

PAPER

View Article Online  
View Journal | View Issue



Cite this: *Environ. Sci.: Nano*, 2022, 9, 2134

# Bioreactivity of a novel poly(epsilon-caprolactone) nanocapsule containing atrazine with human lung alveolar epithelial cells†

Amber J. S. Moore, <sup>‡\*a</sup> Lareb S. N. Dean, <sup>‡\*ab</sup> Aiken N. K. Yam, <sup>a</sup> Renata de Lima, <sup>c</sup> Leonardo F. Fraceto <sup>d</sup> and Teresa D. Tetley <sup>\*ab</sup>

Atrazine (ATZ), a commonly used agricultural herbicide, is potentially harmful to animals and humans. Nanoencapsulation of ATZ (NC-ATZ) within non-toxic, biodegradable poly(ε-caprolactone) (PCL) improves the herbicidal activity of ATZ 10-fold and moderates its environmental persistence, possibly reducing off-target ecological effects. These compounds may reach the pulmonary respiratory units following inhalation; thus, we investigated the effect of ATZ and NC-ATZ on an immortalised human lung alveolar type 1-like epithelial cell model (TT1 cells). The concentration-dependent effect of the compounds was analysed by assessment of viability, reactive oxygen species (ROS) production and inflammatory cytokine release. Confocal microscopy was used to visualise TT1 cell-nanoparticle interactions. NC-ATZ caused cellular effects not observed with ATZ or the PCL nanocapsule alone. NC-ATZ significantly increased lactate dehydrogenase (LDH) release at  $\geq 1 \mu\text{g ml}^{-1}$  after 48 hours of exposure, peaking at  $5 \mu\text{g ml}^{-1}$  (3-fold of the non-treated control,  $***p < 0.001$ ). Moreover,  $\geq 1 \mu\text{g ml}^{-1}$  NC-ATZ was pro-inflammatory at 48 hours, peaking at  $5 \mu\text{g ml}^{-1}$  (IL-6 release  $\sim 125 \text{ pg ml}^{-1}$ ; IL-8 release  $\sim 46 \text{ pg ml}^{-1}$ ). Confocal microscopy of fluorescently-labelled ATZ and NC-ATZ indicated high intensity fluorescence nanoparticle uptake into the cytoplasm and co-localisation in the Golgi, suggesting nanoparticle recycling within 24 hours. We provide evidence that nanoencapsulation of the pesticide ATZ alters bioreactivity, stimulating more necrosis and inflammation in human lung alveolar type 1 epithelial cells in comparison to ATZ or the PCL nanocapsule alone. However, nanoencapsulation improves the efficacy of pesticides, thus lower pesticide concentrations could be used, reducing environmental contamination. Further investigation, particularly with different exposure scenarios, is warranted in order to generate safer nanoencapsulated pesticides.

Received 21st November 2021,  
Accepted 20th April 2022

DOI: 10.1039/d1en01068a

rs.li/es-nano

## Environmental significance

The environment is being inadvertently damaged due to increasing food demands requiring pesticide use. Repeated, high quantities of pesticide use may result in pesticide resistance, reduced soil biodiversity and death of beneficial, non-target organisms. Nanoencapsulation of pesticides can reduce contamination by controlling release and improving efficacy. However, it is important to ensure that there are no unwanted effects in humans and wildlife. Although previous research suggested poly(ε-caprolactone) nanoencapsulation of atrazine was safer to human cells, we discovered adverse bioreactivity with human lung alveolar type 1 epithelial cells compared to ATZ or the nanocapsule alone. While nanoencapsulation reduces environmental exposure to ATZ, further development of safe NC-ATZ formulations is required. This study highlights the importance of determining off-target behaviours of nanomaterials.

<sup>a</sup> Lung Cell Biology, Airways Disease, National Heart and Lung Institute, Imperial College London, London, UK. E-mail: amber.moore14@alumni.imperial.ac.uk, t.tetley@imperial.ac.uk

<sup>b</sup> The National Institute for Health Research Health Protection Research Unit (NIHR HPRU), Health Impact of Environmental Hazards at King's College London in partnership with Public Health England (PHE) in collaboration with Imperial College London, London, UK

<sup>c</sup> LABiToN – Laboratory for Evaluation of Bioactivity and Toxicology of Nanomaterials, University of Sorocaba, São Paulo, Brazil

<sup>d</sup> São Paulo State University (UNESP), Institute of Science and Technology of Sorocaba, Laboratory of Environmental Nanotechnology, Sorocaba, São Paulo, Brazil

† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d1en01068a>

‡ These authors contributed equally to this work.

## 1. Introduction

Estimates suggest that by 2050 the global population will be 9.7 billion; thus, with the additional threats of climate change and conflicts leading to food scarcity, the importance of maximising agricultural productivity is vital.<sup>1,2</sup> Pests and disease are responsible for 20–40% loss of agricultural production worldwide, yet greater damage has been prevented by use of pesticides.<sup>3</sup> However, pesticides are associated with harm to both humans and the environment. Pimentel *et al.* suggested the combined cost of pesticides



(purchase price and harm to health/livestock) is comparable to the economic benefits of improved crop production, hence there is both a moral and economic motive for safer and more cost-effective pesticides.<sup>4</sup>

Atrazine (ATZ) is a herbicide which controls broad-leaved weeds, mainly in the production of maize, sorghum and sugar cane.<sup>5</sup> It is among the most commonly used herbicides worldwide.<sup>6</sup> In the USA, a country responsible for 24% of global pesticide sales, ATZ is the second most highly applied herbicide (500 000 tonnes annually).<sup>7</sup> As well as the herbicidal benefits, the relatively cheap price of ATZ (\$14 per gallon) compared to other alternatives has made it a competitive product in the agricultural sector.<sup>8</sup> In pre-emergence treatment, ATZ is absorbed by the roots and transported to the leaves *via* the xylem. Direct application to the leaves inhibits photosynthesis by displacement of plastoquinone from its D1 binding-site on photosystem II in chloroplasts. This disrupts the electron transport chain and subsequent generation of reactive oxygen species causes lipid and protein damage resulting ultimately in leaf chlorosis, necrosis and plant death.<sup>6,9</sup> Non-target plants are unaffected by rapid outward transport or degradation of ATZ.<sup>10</sup>

Evidence concerning the effects of ATZ on animals and humans is ambiguous. Several *in vivo* rodent studies discovered aerosolised ATZ exposure induced oxidative and nitrosative stress, leading to inflammatory cytokine release, through the nuclear factor-erythroid 2-related factor (Nrf2) pathway; alterations of the Nrf2 pathway in mouse lung and brain results in increased lipid peroxidation, autophagy and apoptosis, leading ultimately to tissue damage, neurodegeneration and pulmonary fibrosis.<sup>11–13</sup> Moreover, multiple studies have found ATZ to cause endocrine disruption in amphibians and rodents. A study in the United States (US) of male frogs (*Xenopus laevis*) reared in contaminated water found low levels of ATZ ( $1 \times 10^{-4}$   $\mu\text{g ml}^{-1}$ ) triggered gonadal dysgenesis and male hermaphroditism.<sup>14</sup> In the same amphibian species, a Canadian group reported the frogs had incomplete testicular development and decreased testicular size and germ cell numbers after 48 hours in water with ATZ ( $0.021 \mu\text{g ml}^{-1}$ ).<sup>15</sup> Changes in circular RNAs have also been implicated in multiple developmental anomalies in male tadpoles exposed to ATZ, with Wnt signalling and progesterone-mediated oocyte maturation pathways identified as differentially upregulated in testis tissue.<sup>16</sup> Additionally, in rodents, it has been demonstrated that ATZ causes delayed reproductive development in both sexes. Recent studies have shown environmentally relevant doses of ATZ ( $0.1\text{--}10 \text{ mg kg}^{-1}$  body weight, oral exposure) had an adverse effect on sperm motility and viability of adult *Calomys laucha* rodents as well as pubescent C57BL/6J male mice.<sup>17,18</sup> Higher doses of ATZ ( $0\text{--}300 \text{ mg kg}^{-1}$  body weight, oral exposure), representative of bioaccumulation, also resulted in reduced sperm dynamics in male albino rats, as well as lowered body weight gains, raised organ weights (except in the testes), and increased  $T_3$  and  $T_4$  hormones (thyroid function disruption).<sup>19</sup> Data from

randomised control trials in rats support evidence of ATZ as a toxicant to the male reproductive system and conclude it may have similar effects on the male human reproductive system.<sup>20</sup> In female rodents, delayed female sexual maturation (timing of uterine growth and vaginal opening) has been demonstrated. Laws *et al.* showed this effect with daily oral gavages of diamino-s-chlorotriazine, a by-product of ATZ, at doses of  $100 \text{ mg kg}^{-1}$  and  $200 \text{ mg kg}^{-1}$ , in peripubertal female Wistar rats.<sup>21</sup> Ashby *et al.* showed a similar response through post-natal oral exposure to  $100 \text{ mg kg}^{-1}$  ATZ in female Sprague-Dawley rats.<sup>22</sup> A number of mechanisms have been suggested to explain these diverse disruptive effects of ATZ including stimulation of aromatase and inhibition of androgen receptors.<sup>23</sup> Although no clear evidence of endocrine disruption has been reported in humans, *in vitro* studies in human cell lines, including the MCF-7 breast cancer cell line and H295R adrenocortical cell line, do support the potential of ATZ to cause adverse effects *via* aromatase induction.<sup>24,25</sup> Furthermore, a US-multi centred case-control study saw poorer sperm quality (reduced concentration and motility) in exposed men; however the association was confounded, as it often is, by other pesticide exposures.<sup>26</sup> Moreover, an association between ATZ exposure and Parkinson's disease (PD), an idiopathic disease of the central nervous system, has been identified. Multiple epidemiological studies suggest a correlation of pesticide use and PD with a recent meta-analysis finding 5 and 10 years of pesticide exposure was associated with 5 and 11% increased risk respectively of PD.<sup>27</sup> Biochemical studies implicate pesticide exposure with the inhibition and loss of dopaminergic neurones, mitochondrial impairment, and oxidative stress in the human brain.<sup>28</sup> Atrazine has been shown to be directly toxic for dopaminergic neurones with some studies identifying miRNA deregulation (*e.g.* miRNA-7, -181 and -34, often studied within the context of PD).<sup>29</sup> In an avian study, neurodegeneration from ATZ exposure has been linked to mitochondrial damage, malformation and apoptosis of neuronal cells.<sup>30</sup>

