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Ecotoxic pharmaceuticals, personal care products, and other emerging contaminants: A review of environmental, receptor-mediated, developmental, and epigenetic toxicity with discussion of proposed toxicity to humans

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Abstract

Pharmaceuticals and personal care products (PPCPs), other emerging contaminants (ECs), and metabolites thereof are ubiquitous in the environment, both built and natural. While such compounds have been environmentally present for some time, new pharmaceuticals and replacements for other ECs phased out due to regulatory limitations are continually being introduced to market. Non-target lower organisms are exposed through affected water, atmospheric emissions, precipitation, sediments, among other routes. Biological disruption/dysfunction (such as endocrine, developmental, and epigenetic disruption) has been reported in lower organisms exposed to trace levels of PPCPs and other ECs. Such disruption/dysfunction may not be exclusively present as traditional toxic response (e.g., cancer or death) but may only slightly alter natural biological processes as a result of exposure to an exogenous chemical (e.g., an increased heart rate or altered size of dorsal fat pads in fish). The epigenome and endocrine system appear to be relatively sensitive to many PPCPs/ECs, particularly during early development.

Humans are exposed to ECs such as plasticizers and perfluorinated compounds (PFCs) mainly through ingestion (food and contaminated liquid) as well as interaction with day-to-day products (detergents, musk compounds in fragrances, etc.). Few, if any, studies have investigated trace-level toxicity of such ECs to humans as direct-exposure trials are highly unethical. However, numerous epidemiological links exist between the presence of contaminants in humans (blood, urine, and tissues) and the occurrence of diseases or other phenotypic alterations. Despite mounting interest and research, such trace-level effects on humans are greatly debated and often criticized.

This paper reviews the current understanding of PPCP/EC toxicity. Discussion of general biological disruption/dysfunction of the following seven classes of PPCP/ECs is included: analgesics, antibiotics, antineoplastic compounds, beta-blockers, endocrine disrupting compounds, PFCs, and plasticizers. A review of receptor-mediated toxicity, non-monotonic dose response relationships, developmental toxicity, and environmental epigenetics is also included. Lastly, an overview of the proposed toxicity to humans is provided including discussion of significant criticism and direction of future research.

Key Words: Pharmaceutical and personal care product (PPCP), emerging contaminant (EC), environmental toxicology, non-monotonicity, epigenetic toxicity, prenatal toxicity

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1. Introduction

Pharmaceuticals, personal care products (PPCPs), and many other synthetic organic compounds have revolutionised modern life and their use is now an indispensable component of a healthy society. Pharmaceutical use in particular is an integral component of establishing and maintaining a healthy population of both humans and livestock. The presence of PPCPs and other emerging contaminants (ECs) have been detected in sewage, surface, ground, sea, estuarine, precipitation, the atmosphere and, at times, even drinking water (Stackelberg et al. 2004; Thomas and Hilton 2004; Fent et al. 2006; Jjemba et al. 2006; Mahmoud et al. 2009; Silva et al. 2011; Lopez-Serna et al. 2013). Table 1 shows environmental concentration ranges for 27 commonly detected PPCPs/ECs. Furthermore, these compounds are also shown to be present in river suspended solids, soil, and river sediment (McClellan and Halden 2010; Walters et al. 2010; Silva et al. 2011). This so-called emerging contamination is defined as any compound, synthetic or natural, present in the environment that shows a potential to cause adverse health effects in humans and/or lower organisms and whose presence is not routinely monitored (Stuart et al. 2012; Raghav et al. 2013; USGS 2014). While such compounds have been environmentally present for some years, the extent to which they are used, and hence appear in nature, continually changes. New pharmaceuticals and replacements for phased-out chemicals in personal care products are continuously introduced to market, constantly demanding new environmental monitoring and toxicological study. The seemingly ubiquitous environmental presence and toxicity of such contaminants as well as their, at times, largely unknown breakdown/transformation products have become of great concern in relatively recent years to the scientific community.

