	-
Acenaphthylene	152	4.6	0.08	0.3 ± 0.01	50.0 ± 0.7	-
Phenanthrene	178	1.8	0.03	0.2 ± 0.04	48.1 ± 3.3	-
Fluoranthene	202	0.2	0.2	0.3 ± 0.01	14.0 ± 0.6	16.1 ± 2.5
Pyrene	202	0.2	0.8	0.2 ± 0.003	26.6 ± 1.4	-
Benzo[ghi]-perylene	276	-	0.009	N/A	N/A	N/A

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Figure 1: Concentration-effect curves used for the derivation of EC values of all inks in all bioassays A) bacterial cytotoxicity, B) mammalian p53 cytotoxicity C) oxidative stress response, D) SOS response for genotoxicity (-S9), E) SOS response for genotoxicity (+S9) and F) p53 response. The concentration-effect curves were from one repeat only and are shown for illustration only.

Figure 2: Comparison of biological effects of tattoo inks with results expressed as A) EC values (g_{ink}/L) in all bioassays and B) fluoranthene-EQ_{bio} ($\mu g_{fluoranthene}/g_{ink}$) for bacterial cytotoxicity, oxidative stress response and p53 response.

Figure 3: Bioanalytical assessment of the detected polycyclic aromatic hydrocarbons A) EC values (mg/L) for bacterial cytotoxicity, mammalian p53 cytotoxicity, oxidative stress response and p53 response and B) fluoranthene-EQ_{chem} ($\mu g_{fluoranthene}/g_{ink}$) for black tattoo ink for bacterial cytotoxicity, oxidative stress response and p53 response.

Figure 4: Tattoo bioanalytical equivalent masses related to the reference compound fluoranthene (fluoranthene-EQ_{tattoo}) ($\mu g_{fluoranthene}$) for bacterial cytotoxicity and oxidative stress response derived from the mass of ink used for a 300 cm² tattoo compared to the estimated annual fluoranthene intake per person ($\mu g_{fluoranthene}/year$) from meat and fish food groups.

Figure 1

Figure 2

Figure 3

Figure 4