Similarly, confounders as well as a lack of sufficient evidence led to the controversial decision by the International Agency for Research on Cancer (IARC) to choose the category of “not classifiable as to its carcinogenicity to humans” (Group 3).<sup>31</sup> *In vitro*, ATZ has been found to promote proliferation, migration and invasion in epithelial ovarian cancer cells (Skov3 and A2780 cell lines) by induction of reactive oxygen species.<sup>32</sup> In the common carp, 24 h *in vitro* exposure caused significant alteration of multiple miRNAs in many pathways, with expression of female-biased genes resulting from decreased miRNAs in the primordial gland; evidence also indicated ATZ can upregulate aromatase through miRNAs.<sup>33</sup> Chronic feeding regimes of  $22.5 \text{ mg per kg per day}$  of ATZ caused early onset of mammary gland tumours in female Sprague-Dawley rats compared to untreated control rats.<sup>34</sup> These results were not replicated in the Fischer 344 rat model due to differences in hormonal environment of Sprague Dawley rats and the mechanisms



that control reproductive senescence and the development of the mammary tumours during aging.<sup>31,35–37</sup> Thus elevated endogenous oestrogen levels were only observed in Sprague-Dawley rats;<sup>34</sup> in this case, ATZ is likely to cause dysfunction of luteinising hormone cycling and premature reproductive senescence leading to increased susceptibility to mammary tumour development.<sup>31</sup> Fischer 334 rats have higher circulatory progesterone levels which influences the effect of oestrogen on the hypothalamic-pituitary axis in the development of mammary tumours.<sup>35,36</sup> Consequently, these mechanisms of carcinogenesis are not relevant to humans where reproductive ageing is related to primary follicle depletion.<sup>31,34</sup> The Agricultural Health Study (AHS), a large human prospective cohort study in the US from 1993–2001, initially observed trends with ATZ exposure relating to increased risk of lung and bladder cancer, non-Hodgkin's lymphoma (NHL) and multiple myeloma.<sup>38</sup> However later analysis (up to 2007; from the initial recruitment timeframe) of double the number of exposed cases saw no significant excess risk with greater duration or intensity of ATZ exposure.<sup>39</sup> Despite a larger sample size, no clear conclusions could be made due to few cancer 'events' (for example 152 exposed cases with NHL compared to 68 cases in the initial study) and a lack of detailed pesticide exposure measurements.<sup>39</sup> These are common limitations for epidemiological studies investigating pesticide exposure.<sup>40</sup> A recent finding of the AHS, in a genome-wide DNA methylation study, found methylation of *PAX6* with ATZ; this implicates ATZ exposure in the development of diabetes and abnormal eye development.<sup>41</sup>

The United States Geological Survey reported ten or more pesticide contaminants in approximately 20% of their tests and two or more in 90% of their tests in streams.<sup>42</sup> Thus, flora and fauna are consistently exposed to multiple pesticides and, indeed, large quantities of ATZ are lost to the environment. ATZ is mobile so it can migrate into groundwater entering streams and rivers *via* runoff.<sup>43</sup> In the US, ATZ is found in approximately 75% of streams and 40% of groundwater samples.<sup>42</sup> 18% of streams tested had atrazine concentrations which exceeded one or more aquatic benchmarks (including the acute and chronic ATZ concentrations above which unacceptable effects to an aquatic community occur).<sup>42</sup> Furthermore ATZ is degraded slowly and can persist for 146 days in soil and 742 days in water.<sup>43</sup> The most recent US Environmental Protection Agency (EPA) report, published in 2018, stated that drinking water wells in only Florida and Wisconsin exceeded the maximum contaminant levels ( $0.003 \mu\text{g ml}^{-1}$  as an annual average) for ATZ in groundwater.<sup>43</sup> However seasonal variations in ATZ exposure, especially related to timing of precipitation and runoff events with ATZ application, can significantly increase the likelihood of periodic high contamination levels in other areas. A study of the White River in Indiana, USA in 1991–1992, demonstrated variation of  $0.009 \mu\text{g ml}^{-1}$  (at the time of the first runoff events following high ATZ usage in May and June) to  $<0.001 \mu\text{g}$

$\text{ml}^{-1}$  (between August to March when less pesticide was used).<sup>44</sup>

ATZ may also remain in the air following application or is volatilized from bodies of water. It can be carried long distances on particles of dust without being broken down, affecting wildlife and human populations far from the site of application.<sup>45</sup> Certain weather conditions (including hot, windy environments) can favour entrance of ATZ into the atmosphere. For this reason, in Midwestern USA, a third of ATZ used enters the atmosphere. In Minnesota, 68.3% of ATZ found in Leech Lake originated from application sites 100–300 km away.<sup>46</sup> Inhalation of ATZ-containing dust or spray particles by humans may cause local effects like asthma (including atopic and non-atopic wheeze)<sup>38,39,47</sup> or they may enter the bloodstream (to cause potential systemic effects).<sup>48</sup> The mode of application of pesticides to the crops, commonly using spray equipment, is a key determinant on the impact on health. A current gap in knowledge exists as to the extent and length of time required for ATZ to be absorbed *via* the lungs. Large particles can deposit in the pharynx or upper airways where they can be coughed up and swallowed *via* the mucociliary escalator.<sup>49</sup> Smaller particles however can be inhaled deeper, into the alveolar region of the lung, the site of gas exchange and mediation of lung homeostasis, where they can interact with and damage the lung epithelial cells.<sup>50,51</sup> Therefore, inhalation toxicity of ATZ is an important consideration both for the farmers during occupational use and for the general population in surrounding areas.

Due to the evidence discussed above, and elsewhere, the US EPA's decision to continue ATZ use has not been without controversy. In 2003, the EU concluded ATZ levels in groundwater would exceed  $0.1 \mu\text{g ml}^{-1}$ , despite appropriate use, and therefore banned its use in member states in 2005.<sup>52</sup>

For the US, Canada and other countries across the world with high ATZ usage, switching to alternative herbicide use could be problematic.<sup>53</sup> The US utilises ATZ on an estimated 55% of corn crop (data from 2014) since ATZ is cheaper than its alternatives.<sup>54</sup> This leads to an additional income of \$1.45 billion annually and 6.8% greater corn yield.<sup>55–57</sup> A ban on ATZ could have devastating impacts, reducing US gross corn sales by as much as one sixth and raising corn prices.<sup>55,56</sup>

Over the last three decades, nanotechnology – the application of materials on the nanoscale – has influenced many fields from medicine and cosmetics to food packaging. Researchers are exploring the beneficial properties of nanomaterials in farming to improve detection of agrochemicals and combat food spoilage using antimicrobial nanoparticle formulations.<sup>58,59</sup> Nanotechnology can also aid agrochemical delivery to target plants. Nanoencapsulation – enclosing an active substance within a nano-sized shell, coating or membrane – has shown promise for this endeavour.<sup>60</sup> Nano-sized particles are likely to be easily internalised by the plant vasculature, however little is known about how nanoencapsulation could affect the bioavailability,



transport and fate of pesticides in the environment, including human and wildlife populations.

In the present study, we used poly( $\epsilon$ -caprolactone) (PCL) – a biodegradable, aliphatic and hydrophobic polyester, to form nanocapsules for ATZ delivery. PCL is biodegradable, harmless to the environment and degrades quickly, thus it is ideal for pesticide delivery.<sup>60</sup> Nanoencapsulation prevents ATZ aggregation, increasing particle number per unit mass and therefore the efficacy of ATZ. The efficacy is further improved by the controlled release of ATZ from the PCL nanocapsule *via* relaxation of polymer chains; 45% and 65% release was observed after 24 and 48 hours respectively with a maximum release of 72% after 5.5 days.<sup>61</sup> Furthermore, nanoencapsulated ATZ (NC-ATZ) is more evenly distributed allowing for greater bioavailability, thus fewer applications would be required to maintain efficacy, lowering cost and potential harm to the environment by reducing accumulation.<sup>62,63</sup> Moreover, efficacy of NC-ATZ is maintained after a 10-fold dilution when applied to target mustard plants (*Brassica* species).<sup>9,61</sup> Regarding the sourgrass plant (*Digitaria insularis*; a highly invasive weed), NC-ATZ induced greater and faster inhibition of photosystem II activity compared with conventional ATZ – even at half-dosage of NC-ATZ.<sup>64</sup> Importantly, NC-ATZ has little genotoxicity and cytogeneticity against human leukocytes, being equivalent to deionised water, unlike toxic ATZ.<sup>65</sup>

Ongoing dermal research is further exploring the toxicity of NC-ATZ; however, inhalation exposure has previously not been investigated. On inhalation, a large percentage of nano-sized particles can reach the alveolar respiratory units, the site of gas exchange. The epithelium of the alveolar respiratory units is comprised of alveolar epithelial type 1 (AT1) and type 2 (AT2) cells. AT1 cells cover 95% of the alveolar surface thus will be a major target of inhaled nano-sized particles.<sup>66</sup>

We hypothesised on inhalation, ATZ would be toxic to human pulmonary alveolar epithelial cells (notably AT1 cells, see above). We further hypothesised that encapsulation of ATZ (NC-ATZ) would subdue the bioreactivity and toxicity of ATZ. We tested this hypothesis using a cell line derived from immortalised primary human alveolar epithelial cells, transduced type 1 (TT1) cells, to mimic the human alveolar epithelium *in vitro*.<sup>67</sup> The ATZ concentrations used in this study were estimations derived from occupational exposures of those spraying the product. Normally 1 mg ml<sup>-1</sup> ATZ is sprayed onto the crops but as a 10-fold dilution, NC-ATZ has the same efficacy. Once sprayed, ATZ becomes diluted in the air thus only a very small percentage of inhaled NCs would reach the alveoli.<sup>68</sup> Consequently, we chose a wide range of concentrations (0.01–10  $\mu$ g ml<sup>-1</sup>) to reflect the occupational and potential far-reaching general exposures. It is worth mentioning that nano-sized particles in the atmosphere take many hours or days to settle and can travel many miles, depending on weather conditions and prevailing winds, possibly impacting on the general population.

## 2. Materials and methods

### 2.1 Materials

Acetone (Sigma Aldrich), poly( $\epsilon$ -caprolactone) – average Mn 80 000 Da (Sigma Aldrich), Myritol® 318 – caprylic/capric triglyceride (BASF), Span® 60 (Sigma Aldrich), Tween® 80 (Sigma Aldrich), atrazine – PESTANAL® (Sigma Aldrich), 18 : 1 Liss Rhod PE [1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt), powder] (Avanti® Polar Lipids).

All other reagents were purchased from Sigma Aldrich unless stated otherwise.

### 2.2 Preparation of poly( $\epsilon$ -caprolactone) (PCL) nanocapsules and chemicals

PCL nanocapsules were prepared according to the interfacial deposition of pre-formed polymer described by Fessi *et al.*<sup>69</sup> Firstly, the organic phase was prepared containing 30 ml of acetone, 100 mg of poly( $\epsilon$ -caprolactone), 200 mg of Myritol, 40 mg of Span 60 and 10 mg of atrazine (ATZ). The aqueous phase was composed of 30 ml of deionised water containing 60 mg of surfactant Tween 80. The organic phase was slowly added into the aqueous phase, with magnetic stirring. Subsequently, the resultant suspension was kept under magnetic stirring for 10 minutes, after which the organic solvent was evaporated using a rotatory evaporator. The suspension was evaporated to a final volume of 10 ml, resulting in a total atrazine concentration, in the nanocapsules, of 1 mg ml<sup>-1</sup> with an encapsulation efficiency of 86.7  $\pm$  0.7% (measured in an acetonitrile diluent, using high-performance liquid chromatography). Characterisation of NC-ATZ, including full methodology of encapsulation efficacy quantification, has been previously published by Grillo *et al.*<sup>61</sup> For labelled nanoparticles, the fluorescent probe (18 : 1 Liss Rhod PE-Avanti® – 0.1% m of probe per m of oil – myritol) was added into the organic phase after polymer dissolution.

An ATZ solution of 1 mg ml<sup>-1</sup> was prepared by dissolving diffuse ATZ in dimethyl sulfoxide (DMSO), ensuring that percentage weight of ATZ matched that of ATZ within the NC (NC-ATZ), so that direct comparison across both treatments could be made.

The nanocapsules in suspension (with and without ATZ) was approximately 7.5  $\times$  10<sup>12</sup> particles per ml. For treatment with the nanocapsule preparation, this was matched in concentration to the nanocapsule component of the NC-ATZ formula.