Virtually every human and veterinary pharmaceutical, either prescription or non-prescription, has been detected in sewage treatment work (STW) effluent and aquatic environments at levels typically not exceeding ng/L (trace-level). Such contamination also

shows a widely recognised persistence in the built aquatic environment, including storage reservoirs and rarely in municipal drinking water (Sackelberg et al. 2004; Eschauzier et al. 2012). In addition to un- and partially-metabolised human and veterinary pharmaceuticals personal care product (PCP) contamination often includes residues of surfactants used in detergents and soaps, plasticisers used in product packaging and linings, musk compounds used as fragrances, personal insecticides, perfluorinated compounds used as domestic anti-stick or hydrophobic textile linings, among others. Other so-called ECs include non-commonly monitored agricultural and industrial chemicals (e.g., perfluorinated compounds, industrial plasticisers, many herbicides, pesticides and so-called 'double use' pharmaceuticals used in animal husbandry) and even residues of recreational drugs and their metabolites. Recent concern regarding groundwater contamination by chemicals used in the hydraulic fracturing of shale could be considered novel emerging contaminants (Gordalla et al. 2013).

Contaminant sources are largely dependent upon their mode of use. Agriculturally focused pharmaceuticals and other agricultural ECs can accumulate in farmland and reach the aquatic environment through leaching and runoff (Thiele-Bruhn 2003; Monteiro and Boxall 2009). Sources of human PPCP contaminants are most notably marked by renal (urine) and biliary (faeces) excretions directed towards wastewater and STW facilities (Jjemba et al. 2006). Many PPCP contaminants persist through STW facilities and are introduced to the aquatic environment through STW effluents (Fent et al. 2006; Jjemba et al. 2006; Silva et al. 2011). Once in the aquatic environment, other than some heavily researched compounds such as bisphenol-A, relatively little is known regarding synergistic toxicity with other compounds, environmental- or bio-accumulation, persistence, transport and partition between solid and liquid phases of the environment. Some research has determined significant metabolites of commonly detected PPCP contaminants (e.g., bisphenol-A) but knowledge is largely limited (Zounkova et al. 2010; Lopez-Serna et al. 2013). The occasional presence of

PPCPs and ECs in municipal drinking water demonstrates standard drinking water treatment may not always be completely capable of removing contaminant residues and is potentially a human exposure source of extremely low amounts of such chemicals and metabolites (Sackelberg et al. 2004; Eschauzier et al. 2012). Many PPCPs and ECs are also present in day-to-day products including food (plasticisers in packaging), clothing (perfluorinated hydrophobic coatings, cleaning products, and stain repellents), soaps (musk compounds and surfactants), and even the atmosphere/ precipitation (volatile compounds) (Mahmoud et al. 2009; Schecter et al. 2010).

Many PPCP and ECs are suggested to be environmental toxins to exposed non-target organisms (Patisaul and Adewale 2009; Vandenberg et al. 2012; Fisher et al. 2014). However, knowledge regarding specific environmental and eco-human toxicological implications is relatively limited, particularly in terms of specific mechanisms causing proposed human health complications. As pharmaceutical compounds are designed to elicit a biological response in target organisms, so too may these contaminants affect the biology of exposed non-target organisms when their presence is of physiological significance. Such toxicity, or biological disruption/dysfunction, may not always arise as overt and classically toxic responses (e.g., cancer). A physiological response to an exogenous compound may result in a natural biological event occurring within an inappropriate biological context and still be considered biological disruption/dysfunction (e.g., increased heart rate in *D. magna* described by Villegas-Navarro et al. (2003), see Section 2.4).

Reported biological implications of PPCPs and ECs on non-target organisms range from interference in plant growth (Jjemba et al. 2002) to suggested epidemiological associations with the development of asthma in prenatally exposed inner-city children (Donohue et al. 2013; Whyatt et al. 2014). Knowledge regarding the toxicity of ECs to exposed humans is emerging but remains limited and highly debated (Willhite et al. 2008;

Goodman et al. 2009; Teeguarden et al. 2011; 2013). Exposure during certain critical periods of development may cause the most significant biological disruption. PPCP and EC exposure to receptors of stem and progenitor cells as well as epigenetic mutations are also possible mechanisms of biological disruption (Skinner et al. 2011; Prins et al. 2014). Many PPCPs and other ECs are currently known to affect endocrine function of lower organisms (Vajda et al. 2011; Fisher et al. 2014) via disruption of estrogenic hormonal pathways and have previously been associated with the stimulation of certain hormonally-stimulated cancers (Maras et al. 2006; Prins et al. 2014).