### 2.3 Evaluation of PCL nanocapsule containing atrazine (NC-ATZ) stability

NC-ATZ was diluted in defined cell culture medium-1 (DCCM-1) (Biological Industries, Israel) and incubated at 37 °C in a growth chamber. Samples were collected at 0 hours (0 h), 24 hours (24 h) and 48 hours (48 h) to evaluate nanoparticle characteristics by dynamic light scattering (DLS), and 0 h and 48 h for atomic force microscopy (AFM).





NC-ATZ in DCCM-1 was evaluated by DLS (ZetaSizer ZS90, Malvern Instruments, UK) using a scattering detection angle of 90 °C to acquire hydrodynamic size (nm), polydispersity index (PDI) and zeta potential (mV). Samples were analysed, in triplicate, at 25 °C.

NC-ATZ in DCCM-1 was diluted in ultrapure water (1:1000) and left to dry for 24 h at room temperature. Images were obtained by AFM (easyScan 2 Basic, Nanosurf, Switzerland), in contact mode using TapAI-G cantilevers (BudgetSensors, Bulgaria). Images were acquired with Gwyddion 2.50 software. Results are presented in ESI† Appendix A and B.

#### 2.4 Culture of immortalised human alveolar type-1 like epithelial (TT1) cells

TT1 cells are an immortal alveolar type 1(AT1)-like cell line derived from primary human alveolar type 2 (AT2) cells, the precursors of AT1 cells *in situ*, as previously described by Kemp *et al.*<sup>67</sup> TT1 cells were cultured in DCCM-1 supplemented with 10% newborn calf serum (NCS) and 1% penicillin-streptomycin-glutamine (PSG). Cells were seeded in 8 or 96 well plates at  $7 \times 10^4$  or  $1 \times 10^5$  cells/well respectively and grown to confluence (48 h) at 37 °C, 5% CO<sub>2</sub>, prior to exposure.

#### 2.5 Sample exposures

TT1 cells were serum starved for 24 hours prior to exposure. PCL nanocapsule alone (NC), ATZ dissolved in DMSO (ATZ) and ATZ within the PCL nanocapsule (NC-ATZ) were diluted in fresh serum-free DCCM-1 to reach desired comparable concentrations (0.01–10 µg ml<sup>-1</sup>). Therefore, the maximum final percentage of DMSO used was 1%, present in the 10 µg ml<sup>-1</sup> sample of ATZ. Vehicle controls included DMSO and Tween 80, using concentrations equivalent to those found in ATZ and NC-ATZ respectively. Serum-free DCCM-1 was used as a negative control. Cells were exposed for 1 hour (1 h), 24 hours (24 h) and 48 hours (48 h).

#### 2.6 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

TT1 cells were exposed to test samples for 24 h and 48 h. Zinc oxide (ZnO) nanoparticles (NPs) are known to induce toxicity to TT1 cells (at 10 µg ml<sup>-1</sup>) thus were used as a positive control.<sup>70</sup> Following sample exposure, cells were incubated with MTT solution (500 µg ml<sup>-1</sup> in DCCM-1) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere for 25 minutes. MTT is reduced by mitochondrial reductases to produce purple formazan, a marker of metabolic activity, which can thus indirectly be used as a measure of cell viability as a loss of MTT reduction.<sup>71</sup> The cells were washed with phosphate-buffered saline (PBS) before formazan solubilisation with ≥99.9% DMSO. The optical density was measured at 570 nm in an Infinite® F50 absorbance microplate reader (Tecan, Switzerland).

#### 2.7 LDH (lactate dehydrogenase) assay

TT1 cells were exposed to test samples for 24 h and 48 h. 45 minutes prior to exposure endpoint, 10% lysis buffer was added to duplicate, unexposed wells, to cause LDH release, for use as a positive control. LDH is released extracellularly as a result of impaired plasma membrane integrity. Following sample exposure, the conditioned media was collected and stored for use at -20 °C. The assay was then carried out according to manufacturer's instructions (Pierce™ LDH Cytotoxicity Assay Kit, Thermo Fisher Scientific).<sup>72</sup> The optical density was measured at 450 nm in an Infinite® F50 absorbance microplate reader (Tecan, Switzerland).

#### 2.8 Enzyme-linked immunosorbent assay (ELISA)

TT1 cells were exposed to test samples for 24 h and 48 h. Following sample exposure, the conditioned media were collected and stored for use at -20 °C. Measurement of IL-6 and IL-8 release was then determined according to the manufacturer's instructions (Human IL-6/8 Standard TMB ELISA Development Kit, Peptotech, Inc.).<sup>73</sup> The protocol was adapted for Greiner Bio-One 96 well half area microplates. The optical density was measured at 492 nm in an Infinite® F50 absorbance microplate reader (Tecan, Switzerland).

#### 2.9 Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS was determined *via* two different assays using cell-permeant reagents: dihydroethidium (DHE; qualitative) and H<sub>2</sub>DCFDA (quantitative). In both cases, TT1 cells were exposed to test samples for 1 h and 24 h in order to capture a possibly early response in ROS production.<sup>74</sup>

For the DHE assay, following test sample exposure, the intact, adherent cells were incubated with DHE (10 µM in DCCM-1) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere for 20 minutes. Cells were subsequently washed with PBS. Oxidation of DHE produces ethidium, resulting in red fluorescence, which was detected using an Axiovert 200 inverted fluorescent microscope (Zeiss, Germany) and captured as an image.

For the H<sub>2</sub>DCFDA assay, following sample exposure, intact, adherent cells were incubated with H<sub>2</sub>DCFDA (25 µM in DCCM-1) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere for 30 minutes. ROS oxidation of H<sub>2</sub>DCFDA results in the formation of fluorescent 2,7-dichlorofluorescein (DCF). Cells were subsequently washed with PBS and disrupted by solubilisation in DMSO. The fluorescence intensity was determined on a FLUOstar OPTIMA microplate reader (BMG Labtech, Germany), at the  $E_x/E_m$  of 485 nm/530 nm, and quantified as relative fluorescence units (RFU).

Results of the ROS formation assays are presented in ESI† Appendix D and E.



## 2.10 Confocal laser scanning microscopy

TT1 cells were exposed for 24 h to NC and NC-ATZ, which were fluorescently labelled with 18:1 Liss Rhod PE [1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-lissamine rhodamine B sulfonyl]. Following sample exposure, cell membranes were stained with wheat germ agglutinin (WGA) Alexa Fluor™ 488 conjugate ( $5 \mu\text{g ml}^{-1}$  in PBS) then fixed with 4% formaldehyde for 10 minutes at 37 °C. Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; 300 nM in PBS) for 10 minutes at 37 °C. A Leica TCS SP5 confocal microscope (Leica Microsystems, UK) was used to visualise  $\geq 15$  cells/well as well as acquire Z stack images at the following wavelengths: rhodamine-derivative  $E_x/E_m$  495 nm/519 nm, WGA  $E_x/E_m$  560 nm/583 nm and DAPI  $E_x/E_m$  358 nm/461 nm.

## 2.11 Statistical analysis

Data are presented as the mean  $\pm$  standard deviation (SD) or standard error of the mean (SEM). A one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* test was used to assess significant differences between all exposure regimens to non-treated controls using GraphPad Prism® software. *p* values  $< 0.05$  were considered statistically significant.

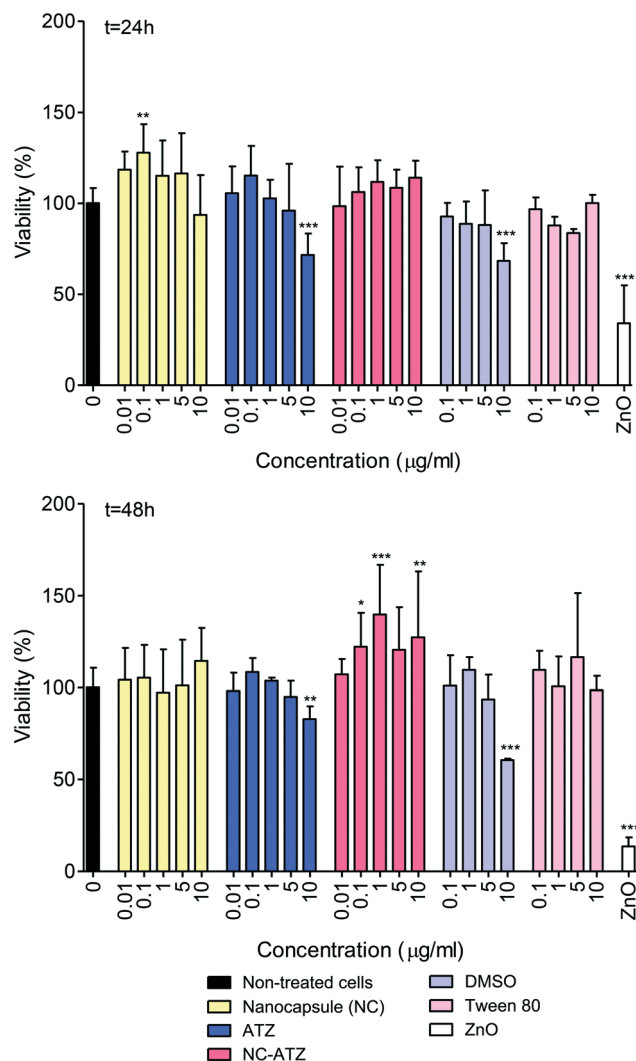
# 3. Results

## 3.1 Effect of nanoencapsulated pesticide and pesticide treatment on TT1 cell viability (MTT assay)

As evidenced in Fig. 1, NC exposure induced a significant ( $**p < 0.01$ ) change in mitochondrial activity at  $0.1 \mu\text{g ml}^{-1}$  only, increasing 28% compared to the control after 24 h. There were no significant changes following NC exposure after 48 h. There was a trend towards a concentration-dependent decrease in cell viability at 24 h and 48 h post-ATZ exposure although only  $10 \mu\text{g ml}^{-1}$  caused a significant reduction (28%,  $t = 24 \text{ h}$ ,  $***p < 0.001$ ; 19%,  $t = 48 \text{ h}$ ,  $**p < 0.01$ ). However, DMSO (1%) alone caused a similar decline (approx. 30%,  $***p < 0.001$ ) at both time points, likely a reflection of its well-documented toxicity, and most likely this was the chief determinant of the cytotoxicity of ATZ alone. Interestingly, NC-ATZ stimulated MTT/mitochondrial activity 48 h post-exposure at  $0.1 \mu\text{g ml}^{-1}$  ( $*p < 0.05$ ),  $1 \mu\text{g ml}^{-1}$  ( $***p < 0.001$ ) and  $10 \mu\text{g ml}^{-1}$  ( $**p < 0.01$ ) suggesting cell activation and/or proliferation may have occurred. There were no significant changes in viability in the cells exposed to the Tween 80 vehicle control compared to the non-treated control cells. Zinc oxide (ZnO), a cytotoxic nanoparticle, was used as a positive control and caused a significant decrease in cell viability at  $10 \mu\text{g ml}^{-1}$  ( $***p < 0.001$ ) at both time points.

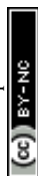
## 3.2 Effect of nanoencapsulated pesticide and pesticide treatment on TT1 cell viability (LDH assay)

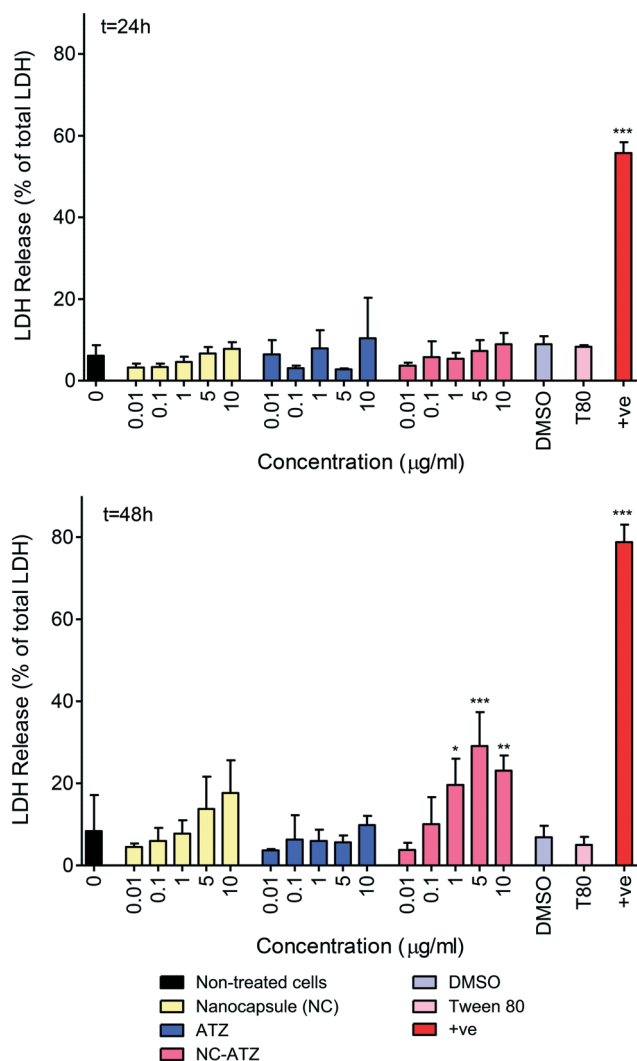
As lactate dehydrogenase (LDH) release indicates impaired plasma membrane integrity, it indicates necrotic cell death.



**Fig. 1** Cell viability of TT1 cells exposed to increasing concentrations of a PCL nanocapsule (NC), atrazine (ATZ) in DMSO, a PCL NC containing ATZ (NC-ATZ), vehicles DMSO and Tween 80 (T80), and Zinc Oxide (ZnO) nanoparticles for 24 h and 48 h. Concentrations of NC and ATZ were matched to the equivalent percentage weights in the NC-ATZ treatment. Concentrations of DMSO and T80 were matched to the equivalent percentage weights in the ATZ and NC-ATZ treatments respectively. Data are presented as a percentage of the non-treated control cells. Error bars are mean  $\pm$  SD ( $n = 4-7$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

All data were compared to maximal potential LDH release due to 100% necrotic cell death (generated from lysed cells). As can be observed in Fig. 2, there were no significant increases in LDH release following exposure to any of the conditions at 24 h. NC-ATZ caused increased LDH release at higher concentrations ( $1 \mu\text{g ml}^{-1}$ ,  $*p < 0.05$ ;  $5 \mu\text{g ml}^{-1}$ ,  $***p < 0.001$  and  $10 \mu\text{g ml}^{-1}$ ,  $**p < 0.01$ ) peaking at  $5 \mu\text{g ml}^{-1}$ , with 3-fold greater LDH release compared to the non-treated cells, after 48 h exposure. Since NC-ATZ induced mitochondrial activity, NC-ATZ-induced necrosis is likely accompanied by activation and/or proliferation of residual cells. The positive assay control (+ve) showed a significant





**Fig. 2** LDH release from TT1 cells exposed to increasing concentrations of a PCL nanocapsule (NC), atrazine (ATZ) in DMSO, a PCL NC containing ATZ (NC-ATZ), vehicles DMSO and Tween 80 (T80) and a positive control (+ve) for 24 h and 48 h. Concentrations of NC and ATZ were matched to the equivalent percentage weights in the NC-ATZ treatment. Concentrations of DMSO and T80 were matched to the equivalent percentage weights in the ATZ and NC-ATZ treatments respectively. Data are presented as a percentage of total cellular LDH (determined by LDH release from lysed control cells). Error bars are mean  $\pm$  SD ( $n = 4-6$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

increase in LDH release (\*\* $p < 0.001$ ) validating the assay reagents and methodology.

### 3.3 Effect of nanoencapsulated pesticide and pesticide treatment on inflammatory mediator release from TT1 cells

Release of inflammatory mediators, IL-6 and IL-8, by TT1 cells was quantified by ELISA (Fig. 3). NC-ATZ treatment induced a concentration-dependent increase with significant IL-6 release at  $1 \mu\text{g ml}^{-1}$ ,  $5 \mu\text{g ml}^{-1}$  and  $10 \mu\text{g ml}^{-1}$  NC-ATZ releasing  $8 \text{ pg ml}^{-1}$ ,  $9 \text{ pg ml}^{-1}$  and  $8 \text{ pg ml}^{-1}$  of IL-6 respectively (\* $p < 0.05$ ). This was amplified at 48 h ( $1 \mu\text{g}$

$\text{ml}^{-1}$ , \* $p < 0.05$ ;  $5 \mu\text{g ml}^{-1}$ , \*\*\* $p < 0.001$ ;  $10 \mu\text{g ml}^{-1}$ , \*\* $p < 0.01$ ) peaking at  $5 \mu\text{g ml}^{-1}$  NC-ATZ exposure with  $125 \text{ pg ml}^{-1}$  IL-6 release. There was no significant change in IL-6 release by NC and ATZ exposed TT1 cells at either time point. IL-6 release increased significantly, compared to control, from TT1 cells exposed to DMSO (1%) at 24 h (\* $p < 0.05$ ); IL-6 release was also significantly stimulated in TT1 cells exposed to DMSO (1%; \* $p < 0.05$ ) at 48 h (Fig. 3 and see ESI† Appendix C, Fig. C).

Concentration-dependent trends of increasing IL-8 release by TT1 cells were observed after 24 h of exposure to NC, ATZ and NC-ATZ (Fig. 3). Only  $10 \mu\text{g ml}^{-1}$  NC (\* $p < 0.05$ ) and most concentrations of NC-ATZ ( $0.1 \mu\text{g ml}^{-1}$  and  $10 \mu\text{g ml}^{-1}$ , \* $p < 0.05$ ;  $1 \mu\text{g ml}^{-1}$ , \*\* $p < 0.01$ ;  $5 \mu\text{g ml}^{-1}$ , \*\*\* $p < 0.001$ ) were significantly increased, with NC-ATZ treatment peaking at  $5 \mu\text{g ml}^{-1}$  with  $5 \text{ pg ml}^{-1}$  IL-8 release at 24 h. After 48 h of exposure, overall, NC did not stimulate significant IL-8 release (Fig. 3); however, there were significant decreases in IL-8 release caused by ATZ ( $0.01 \mu\text{g ml}^{-1}$ ,  $5 \mu\text{g ml}^{-1}$  and  $10 \mu\text{g ml}^{-1}$ , \*\* $p < 0.01$ ;  $1 \mu\text{g ml}^{-1}$ , \* $p < 0.05$ ). NC-ATZ treatment ( $1 \mu\text{g ml}^{-1}$ , \* $p < 0.05$ ;  $5 \mu\text{g ml}^{-1}$ , \*\* $p < 0.01$ ) significantly increased levels of IL-8 release, with levels peaking at  $5 \mu\text{g ml}^{-1}$  with  $46 \text{ pg ml}^{-1}$  of NC-ATZ (\*\* $p < 0.01$ ). Furthermore, significant IL-8 release was observed in response to  $10 \mu\text{g ml}^{-1}$  Tween 80 exposure at 24 h (\* $p < 0.05$ ) and DMSO (1%) at 48 h (\*\* $p < 0.001$ ), perhaps as a result of cell death (Fig. 3 and see ESI† Appendix C, Fig. C).

### 3.4 Uptake of PCL nanoencapsulated pesticide by TT1 cells

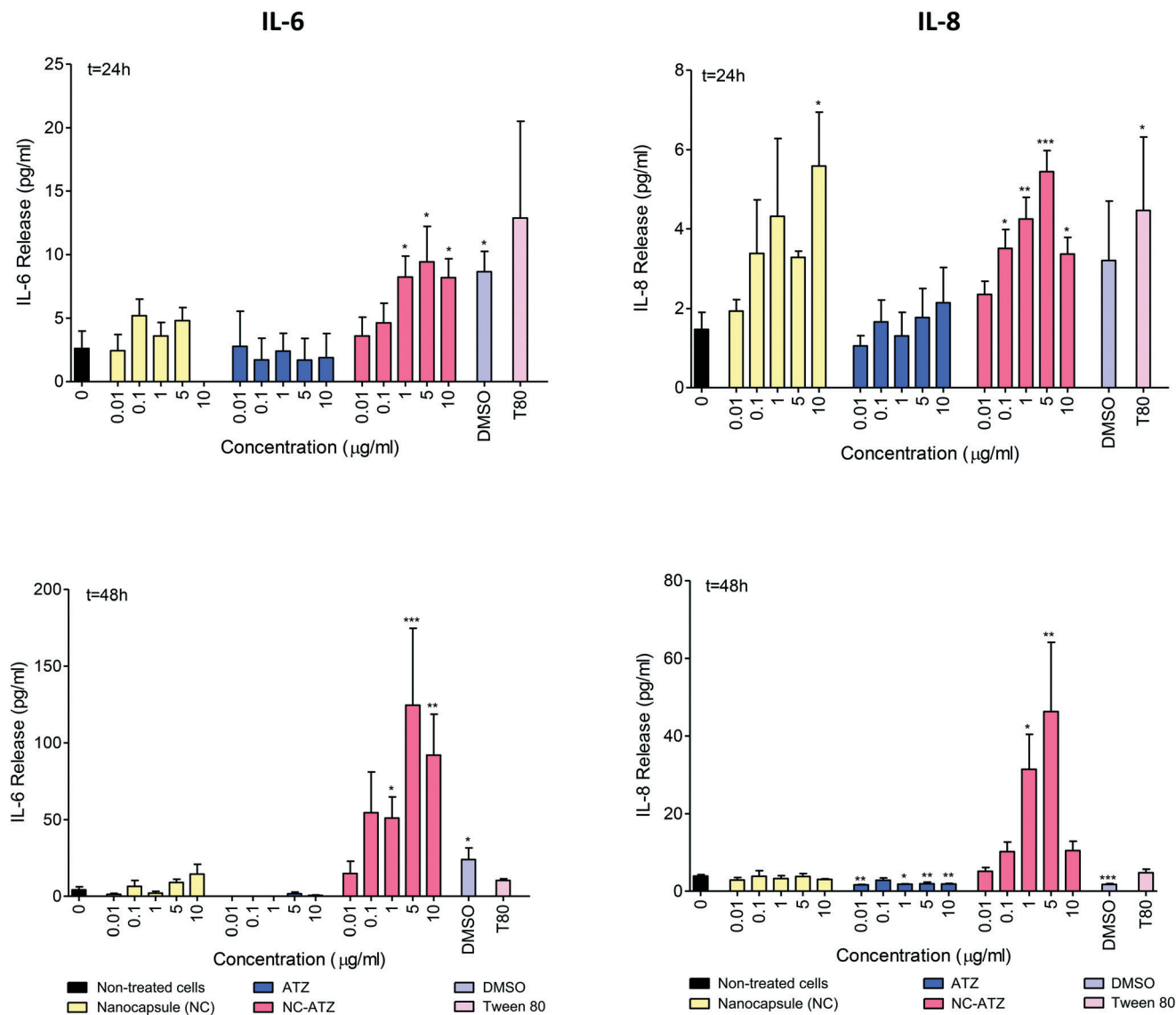
Uptake of fluorescently-labelled NC and NC-ATZ was captured by confocal microscopy after 24 h of exposure (Fig. 4 and 5). NC (red) was readily internalised by TT1 cells, confirmed by visualising the NC inside the cells on the middle focal plane. The pan-staining indicates intracellular polymer degradation of the NC. Red fluorescence is visible in similar quantities at  $0.01 \mu\text{g ml}^{-1}$  and  $1 \mu\text{g ml}^{-1}$  NC, but less evenly distributed among cells at  $10 \mu\text{g ml}^{-1}$  NC. Interestingly, when comparing the distribution of red fluorescent particles following NC and NC-ATZ exposure, NC appears to have more pan-staining at lower concentrations and overall.