This review examines the ecotoxicological potential of common PPCPs and other ECs. In addition to discussion of reported toxicity and mechanisms thereof in lower organisms, an overview of suggested implications to human biology is included. It should be noted however that unlike exposed non-target organisms, humans are not likely exposed to a significant amount of such contaminants through water. Rather discussion of proposed biological disruption/dysfunction in humans regards exposure to the discussed non-pharmaceutical contaminants present in day-to-day life (e.g., plasticisers in packaging, can linings and in food).

2. PPCP and EC exposure and reported biological disruption/dysfunction

Pharmaceutical compounds, being specifically designed and administered to elicit biological responses in target organisms, may also elicit a biological response in non-target organisms exposed to an almost exclusively trace-level (e.g., ng/L) environmental presence of such contaminants. The effects of chronic trace-level exposure and specifically exposure at certain sensitive stages of development, is more likely than acute high dose exposure to explain observed abnormalities within exposed organisms (Fent et al. 2006). In this regard, Jjemba et al. (2006) suggest an approach to assessing the potential ecotoxicity of these

contaminants based upon three criteria: chronic toxicity, duration of exposure to non-target organisms, and bioavailability.

Current knowledge regarding the ecotoxicological potential of PPCP and other emerging contamination is largely limited to the biological and physiological effects on non-target aquatic organisms (Isidori et al. 2005; Fent et al. 2006). In vitro assays are the main mechanism by which the acute toxicity of PPCP contaminants, and their toxic potential at large, is determined (Fent et al. 2006). Aquatic biota is exposed to some level of PPCP contamination throughout their respective life cycles and contaminants can interact with cellular receptors of lower organisms in identical or similar ways as in humans (Fent et al. 2006). Although highly debated, study of toxicity to highly conserved biological mechanisms in lower organisms may offer insight into the outcomes and mechanisms of toxicity in humans exposed to such compounds through means likely other than water.

The toxicity of certain PPCPs and other emerging contaminants has been shown to vary depending on the exposed organism, duration of exposure, contaminant concentration, and developmental window in which exposed. For example, testicular oogenesis is reported in American Leopard frogs (*Rana pipiens*) exposed to 100ng/L of the herbicide atrazine (Hayes et al. 2003) but the same compound may not disrupt the biosynthesis of aquatic plants below a concentration of 2.59mg/L (Białk-Bielińska et al. 2011). Often, a compound's toxicity is calculated by extrapolation based upon high-dose and relatively acute toxicity in one species (e.g., EC_{50} determination) despite the contaminant's ability to affect different organisms with different toxic outcomes and at times with suggested non-monotonic dose response relationships (Fent et al. 2006; Vandenberg et al. 2012; Petrie et al. 2015).

Trace concentrations (e.g., ng/L) of parent compounds in isolation are not a realistic indication of the total synergistic activity of environmental PPCP and EC pollution. As many pharmaceutical contaminants are environmentally introduced after human or veterinary use,

metabolite concentrations may be more significant than that of parent compounds. Often, studies do not evaluate ecotoxicity of PPCP and EC metabolites or conjugated transformation products, which are at times still suggested to be biologically disruptive (Isidori et al. 2005; Boucher et al. 2015). Isidori et al. (2005) suggest that the phototransformation products of naproxen are more toxic than the parent compound for algae, rotifers, and microcrustaceans. The pH range within which contaminants are potentially bioavailable can also be of particular importance when considering acidic pharmaceutical compounds that may elicit different toxicological responses at different pH levels in exposed organisms (Fent et al. 2005). Furthermore, the distribution of contaminants among different components of the environment can potentially disproportionately affect certain tropic levels. For example, metals shown to accumulate in river biofilms have been indicated to increase the toxicity of certain antibiotic contaminants in an additive manner (see Section 3.2) (Morin et al. 2008; Zhang et al. 2012). Table 1 summarises several examples of biological disruption in humans and lower organisms associated with specific contaminant classes and more detailed discussion is included in section 3. This section reviews specific toxicity reported of seven groups of common PPCP and ECs: analgesics, antibiotics, antineoplastic compounds, beta-blockers, endocrine disrupting compounds, perfluorinated compounds, and plasticisers.