Co-localisation of the NP and membranes (yellow) suggests NC uptake into the membrane-rich Golgi-apparatus, in close apposition to the nucleus. This is shown more explicitly within magnified inserts (Fig. 4 and 5, yellow arrows).

## 4. Discussion

This study revealed some very striking differences between the bioreactivity of ATZ and nanoencapsulated ATZ (NC-ATZ) with human lung alveolar epithelial type 1-like cells. Over a period of 48 h, NC-ATZ caused increased TT1 cell mitochondrial activity, indicative of either cell activation and/or cell proliferation, and yet induced LDH release – a marker of necrotic cell death – whilst stimulating release of





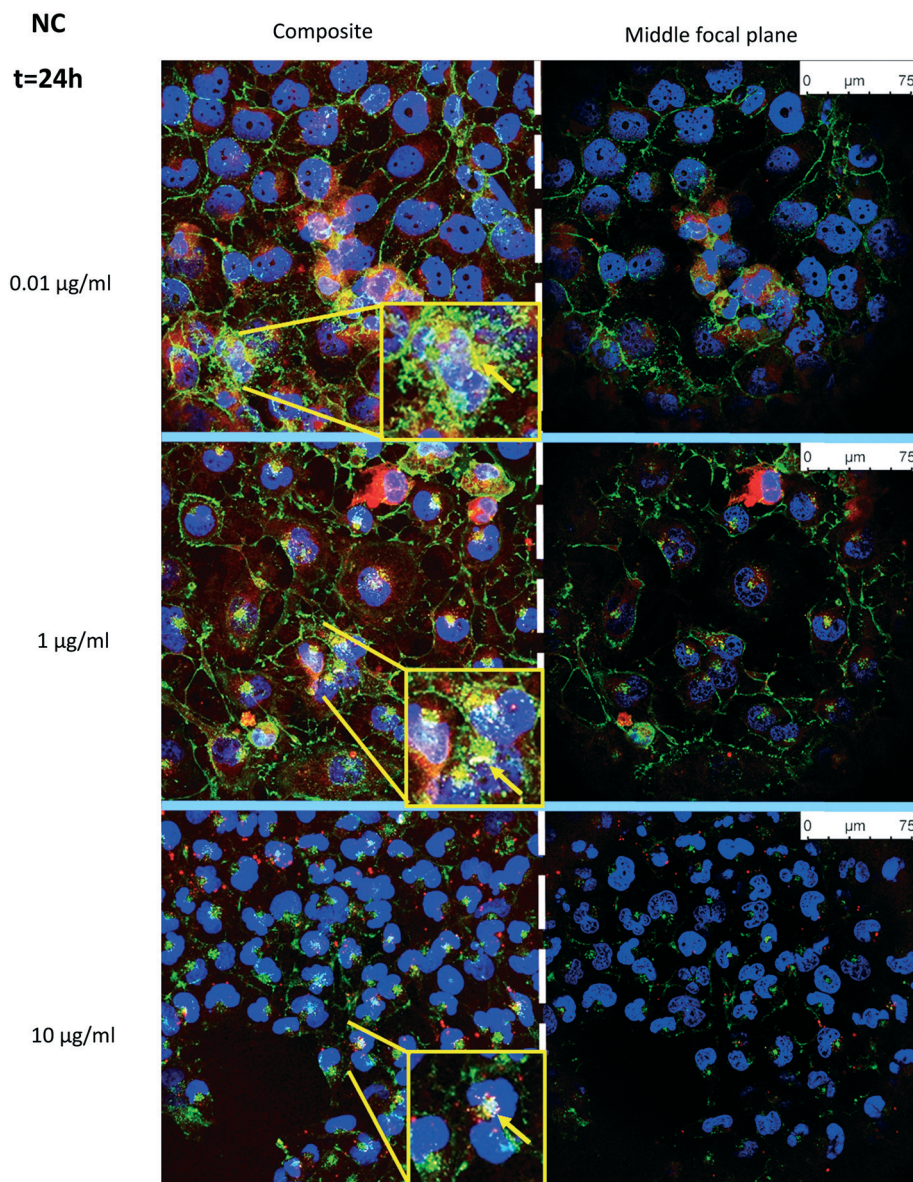
**Fig. 3** IL-6 and IL-8 release from TT1 cells exposed to increasing concentrations of a PCL nanocapsule (NC), atrazine (ATZ) in DMSO, a PCL NC containing ATZ (NC-ATZ), vehicles DMSO and Tween 80 (T80) for 24 h and 48 h. Concentrations of NC and ATZ were matched to the equivalent percentage weights in the NC-ATZ treatment. Concentrations of DMSO and T80 were matched to the equivalent percentage weights in the ATZ and NC-ATZ treatments respectively. Error bars are mean  $\pm$  SEM ( $n = 3-5$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

proinflammatory mediators. Different response patterns were observed with the NC and ATZ alone. TT1 cells readily internalised the NC as well as NC-ATZ, which produced a staining pattern indicative of cytoplasmic dispersal and recycling of the capsules, presumably releasing the drug intracellularly. Interestingly, there were no changes in ROS during the first 24 h despite significantly elevated levels of IL-6 and IL-8 in response to NC-ATZ exposure.

The bioreactivity of nanoencapsulated ATZ is likely to be dependent on size, shape, composition, charge and surface chemistry of the NC, as has been demonstrated by studies of nanoparticle toxicity.<sup>75-77</sup> Grillo *et al.* demonstrated NC-ATZ size and shape remains similar to NC, however, other properties may be different due to the process of combining the NC and ATZ.<sup>61</sup> Characteristics of NC-ATZ may also differ

in the cell culture media used in this study. Previous research on human leukocytes, algae and plants cells showed NC-ATZ to be safer than ATZ alone, although microcrustaceans were more sensitive to NC-ATZ toxicity.<sup>61,62,65</sup> Thus, it is likely that NC-ATZ has differential effects depending on the organism and cell targets. It is important to appreciate that TT1 cells have been shown to internalise nanoparticles *via* both passive (passing through cell membranes without using specific processes or cell energy) and active (largely endocytic) processes.<sup>74,78,79</sup> *In situ*, such processes enhance transportation of small molecules across the alveolar barrier. Thus, the observation of NC uptake aligns with our previous findings.<sup>74,80,81</sup> Our other studies of TT1 cells have shown that surface chemistry and charge of nanoparticles impacts on cell death.<sup>80,81</sup>





**Fig. 4** Visualisation of nanoparticle uptake by TT1 cells after 24 h of exposure to increasing concentrations of a fluorescently-labelled PCL nanocapsule (NC) (red). Concentrations of NC were matched to the equivalent percentage weights in the NC-ATZ treatment. Nuclei are stained with DAPI (blue) and membranes with WGA (green). Images on the left are a composite of all sections at any given concentration (demonstrating uptake) whilst images on the right depict the corresponding middle focal plane through the cells (confirming internalisation) ( $n = 1$ ).

Amine-modified polymer NPs permeabilise TT1 cell plasma membranes, inducing ROS and apoptotic cell death, whereas the same concentrations of neutral and negatively-charged NPs have little effect on TT1 cells. On the other hand, negatively charged NPs induce apoptotic cell death in cardiomyocytes by inducing ROS and mitochondrial damage.<sup>80,81</sup> Thus, altered NC surface chemistry during processing could account for the heightened bioreactivity of NC-ATZ seen here. Alternatively, since polymer NPs degrade within 24 h and significant effects on cell viability and LDH release are only seen after 48 h (Fig. 1 and 2), perhaps a degradation product is causing this increased harmful effect compared to ATZ alone.<sup>82–84</sup>

IL-6 is released acutely in response to pathogen-associated molecular patterns through signalling pathways and activation of transcription factors *e.g.* NF- $\kappa$ B. IL-8 is an important neutrophil chemoattractant, inducing neutrophil migration and activation, *e.g.* degranulation and ROS production. These cytokines were investigated due to their role in many inflammatory lung diseases. NC-ATZ was proinflammatory at 24 h and 48 h (Fig. 3), reflecting activation of pattern recognition receptors, *e.g.* toll-like receptors.<sup>85</sup> Acute increases in IL-6 and IL-8 have been associated with lung injury and, in severe cases, acute respiratory distress syndrome.<sup>86,87</sup> However the relatively low concentrations released following NC-ATZ exposure may not

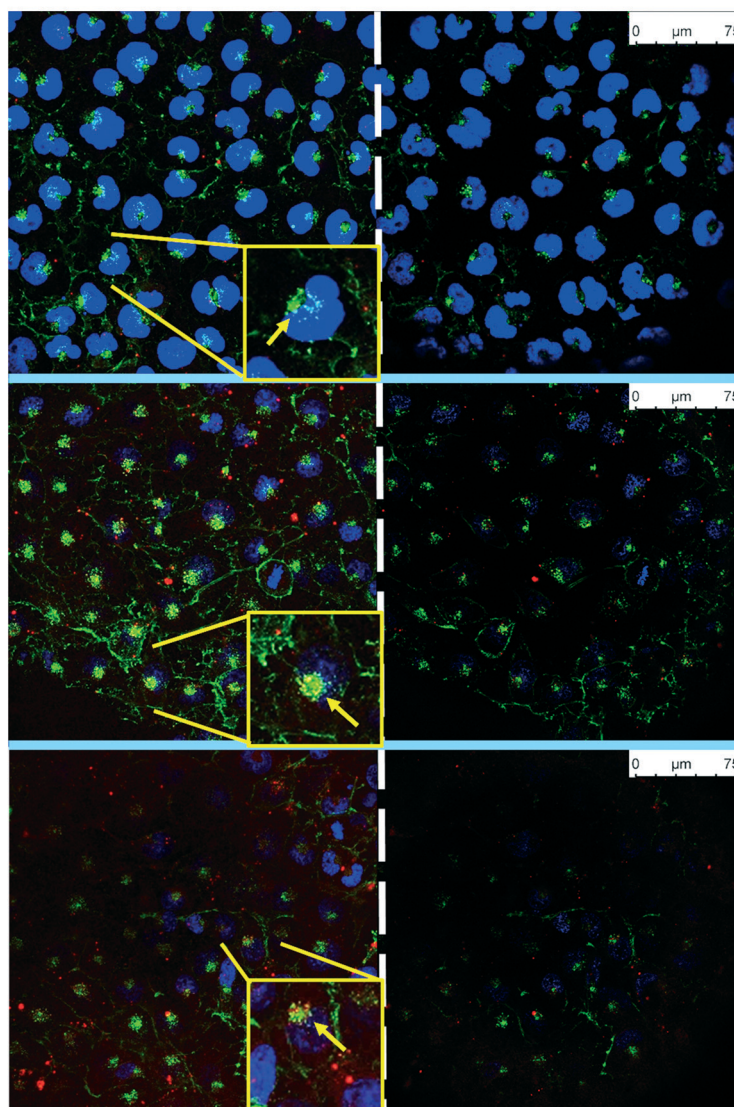


## NC-ATZ

t=24h

Composite

Middle focal plane

0.01  $\mu\text{g/ml}$ 1  $\mu\text{g/ml}$ 10  $\mu\text{g/ml}$ 

**Fig. 5** Visualisation of nanoparticle uptake by TT1 cells after 24 h of exposure to increasing concentrations of a fluorescently-labelled PCL nanocapsule (NC) (red) containing ATZ. Nuclei are stained with DAPI (blue) and membranes with WGA (green). Images on the left are a composite of all sections at any given concentration (demonstrating uptake) whilst images on the right depict the corresponding middle focal plane through the cells (confirming internalisation) ( $n = 1$ ).

be physiologically relevant. Conversely, AT1 cells cover 95% of the alveolar surface area and such a marked increase in release of IL-6 and IL-8 at the gas liquid interface of the lung as observed here, may be problematic. Moreover, the increase in mitochondrial activity seen following exposure to NC-ATZ for 48 h supports the suggestion that the cells have been activated. Chronic levels of IL-6 and IL-8 are associated with inflammatory lung diseases, therefore the effect of chronic exposure to NC-ATZ on cytokine release from TT1 cells is an important consideration.<sup>88,89</sup>

Inflammatory signalling often involves the generation of ROS; interestingly, ROS was unchanged compared to non-treated controls (see ESI† Appendix E, Fig. E). Visual

assessment indicated that the DHE assay succeeded, showing deep red cells at baseline levels (see Appendix D, Fig. D1 and D2).

Holes in the monolayer appeared following exposure to NC-ATZ for 24 h, which subsequently were observed at higher concentrations of NC and NC-ATZ treatment after 48 h (see Appendix D, Fig. D1 and D2). Necrotic cell death may have contributed to the impaired monolayer observed, particularly for NC-ATZ 48 h exposure (Fig. 2). Cell death, as indicated by LDH release, and loss of adherent cells may account for the low detection of cellular ROS. In addition, these NCs may interfere with TT1 tight junctions, as observed with 300 nm carbon nanotubes.<sup>78</sup> Decrease of alveolar barrier





integrity would be problematic *in vivo*, possibly leading to increased risk of respiratory infections as well as unwanted access to the bloodstream and other organs.<sup>75</sup>

Confocal microscopy showed pan staining of fluorescently-labelled NC uptake by TT1 cells indicating intracellular polymer degradation and cytosolic dispersion of the NC constituents (Fig. 4 and 5), which previous studies suggest occurs within 24 h.<sup>80,81</sup> These NCs are 240–260 nm thus are likely to be endocytosed *via* clathrin-coated pits or micropinocytosis.<sup>61,75,79</sup> Co-localisation of the NC and membrane (yellow) suggests uptake of NC into the Golgi apparatus and subsequent recycling (Fig. 4 and 5). The contrasting confocal images for NC and NC-ATZ are evidence of differential TT1 cell interactions. Lower fluorescence following NC-ATZ uptake compared to NC uptake suggests differing uptake mechanisms and cellular dynamics, where the proinflammatory processes might have (as yet unknown) knock-on effects.<sup>80,81</sup>

Overall, these experiments suggest NC-ATZ is more harmful compared to NC or ATZ alone. This highlights the importance of *in vitro* research prior to experimentation *ex vivo* or in animal models. Further work could investigate other factors influencing the effect of NC-ATZ *in vivo* using different exposure scenarios. Within the alveoli, macrophages are likely to take up a large proportion of NC-ATZ and AT2 cells produce surfactant, which will further interact with the NCs. Surfactant may alter alveolar macrophage uptake and toxicity of NC-ATZ, whilst the nanoparticle may alter the function of surfactant (including its ability to provide a biological barrier) and precipitate disease.<sup>90</sup> To investigate the impact of surfactant, air-liquid interface conditions would be required (compared to the submerged culture conditions in this study) and would be provide a greater understanding of the physiological interactions of NC-ATZ within the alveoli.<sup>91</sup> Additionally, other body systems will be exposed to NC-ATZ. Modification of the NC properties and construction of the NC-ATZ particles could also be investigated to provide a safer product. Establishing the effects of novel nanoencapsulated pesticides on alveolar epithelial co-cultures and other body systems (primarily dermal or gastrointestinal) would further our understanding of the possible effects *in vivo*. Further characterisation of NC-ATZ within these models is likely to provide a better understanding of the mechanisms underpinning its bioreactivity. Finally, comparing the results obtained with NC-ATZ with commercial formulations would be useful to better understand the effects of surfactants and additives used in typical atrazine herbicide formulations in comparison to those used in NC-ATZ preparations.

## 5. Conclusion

Although previous research suggested nanoencapsulation of ATZ was safer (less cytotoxic and genotoxic) to human cells, this study revealed that it may be more bioreactive compared

to ATZ alone if inhaled. The differences observed between NC and NC-ATZ cannot be attributed to ATZ alone, suggesting altered properties of the nanoencapsulated pesticide. A change in the physicochemical properties of the NC may be responsible. To better understand/modify mechanisms of NC-ATZ toxicity, different nanomaterial properties of NC and NC-ATZ must be determined/modified. Nanomaterials have brought many advantages to science, but we are yet to be fully cognisant of their behaviours.

Although this study suggests nanoencapsulation is more harmful compared to ATZ alone, the improved herbicidal efficacy with nanoencapsulation could significantly reduce environmental contamination and respiratory exposure. This may be of vital importance in countries where pesticide regulation and respiratory protection is less stringent. Therefore, research into PCL nanoencapsulation of ATZ should be continued and further developed, to improve its biocompatibility in humans and other organisms.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgements

Dr. Renata de Lima and Dr. Leonardo F. Fraceto would like to thank São Paulo Research Foundation (FAPESP, #16/50003-4 and #17/21004-5) and CNPq, and also Dr. Estefânia V. Ramos Campos for the nanocapsule preparation and Dr. Anderson do Espírito Santo Pereira for nanoparticle characterization. Professor Teresa Tetley and Dr Lareb Dean received funding from the National Institute for Health Research Health Protection Research Unit (NIHR HPRU) in Health Impact of Environmental Hazards at King's College London in partnership with Public Health England (PHE) in collaboration with Imperial College London, London, UK.

## References

- 1 United Nations, Department of Economic and Social Affairs, Population Division, World Population Prospects: The 2015 Revision, Key Findings and Advance Tables. United Nations, New York, Working paper number: ESA/P/WP.241. 2015, [https://esa.un.org/unpd/wpp/publications/files/key\\_findings\\_wpp\\_2015.pdf](https://esa.un.org/unpd/wpp/publications/files/key_findings_wpp_2015.pdf), (accessed November 2021).
- 2 F. P. Carvalho, Pesticides, environment, and food safety, *Food Energy Secur.*, 2017, **6**, 48–60.
- 3 S. Savary, A. Ficke, J. N. Aubertot and C. Hollier, Crop losses due to diseases and their implications for global food production losses and food security, *Food Secur.*, 2012, **4**, 519–537.
- 4 D. Pimentel and M. Burgess, Environmental and economic costs of the application of pesticides primarily in the United States, *Integrated Pest Management*, ed. D. Pimentel and R. Peshin, Springer, Dordrecht, 2014, vol. 3, pp. 47–71.
- 5 M. Salazar-Ledesma, B. Prado, O. Zamora and C. Siebe, Mobility of atrazine in soils of a wastewater irrigated maize field, *Agric., Ecosyst. Environ.*, 2018, **255**, 73–83.