2.1 Analgesics

The known ecotoxic potential of analgesic contaminants varies greatly between compounds. Acute toxicity has been evaluated for selected analgesic compounds mainly in algae and plankton (Ferrari et al. 2004). Diclofenac is suggested to be among the most toxic analgesics in acute exposure studies with EC_{50s} commonly reported below 100mg/L (Fent et al. 2006). Ferrari et al. (2004) found phytoplankton highly sensitive to diclofenac in acute, high-level exposure with an EC_{50} value of 14.5mg/L at 96 hours.

Diclofenac is a commonly studied analgesic often detected at trace-level (e.g., ng/L) in the environment. A high death rate of vultures in India and Pakistan reported between 2000 and 2003 was attributed to diclofenac exposure (Oaks et al. 2004). Upon closer examination, visceral gout caused by acute renal failure as a consequence of feeding on veterinary diclofenac-exposed deceased livestock was determined to cause the significant decline in regional vulture populations (Oaks et al. 2004). Renal damage due to extremely low concentrations of diclofenac in fish has also been reported. Aquatic concentrations as low as 5µg/L diclofenac caused renal lesions and gill alterations in rainbow trout after 28 days of exposure (Schwaiger et al. 2004). Reproduction does not appear to be greatly affected by trace-level analgesic contamination as diclofenac was only observed to delay zebrafish hatching at concentrations of 1000-2000µg/L (Hallare et al. 2004). At a much lower, and more environmentally appropriate concentration, ibuprofen was indicated to increase in the prevalence of micronuclei in *Oreochromis niloticus* (tilapia) exposed to 0.3µg/L for 10 days (Ragugnetti et al. 2011). Recent evidence suggests naproxen bound to aquatic sediment at levels of 1/10th previously demonstrated LC_{50} and NOAEL (339.2mg/kg and 76.6mg/kg respectively) increased protein and lipid oxidation and oxidative DNA damage in the amphipod crustacean *H. Azteca* (Lucero et al. 2015).

2.2 Antibiotics

The use of antibiotic medications in human and veterinary populations has been of significant importance since their use became widespread in the mid-20th century. In recent years, the presence of these compounds in the aquatic environment has caused concern within the scientific community. It is now understood that antibiotic contamination of the aquatic environment is common and the potential effects are poorly understood at large (Brosche et al. 2010; Białk-Bielińska et al. 2011; González-Pleiter et al. 2013). Combinations of environmentally-relevant levels of antibiotics have been suggested to increase the likelihood

of biological disruption. Gonzalez-Pleiter et al. (2013) suggest the ratio between environmental concentrations and those predicted to effect the cyanobacterium *Anabaena* CPB4337 and green alga was greater than 1 for the binary combination of erythromycin and tetracycline.

A noteworthy systematic analysis of the ecotoxicological potential of 12 sulfonamide antibiotics commonly detected in the environment was carried out by Bialk-Bielinska et al. (2011) on both bacteria and non-target plants and alga. Here, EC_{50}-values were calculated using enzyme inhibition assays (using acetylcholine sterase and glutathione), luminescence inhibition assays (*Vibrio Fischeri*), sediment contact assay (*Arthrobacter globiformis*), reproduction inhibition assay (limnic green algae) and growth inhibition assay (duckweed, aka *Lemna minor*). EC_{50}-values ranged from >250mg/L for all 12 sulfonamides in enzyme inhibition assays to 0.02mg/L for sulfadimethoxine in the inhibition of duckweed growth (Bialk-Bielinska et al. 2011). Some acetylated metabolites of antibiotics (such as N^4-acetylsulfapyridine) have been indicated more toxic than the parent compound (sulfapyridine) through bioluminescent inhibition assays with reported EC_{50}-values of 8.2mg/L and 27.4mg/L respectively (García-Galán et al. 2012). In addition, other environmentally present elements can potentially increase the toxicity of antibiotic contaminants. Zhang et al. (2012) showed that co-contamination of ligand-like antibiotics, such as tetracyclines and quinolones, with heavy metals copper, zinc, and cadmium may result in a more toxic metal-antibiotic association. Oxytetracycline (OTC) and ciprofloxacin were shown to interact with heavy metals through binding multiple coordination sites in this way (Zhang et al. 2012). Here, EC_{50}-values were calculated using luminescence inhibition of *Vibro fischeri* and growth inhibition of green algae *Scenedesmus obliquus*. Reported EC_{50}-values decreased from 1.17mmol/L (OTC) to 0.06mmol/L (OTC-Zn complex) in luminescence inhibition assays and from 0.38mmol/L (OTC) to >0.01mmol/L (OTC-Zn) in

algal growth inhibition when they were associated with metal (Cu, Zn and Cd) contaminants (Zhang et al. 2012). It is suggested that environmental risks may be misclassified by ignoring such associations.

Concern exists that antibiotics present at trace concentrations in the environment can result in the development of antibiotic-resistant strains of both environmental and pathogenic bacteria (Baquero et al. 2008; Martinez 2009; Hong et al. 2013). Positive correlations have been found between antibiotic-resistant microorganisms and trace concentrations of aquatic antibiotic contaminants (Novo et al. 2013; Berglund 2014). This research was mainly conducted through the detection of antibiotic resistant genes (ARGs) on mobile plasmids and integrons, thus capable of affecting taxonomically unrelated species (Berglund 2014). Jiang et al. (2013) found 11 ARGs and residues of corresponding antibiotics with detection frequencies of 42-100% and at concentrations ranging from 3.66 (Tetracycline ARG B) to 162000copies/mL (Sulphonamide ARG II) in the water of the Huangpu River (Shanghi, China) using quantitative real-time PCR. In the same study, such ARG contamination was also observed in raw drinking water sources (Jiang et al. 2013). Such environmental presence of ARGs may ultimately affect the use and effectiveness of antibiotic pharmaceuticals on human populations.

2.3 Antineoplastic compounds (ACs)

The presence of antineoplastic (anti-cancer) pharmaceuticals in the aquatic environment is of emerging concern. Antineoplastic compounds are being increasingly used with a concomitant increase in the incidence of cancers (Brezovsek et al. 2014). Due to their specific mechanisms of action, such contaminants may affect conserved biological pathways in lower organisms in similar ways as in humans and currently very little is known regarding their possible ecotoxicological effects at environmentally-relevant concentrations of often less than 0.2ng/L (see Table 1) (Brezovsek et al. 2014). Individual and mixtures of ACs are

reported to show differing organism-specific toxicity (Toolaram et al. 2014) at varying concentrations (Brezovsek et al. 2014). However, cisplatin and 5-fluorouracil have been demonstrated using standard growth inhibition assays to be particularly toxic to aquatic organisms such as algae *Pseufokirchneriella subcapitata* (EC_{50} 1.52 and 0.13mg/L respectively) and Cyanobacteria *Synechococcus leopoliensis* (EC_{50} 0.67 and 1.20mg/L respectively) (Brezovsek et al. 2014). Similar EC_{50}-values have been reported elsewhere for 5-fluorouracil and cytarabine in the growth inhibition of *P. putida* (EC_{50} 0.044mg/L and 17mg/L respectively) and reproduction of *D. magna* (EC_{50} 0.1 and 10mg/L respectively) (Zounkova et al. 2010). Major metabolites of ACs are often suggested to be less toxic than parent compounds. Zounkova et al. (2010) report EC_{50} values for 5-fluorouracil metabolite a-fluoro-B-alanine of 100mg/L and >10mg/L for the disruption of *P. putida* growth and the reproduction of *D. magna* respectively as well as >500mg/L and >10mg/ml for uracil-1-B-Darabinofuranoside. Zounkova et al. (2010) suggest many antineoplastic compounds appear to be present in hospital effluents at concentrations near the EC_{50} values determined for common aquatic bacteria. Further research is needed to determine the potential eco- and genotoxic effects of antineoplastic contaminants on non-target organisms as little is presently known at environmentally-relevant concentrations (Toolaram et al. 2014).