- 6 J. Liu, J. H. Zhou, Q. N. Guo, L. Y. Ma and H. H. Yang, Physiochemical assessment of environmental behaviours of herbicide atrazine in soils associated with its degradation and bioavailability to weeds, *Chemosphere*, 2021, **262**, 127830.
- 7 D. Atwood and C. Paisley Jones, Pesticides Industry Sales and Usage, 2008–2012 Market Estimates, U.S. Environmental Protection Agency, [https://www.epa.gov/sites/production/files/2017-01/documents/pesticides-industry-sales-usage-2016\\_0.pdf](https://www.epa.gov/sites/production/files/2017-01/documents/pesticides-industry-sales-usage-2016_0.pdf), (accessed November 2021).
- 8 J. A. Ferrell and B. A. Sellers, Approximate Herbicide Pricing, The Institute of Food and Agricultural Sciences, University of Florida, Publication #SS-AGR-16, <https://edis.ifas.ufl.edu/wg056>, (accessed November 2021).
- 9 H. C. Oliveira, R. Stolf-Moreira, C. B. R. Martinez, R. Grillo, M. B. de Jesus and L. F. Fraceto, Nanoencapsulation enhances the post-emergence herbicidal activity of atrazine against mustard plants, *PLoS One*, 2015, **10**, e0132971.
- 10 W. C. Koskinen and S. A. Clay, Factors affecting atrazine fate in north central US soils, *Rev. Environ. Contam. Toxicol.*, 1997, **151**, 117–165.
- 11 R. D'Amico, F. Monaco, R. Fusco, R. Siracusa, D. Impellizzeri, A. F. Peritore, R. Crupi, E. Gugliandolo, S. Cuzzocrea, R. Di Paola, T. Genovese and M. Cordaro, Atrazine inhalation worsens pulmonary fibrosis regulating the Nuclear Factor-Erythroid 2-Related Factor (Nrf2) pathways inducing brain comorbidities, *Cell. Physiol. Biochem.*, 2021, **55**, 704–725.
- 12 R. D'Amico, F. Monaco, R. Fusco, A. F. Peritore, T. Genovese, D. Impellizzeri, R. Crupi, L. Interdonato, A. M. Sforza, E. Gugliandolo, R. Siracusa, S. Cuzzocrea, M. Cordaro and R. Di Paola, Exposure to atrazine induces lung inflammation through Nrf2-HO1 and Beclin 1/LC3 Pathways, *Cell. Physiol. Biochem.*, 2021, **55**, 413–427.
- 13 T. Genovese, R. Siracusa, R. Fusco, R. D'Amico, D. Impellizzeri, A. F. Peritore, R. Crupi, E. Gugliandolo, R. Morabito, S. Cuzzocrea, A. T. Salinaro, M. Cordaro and R. Di Paola, Atrazine inhalation causes neuroinflammation, apoptosis and accelerating brain aging, *Int. J. Mol. Sci.*, 2021, **22**, 7938.
- 14 T. Hayes, K. Haston, M. Tsui, A. Hoang, C. Haeffele and A. Vonk, Atrazine-induced hermaphroditism at 0.1 ppb in American leopard frogs (*Rana pipiens*): laboratory and field evidence, *Environ. Health Perspect.*, 2003, **111**, 568.
- 15 L. Tavera-Mendoza, S. Ruby, P. Brousseau, M. Fournier, D. Cyr and D. Marcogliese, Response of the amphibian tadpole (*Xenopus laevis*) to atrazine during sexual differentiation of the testis, *Environ. Toxicol. Chem.*, 2002, **21**, 527–531.
- 16 L. Sai, L. Li, C. Hu, B. Qu, Q. Guo, Q. Jia, Y. Zhang, C. Bo, X. Li, H. Shao, J. C. Ng and C. Peng, Identification of circular RNAs and their alterations involved in developing male *Xenopus laevis* chronically exposed to atrazine, *Chemosphere*, 2018, **200**, 295–301.
- 17 G. Q. Saalfeld, A. S. V. Junior, T. Castro, F. A. Pereira, S. M. M. Gheller, A. C. da Silva, C. D. Corcini, C. E. da Rosa and E. P. Colares, Low atrazine dosages reduce sperm quality of *Calomys laucha* mice, *Environ. Sci. Pollut. Res.*, 2018, **25**, 2924–2931.
- 18 L. E. Cook, B. J. Finger, M. P. Green and A. J. Pask, Exposure to atrazine during puberty reduces sperm viability, increases weight gain and alters the expression of key metabolic genes in the liver of male mice, *Reprod., Fertil. Dev.*, 2019, **31**, 920–931.
- 19 A. M. Khozimy, H. El-Danasoury and M. Abuzeid, Biochemical effects of treatments with herbicide atrazine in male albino rats, *Journal of the Advances in Agricultural Researches*, 2022, **4**, 43–57.
- 20 S. Zhu, T. Zhang, Y. Wang, X. Zhou, S. Wang and Z. Wang, Meta-analysis and experimental validation identified atrazine as a toxicant in the male reproductive system, *Environ. Sci. Pollut. Res.*, 2021, **28**, 37482–37497.
- 21 S. C. Laws, J. M. Ferrell, T. E. Stoker and R. L. Cooper, Pubertal development in female Wistar rats following exposure to propazine and atrazine biotransformation by-products, diamino-S-chlorotriazine and hydroxyatrazine, *Toxicol. Sci.*, 2003, **76**, 190–200.
- 22 J. Ashby, H. Tinwell, J. Stevens, T. Pastoor and C. B. Breckenridge, The effects of atrazine on the sexual maturation of female rats, *Regul. Toxicol. Pharmacol.*, 2002, **35**, 468–473.
- 23 J. B. Sass and A. Colangelo, European Union bans atrazine while the United States negotiates continued use, *Int. J. Occup. Environ. Health*, 2006, **12**, 260–267.
- 24 J. T. Sanderson, R. J. Letcher, M. Heneweer, J. P. Giesy and M. van den Berg, Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes, *Environ. Health Perspect.*, 2001, **109**, 1027–1031.
- 25 W. Q. Fan, T. Yanase, H. Morinaga, S. Gondo, T. Okabe, M. Nomura, T. Komatsu, K. I. Morohashi, T. B. Hayes, R. Takayanagi and H. Nawata, Atrazine-induced aromatase expression is SF-1 dependent: Implications for endocrine disruption in wildlife and reproductive cancers in humans, *Environ. Health Perspect.*, 2007, **115**, 720–727.
- 26 S. H. Swan, R. L. Kruse, F. Liu, D. B. Barr, E. Z. Drobnis, J. B. Redmon, C. Wang, C. Brazil and J. W. Overstreet, Study for Future Families Research Group, Semen quality in relation to biomarkers of pesticide exposure, *Environ. Health Perspect.*, 2003, **111**, 1478–1484.
- 27 D. Yan, Y. Zhang, L. Liu, N. Shi and H. Yan, Pesticide exposure and risk of Parkinson's disease: Dose-response meta-analysis of observational studies, *Regul. Toxicol. Pharmacol.*, 2018, **96**, 57–63.
- 28 M. S. Islam, F. Azim, H. Saju, A. Zargar, M. Shirzad, M. Kamal, K. Fatema, S. Rehman, M. A. M. Azad and S. E. Barough, Pesticides and Parkinson's disease: Current and future perspective, *J. Chem. Neuroanat.*, 2021, **115**, 101966.
- 29 A.-M. Aloizou, V. Siokas, E.-M. Sapouni, N. Sita, I. Liampa, A. G. Brotis, V. N. Rakitskii, T. I. Burykina, M. Aschner, D. P. Bogdanos, A. Tsatsakis, G. M. Hadjigeorgiou and E. Dardiotis, Parkinson's disease and pesticides: Are microRNAs the missing link?, *Sci. Total Environ.*, 2020, **744**, 140591.





- 30 J. Lin, H.-S. Zhao, L. Qin, X.-N. Li, C. Zhang, J. Xia and J.-L. Li, Atrazine triggers mitochondrial dysfunction and oxidative stress in quail (*Coturnix C. coturnix*) cerebrum via activating xenobiotic-sensing nuclear receptors and modulating cytochrome P450 systems, *J. Agric. Food Chem.*, 2018, **66**, 6402–6413.
- 31 E. D. Kuempel and T. Sorahan, *Identification of Research Needs to Resolve the Carcinogenicity of High-priority IARC Carcinogens, Views and Expert Opinions of an IARC/NORA Expert Group Meeting*, Lyon, France, IARC Technical Publication No. 42, 2010.
- 32 J. Chen, J. Liu, S. Wu, W. Lu, Y. Xia, J. Zhao, Y. Yang, Y. Wang, Y. Peng and S. Zhao, Atrazine promoted epithelial ovarian cancer cells proliferation and metastasis by inducing low dose Reactive Oxygen Species (ROS), *Iran. J. Biotechnol.*, 2021, **19**, e2623.
- 33 F. Wang, Q.-W. Yang, W.-J. Zhao, Q.-Y. Du and Z.-J. Chang, Effects of short-time exposure to atrazine on miRNA expression profiles in the gonad of common carp (*Cyprinus carpio*), *BMC Genomics*, 2019, **20**, 587.
- 34 L. T. Wetzel, L. G. Luempert III, C. B. Breckenridge, M. O. Tisdell, J. T. Stevens, A. K. Thakur, P. J. Extrom and J. C. Eldridge, Chronic effects of atrazine on estrus and mammary tumor formation in female Sprague-Dawley and Fischer 344 rats, *J. Toxicol. Environ. Health, Part A*, 1994, **43**, 169–182.
- 35 R. L. Cooper, S. C. Laws, P. C. Das, M. G. Narotsky, J. M. Goldman, E. L. Tyrey and T. E. Stoker, Atrazine and reproductive function: Mode and mechanism of action studies, *Birth Defects Res., Part B*, 2007, **80**, 98–112.
- 36 K. S. Estes, J. W. Simpkins and S. P. Kalra, Normal LHRH neuronal function and hyperprolactinemia in old pseudopregnant Fischer 344 rats, *Neurobiol. Aging*, 1982, **3**, 247–252.
- 37 J. C. Eldridge, M. K. Tennant, L. T. Wetzel, C. B. Breckenridge and J. T. Stevens, Factors affecting mammary tumor incidence in chlorotriazine-treated female rats: Hormonal properties, dosage, and animal strain, *Environ. Health Perspect.*, 1994, **102**, 29–36.
- 38 J. A. Rusiecki, A. De Roos, W. J. Lee, M. Dosemeci, J. H. Lubin, J. A. Hoppin, A. Blair and M. C. Alavanja, Cancer incidence among pesticide applicators exposed to atrazine in the Agricultural Health Study, *J. Natl. Cancer Inst.*, 2004, **96**, 1375–1382.
- 39 L. E. B. Freeman, J. A. Rusiecki, J. A. Hoppin, J. H. Lubin, S. Koutros, G. Andreotti, S. H. Zahm, C. J. Hines, J. B. Coble, F. Barone-Adesi, J. Sloan, D. P. Sandler, A. Blair and M. C. R. Alavanja, Atrazine and cancer incidence among pesticide applicators in the Agricultural Health Study (1994–2007), *Environ. Health Perspect.*, 2011, **119**, 1253–1259.
- 40 A. J. De Roos, S. Zahm, K. Cantor, D. Weisenburger, F. Holmes, L. Burmeister and A. Blair, Integrative assessment of multiple pesticides as risk factors for non-Hodgkin's lymphoma among men, *Occup. Environ. Med.*, 2003, **60**, e11.
- 41 T. T. Hoang, C. Qi, K. C. Paul, M. Lee, J. D. White, M. Richards, S. S. Auerbach, S. Long, S. Shrestha, T. Wang, L. E. B. Freeman, J. N. Hoffman, C. Parks, BIOS Consortium, C.-J. Xu, B. Ritz, G. H. Koppelman and S. J. London, Epigenome-wide DNA methylation and pesticide use in the Agricultural Lung Health study, *Environ. Health Perspect.*, 2021, **129**, 97008.
- 42 R. J. Gilliom, J. E. Barbash, C. G. Crawford, P. A. Hamilton, J. D. Martin, N. Nakagaki, L. H. Nowell, J. C. Scott, P. E. Stackelberg, G. P. Thelin and D. M. Wolock, Pesticides in the nation's streams and ground water 1992–2001, National Water-Quality Assessment Program, U.S. Geological Survey Circular 1291, 2006, <https://pubs.usgs.gov/circ/2005/1291/pdf/circ1291.pdf>, (accessed November 2021).
- 43 United States Environmental Protection Agency, Washington D.C 20460, Atrazine, Draft Human Health Risk Assessment, 2018, <https://www.regulations.gov/document?D=EPA-HQ-OPP-2013-0266-1159>, (accessed November 2021).
- 44 D. S. Carter, M. J. Lydy and C. G. Crawford, Water-quality assessment of the White River Basin, Indiana: Analysis of available information on pesticides, US Geological Survey 1972–92, 1995, [https://www.in.gov/dnr/water/files/WFWR\\_web50-119.pdf](https://www.in.gov/dnr/water/files/WFWR_web50-119.pdf), (accessed November 2021).
- 45 U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry (ATSDR), Toxicological profile for atrazine, 2003, <https://www.atsdr.cdc.gov/ToxProfiles/tp153.pdf>, (accessed November 2021).
- 46 M. Cohen, B. Commoner, P. Bartlett, P. Cooney and H. Eisl, Exposure to endocrine disruptors from long-range air transport of pesticides. Center for the Biology of Natural Systems, Queens College, 1997, [https://www.academia.edu/5712627/Exposure\\_of\\_endocrine\\_disruptors\\_from\\_long-range\\_air\\_transport\\_of\\_pesticides\\_Cohen\\_M\\_Commoner\\_B\\_Bartlett\\_P.W\\_Cooney\\_P\\_Eisl\\_H](https://www.academia.edu/5712627/Exposure_of_endocrine_disruptors_from_long-range_air_transport_of_pesticides_Cohen_M_Commoner_B_Bartlett_P.W_Cooney_P_Eisl_H), (accessed November 2021).
- 47 J. A. Hoppin, D. M. Umbach, S. Long, S. J. London, P. K. Henneberger, A. Blair and D. P. Sandler, Pesticides are associated with allergic and non-allergic wheeze among male farmers, *Environ. Health Perspect.*, 2017, **125**, 535–543.
- 48 R. Ikonen, J. Kangas and H. Savolainen, Urinary atrazine metabolites as indicators for rat and human exposure to atrazine, *Toxicol. Lett.*, 1988, **44**, 109–112.
- 49 S. Ganesan, A. T. Comstock and U. S. Sajjan, Barrier function of airway tract epithelium, *Tissue Barriers*, 2013, **1**, e24997.
- 50 K. Donaldson, L. Tran, L. A. Jimenez, R. Duffin, D. E. Newby, N. Mills, W. MacNee and V. Stone, Combustion-derived nanoparticles: A review of their toxicology following inhalation exposure, *Part. Fibre Toxicol.*, 2005, **2**, 10.
- 51 H. Schulz, Mechanisms and factors affecting intrapulmonary particle deposition: Implications for efficient inhalation and therapies, *Pharm. Sci. Technol. Today*, 1998, **1**, 326–344.



- 52 Health and Consumer Protection Directorate-General, Scientific Committee on Plants, Opinion of the Scientific Committee on Plants on specific questions from the Commission concerning the evaluation of atrazine in the context of council directive 91/414/EEC, Brussels, Belgium: European Commission, 2003, <https://ec.europa.eu/transparency/regdoc/rep/10102/2016/EN/SWD-2016-211-F1-EN-MAIN-PART-10.PDF>, (accessed November 2021).
- 53 N. D. Jablonowski, A. Schaffer and P. Burauel, Still present after all these years: Persistence plus potential toxicity raise questions about the use of atrazine, *Environ. Sci. Pollut. Res.*, 2011, **18**, 328–331.
- 54 C. M. Benbrook, Trends in glyphosate herbicide use in the United States and globally, *Environ. Sci. Eur.*, 2016, **28**, 3.
- 55 F. Ackerman, The economics of atrazine, *Int. J. Occup. Environ. Health*, 2007, **13**, 437–445.
- 56 United States Environmental Protection Agency, Biological and Economic Analysis Division, Assessment of Potential Mitigation Measures for Atrazine, 2002, <https://ppp.purdue.edu/wp-content/uploads/2016/08/ABA-02-03.pdf>, (accessed November 2021).
- 57 R. S. Fawcett and I. A. Huxley, Two Decades of Atrazine Yield Benefits Research, Triazine Network, 2006.
- 58 S. M. Rodrigues, P. Demokritou, N. Dokoozlian, C. O. Hendren, B. Karn, M. S. Mauter, O. A. Sadik, M. Safarpour, J. M. Unrine, J. Viers, P. Welle, J. C. White, M. R. Wiesner and G. V. Lowry, Nanotechnology for sustainable food production: Promising opportunities and scientific challenges, *Environ. Sci.: Nano*, 2017, **4**, 767–781.
- 59 J. Machell, K. Prior, R. Allan and J. M. Andresen, The water energy food nexus—challenges and emerging solutions, *Environ. Sci.: Water Res. Technol.*, 2015, **1**, 15–16.
- 60 M. Nuruzzaman, M. M. Rahman, Y. Liu and R. Naidu, Nanoencapsulation, nano-guard for pesticides: a new window for safe application, *J. Agric. Food Chem.*, 2016, **64**, 1447–1483.
- 61 R. Grillo, N. Z. dos Santos, C. R. Maruyama, A. H. Rosa, R. de Lima and L. F. Fraceto, Poly ( $\epsilon$ -caprolactone) nanocapsules as carrier systems for herbicides: Physico-chemical characterization and genotoxicity evaluation, *J. Hazard. Mater.*, 2012, **231–232**, 1–9.
- 62 A. E. Pereira, R. Grillo, N. F. Mello, A. H. Rosa and L. F. Fraceto, Application of poly (epsilon-caprolactone) nanoparticles containing atrazine herbicide as an alternative technique to control weeds and reduce damage to the environment, *J. Hazard. Mater.*, 2014, **268**, 207–215.
- 63 S. N. M. Yusoff, A. Kamari and N. F. A. Aljafree, A review of materials used as carrier agents in pesticide formulations, *Int. J. Environ. Sci. Technol.*, 2016, **13**, 2977–2994.
- 64 B. T. Sousa, A. E. S. Pererira, L. F. Fraceto, H. C. de Oliveira and G. Dalazen, Effectiveness of nanoatrazine in post-emergent control of the tolerant weed *Digitaria insularis*, *J. Plant Prot. Res.*, 2020, **60**, 185–192.
- 65 Z. Clemente, R. Grillo, M. Jonsson, N. Santos, L. O. Feitosa, R. Lima and L. F. Fraceto, Ecotoxicological evaluation of poly ( $\epsilon$ -caprolactone) nanocapsules containing triazine herbicides, *J. Nanosci. Nanotechnol.*, 2014, **14**, 4911–4917.
- 66 J. D. Crapo, B. E. Barry, P. Gehr, M. Bachofen and E. R. Weibel, Cell number and cell characteristics of the normal human lung, *Am. Rev. Respir. Dis.*, 1982, **126**, 332–337.
- 67 S. J. Kemp, A. J. Thorley, J. Gorelik, M. J. Seckl, M. J. O'Hare, A. Arcaro, Y. Korchev, P. Goldstraw and T. D. Tetley, Immortalization of human alveolar epithelial cells to investigate nanoparticle uptake, *Am. J. Respir. Cell Mol. Biol.*, 2008, **39**, 591–597.
- 68 W. Yang, J. I. Peters and R. O. Williams III, Inhaled nanoparticles - a current review, *Int. J. Pharm.*, 2008, **356**, 239–247.
- 69 H. Fessi, F. Puisieux and J. P. Devissaguet, Procédé de préparation de systèmes colloïdaux dispersibles d'une substance sous forme de nanocapsules, *European Pat.*, 274961A1, 1988.
- 70 J. Zhang, X. Qin, B. Wang, G. Xu, Z. Qin, J. Wang, L. Wu, X. Ju, D. D. Bose, F. Qiu, H. Zhou and Z. Zou, Zinc oxide nanoparticles harness autophagy to induce cell death in lung epithelial cells, *Cell Death Dis.*, 2017, **8**, e2954.
- 71 T. L. Riss, R. A. Moravec, A. L. Niles, S. Duellman, H. A. Benink, T. J. Worzella and L. Minor, *Cell viability assays, Assay Guidance Manual*, ed. G. S. Sittampalam, K. Brimacombe, A. Grossman, M. Arkin, D. D. Auld, C. Austin, J. Baell, B. Bejcek, J. M. M. Caaveiro, T. D. Y. Chung, N. P. Cousens, J. L. Dahlin, V. Devanaryan, T. L. Foley, M. Glicksman, M. D. Hall, J. V. Haas, S. R. J. Hoare, J. Inglese, P. W. Iversen, S. D. Kahl, S. C. Kales, S. Kirshner, M. Lal-Nag, Z. Li, J. McGee, O. McManus, T. Riss, O. J. Trask, J. R. Weidner, M. J. Wildey, M. Xia and X. Xu, 2013, <https://www.ncbi.nlm.nih.gov/books/NBK144065/>, (accessed November 2021).
- 72 Thermo Fisher Scientific, Pierce LDH Cytotoxicity Assay Kit, <https://www.thermofisher.com/order/catalog/product/88953>, (accessed May 2018).
- 73 Peprotech, ELISA: Sandwich TMB, <https://www.peprotech.com/ELISA-Sandwich-TMB>, (accessed May 2018).
- 74 A. Katsumiti, A. J. Thorley, I. Arostegui, P. Reip, E. Valsami-Jones, T. D. Tetley and M. P. Cajaraville, Cytotoxicity and cellular mechanisms of toxicity of CuO NPs in mussel cells in vitro and comparative sensitivity with human cells, *Toxicol. In Vitro*, 2018, **48**, 146–158.
- 75 K. L. Aillon, Y. Xie, N. El-Gendy, C. J. Berkland and M. L. Forrest, Effects of nanomaterial physicochemical properties on in vivo toxicity, *Adv. Drug Delivery Rev.*, 2009, **61**, 457–466.
- 76 H. C. Fischer and W. C. Chan, Nanotoxicity: the growing need for in vivo study, *Curr. Opin. Biotechnol.*, 2007, **18**, 565–571.
- 77 H. Sun, J. Cuijuan, L. Wu, X. Bai and S. Zhai, Cytotoxicity-related bioeffects induced by nanoparticles: the role of surface chemistry, *Front. Bioeng. Biotechnol.*, 2019, **7**, 414.
- 78 P. Ruenraroengsak, S. Chen, S. Hu, J. Melbourne, S. Sweeney, A. J. Thorley, J. N. Skepper, M. S. Shaffer, T. D. Tetley and A. E. Porter, Translocation of functionalized multi-walled carbon nanotubes across human pulmonary alveolar epithelium: dominant role of epithelial type 1 cells, *ACS Nano*, 2016, **10**, 5070–5085.



- 79 A. J. Thorley, P. Ruenaroengsak, T. E. Potter and T. D. Tetley, Critical determinants of uptake and translocation of nanoparticles by the human pulmonary alveolar epithelium, *ACS Nano*, 2014, **8**, 11778–11789.
- 80 P. Ruenaroengsak, P. Novak, D. Berhanu, A. J. Thorley, E. Valsami-Jones, J. Gorelik, Y. E. Korchev and T. D. Tetley, Respiratory epithelial cytotoxicity and membrane damage (holes) caused by amine-modified nanoparticles, *Nanotoxicology*, 2012, **6**, 94–108.
- 81 P. Ruenaroengsak and T. D. Tetley, Differential bioreactivity of neutral, cationic and anionic polystyrene nanoparticles with cells from the human alveolar compartment: robust response of alveolar type 1 epithelial cells, *Part. Fibre Toxicol.*, 2015, **12**, 19.
- 82 T. Akagi, F. Shima and M. Akashi, Intracellular degradation and distribution of protein-encapsulated amphiphilic poly (amino acid) nanoparticles, *Biomaterials*, 2011, **32**, 4959–4967.
- 83 A. Barthel, M. Dass, M. Droge, J. M. Cramer, D. Baumann, M. Urban, K. Landfester, V. Mailander and I. Lieberwirth, Imaging the intracellular degradation of biodegradable polymer nanoparticles, *Beilstein J. Nanotechnol.*, 2014, **5**, 1905–1917.
- 84 A. K. Mohammad and J. J. Reineke, Quantitative detection of PLGA nanoparticle degradation in tissues following intravenous administration, *Mol. Pharmaceutics*, 2013, **10**, 2183–2189.
- 85 C. Buzea, I. I. Pacheco and K. Robbie, Nanomaterials and nanoparticles: Sources and toxicity, *Biointerphases*, 2007, **2**, MR17–71.
- 86 B. T. Thompson, R. C. Chambers and K. D. Liu, Acute respiratory distress syndrome, *N. Engl. J. Med.*, 2017, **377**, 562–572.
- 87 G. U. Meduri, G. Kohler, S. Headley, E. Tolley, F. Stentz and A. Postlethwaite, Inflammatory cytokines in the BAL of patients with ARDS: Persistent elevation over time predicts poor outcome, *Chest*, 1995, **108**, 1303–1314.
- 88 N. Mukaida, Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases, *Am. J. Physiol. Lung Cell Mol. Physiol.*, 2003, **284**, L566–L577.
- 89 W. Stankiewicz, M. P. Dabrowski, A. Chcialowski and T. Plusa, Cellular and cytokine immunoregulation in patients with chronic obstructive pulmonary disease and bronchial asthma, *Mediators Inflamm.*, 2002, **11**, 307–312.
- 90 C. A. Ruge, U. F. Schaefer, J. Herrmann, J. Kirch, O. Canadas, M. Echaide, J. Perez-Gil, C. Casals, R. Müller and C. M. Lehr, The interplay of lung surfactant proteins and lipids assimilates the macrophage clearance of nanoparticles, *PLoS One*, 2012, **7**, e40775.
- 91 G. Wang, X. Zhang, X. Liu and J. Zheng, Co-culture of human alveolar epithelial (A549) and macrophage (THP-1) cells to study the potential toxicity of ambient PM2.5: a comparison of growth under ALI and submerged conditions, *Toxicol. Res.*, 2020, **9**, 636–651.