2.4 Beta-Blockers

Research indicates the toxicity of beta-blockers is dose and species specific and can show non-monotonic trends. Beta-blockers may exert biological disruption by interaction with β_2-receptors. As in humans, fish contain β_2-receptors in reproductive, heart, and liver tissue which increase the likelihood of ecotoxic action by these compounds in these lower organisms (Haider and Baqri, 2000; Fent et al., 2006). Villegas-Navarro et al. (2003) showed that metoprolol at low concentrations (10^{-8},10^{-7} and 10^{-6} M) induced a positive chronotropic

effect on the heart of *D. magna* while exposure at higher concentrations (10^{-4}M) induced negative chronotropy. Huggett et al. (2002) found no observed effect concentrations (NOEC) and lowest observed effects concentrations (LOEC) of 1.0µg/L and 100µg/L respectively for reproduction of *H. azteca* and 125µg/L and 250µg/L respectively for the reproduction of *C. dubia*. In a 4-week reproductive function study, propranol exposure to Japanese rice fish at a concentration as low as 0.5µg/L was shown to decrease the number of eggs released, possibly due to decreased sex steroids and oxytocin (Huggett et al. 2002). Maszkowska et al. (2014) suggest that due to high sorption to sediments the bioavailable fraction of beta-blockers in water is reduced, thereby reducing the toxicity to aquatic organisms. Environmental concentrations of both metoprolol and propranol are reported with maximum values reaching 25.1µg/L and 6.5µg/L respectively in hospital effluent samples (Orias and Perrodin 2013); however their surface water levels are typically much lower (up to 0.01µg/L and 0.56µg/L respectively) (Petrie et al. 2015). Generally, beta-blockers are thought to pose little risk to aquatic organisms due to the extremely low levels they are found in the environment and sorption to sediment (Huggett et al. 2002; Maszkowska et al. 2014).

2.5 Endocrine disrupting compounds (EDCs)

Many aspects of endocrine disruption (ED) in both humans and lower organisms by exposure to PPCP and other ECs have been widely debated over recent years. Where debate currently exists often regards specific conditions leading to ED in humans and lower organisms rather than the concept as a whole. Much criticism regarding ED has been centred on the compound BPA (see section 3.7) (Willhite et al. 2008; Tyl 2009). It should be noted that while ED as a concept is widely recognised, specific ED issues are not without critics. When considering endocrine disruption, especially in the human model, extreme caution must be taken. Careful consideration of the conditions leading to ED in each study must not

be ignored or results may easily be misinterpreted. In this regard, variables such as biology of the concerned organism, gender, contaminant concentration, organism lifecycle in which exposed (e.g., adult vs. prenatal), and the specific biological endpoints being disrupted become even more essential in the interpretation of results.

Endocrine disruption as a result of PPCP and EC exposure is perhaps among the most common disruptive effects of PPCP and EC exposure. Many contaminants have been shown to mimic the action of natural hormonal signals at extremely low levels, most commonly in estrogenic hormonal pathways. The amount of an exogenous endocrine disrupting compound needed to surpass an effective threshold of occupied receptors may be lower in organisms that are already physiologically active, particularly during certain sensitive periods of development. In that regard, many environmental PPCPs and other ECs are suggested as both endocrine and developmental disrupters (see Section 4.2), with varying degrees of potency. EDCs show numerous disruptive capabilities ranging from alterations in reproductive physiology and behaviour (Vajda et al. 2008; Patisaul and Adewale 2009) to an association with the stimulation of hormonally mediated cancers (Maras et al. 2006; Prins et al. 2014). Endocrine disruption in humans has been suggested and effects in lower organisms offer additional insight into possible human toxicity although effects on humans are highly debated (see Section 5) (Teeguarden et al. 2013; Fisher et al., 2014; Prins et al. 2014; Ruiz-Hernandez et al 2015). The full study of environmental and aquatic EDCs will not be discussed here and is specifically reviewed in greater scope and detail elsewhere (Patisaul and Adewale 2009; Fisher et al. 2014).

Much concern is centered on disruption of the hypothalamic-pituitary-gonadal (HPG) axis which can potentially result in numerous reproductive and developmental complications. In the aquatic environment, endocrine disruptors have been shown to alter physiological development of fish and frog reproductive tissues (gonadal intersex) (Hayes et al. 2003,

2010; Vajda et al. 2008, 2011), create female biased sex ratios in fish populations (Wooding et al. 2006), and influence the development of fish secondary sexual characteristics (dorsal fat pad development) (Fisher et al. 2014). Wooding et al. (2006) found an 83% female sex ratio of white suckerfish collected downstream of STW facilities in greater Denver (USA) compared to 45% upstream. In the same study, gonadal deformities, such as intersex, and delayed follicular maturation were noted only downstream of STW effluents. Vajda et al. (2011) showed, under controlled conditions, that water containing a volume of 25% STW effluent (from the same location as Wooding et al. (2006)) with estrone, 17β-estradiol, estriol, 17α-ethynylestradiol, alkylphenols, and bisphenol-A (BPA) (a total estrogen equivalence of 31ng/L) was capable of elevating plasma vitellogenin expression, decreasing sperm abundance, and demasculinisation of dorsal fat pads and nuptial tubercles of adult flathead minnow fish.

Numerous anthropogenic contaminants have demonstrated endocrine disrupting capabilities at extremely low levels (e.g., low ng/L). Plasticisers such as BPA, bisphenol-AF (BPAF), and bisphenol-S (BPS), alkylphenols such as 4-nonylphenol used in detergents, herbicides, PFCs, and oral contraceptives such as 17α-ethynylestradiol (EE2) have all shown distinct EDC activity. Such compounds have been shown to elicit biological responses at concentrations far below that of other contaminants and at times in a non-monotonic profile. Welshons et al. (2003) suggest when endocrine disrupting chemicals interact with ultra-sensitive cellular receptors (such as the estrogen receptor) biological response can present at levels as low as upper parts per quadrillion concentrations. For example, Parrott and Blunt (2005) report a reduction in egg fertilisation success and the occurrence of a female-biased sex ratio in flathead minnow fish exposed to 320pg/L EE2 for 150 days post-hatching. It should be noted that this response is only considered adverse if it occurs outside a

biologically appropriate situation and may not in every case be detrimental to the organism but simply occurs as a response to an exogenous chemical.

Patisaul and Adewale (2009) outline three key concepts regarding endocrine disruption. First a latency period exists between EDC exposure and the manifestation of physiological or behavioural dysfunction (which may be as long as decades in proposed human biological disruption). Second, unlike many other contaminants, the dose response of EDCs and hormones may be non-monotonic (see Section 4.1 on non-monotonic dose responses). Third, the timing of exposure is critical to the type of endocrine disruption caused. During certain critical periods of early neural and reproductive development, sensitivity to hormones is heightened to a level where EDCs have been suggested to disrupt natural hormonal signaling. Of critical importance are the timing of first exposure, total dose, and duration of exposure. EDCs have been implicated in directly targeting various tissue stem and early progenitor cells during developmental stages in such a way that results in life-long signal pathway reprogramming and alterations of both structure and gene expression (Hu et al. 2012). Such mechanisms may prove vital to the understanding of how PPCP and EC endocrine disruption actually occurs. Research implications on disciplines including human behavioural, developmental and neuroendocrinology are increasingly apparent, highly debated, and almost exclusively limited to epidemiological study (see Section 5).

2.6 Perfluorinated compounds (PFCs)

Perfluorinated compounds (PFCs) are widely used and have been detected in both the aquatic environment and in biological tissues. Due to their affinity for proteins, PFCs are widely detected and bioaccumulate in humans (hair, blood, nails, urine) and other lower organisms (Seacat et al. 2002; Perez et al. 2012). Research suggests that longer C-F chains (8-carbons or more) are more likely to be bioaccumulative, toxic, and persistent than shorter-chained alternatives (Renner 2006). Observed half-lives of many PFC compounds in the

tissues of some mammals can exceed 200 days (Seacat et al. 2002). PFCs are commonly used in many personal care products including surfactants, cosmetics, and in hydrophobic textile coatings (Inoue et al. 2004). Study indicates that PFCs, particularly perfluorooctane sulfonate (PFOS- the end product of the breakdown of many PFCs), may affect thyroid hormone levels, foetal development, mitochondrial bioenergetics, cell-to-cell communication, neuroendocrine disruption, and birth weight (Seacat et al. 2002; Austin et al. 2003; Hu et al. 2002; Inoue et al. 2004). However, little is known regarding potential biological disruption/dysfunction of PFCs at levels commonly observed in waste, surface and, at times, drinking water. At levels higher than likely environmental exposure, an oral PFOS exposure level of 0.75mg/kg body weight/day for 182 days showed Rhesus and Cynomolgus monkeys becoming listless, uninterested in food, decreased body weight, increased liver weight, lower estradiol levels, and eventually mortality in 2 of 6 monkeys (Seacat et al. 2002). While exposure to PFOS at this level is extremely unlikely, such studies illustrate the toxic potential of PFCs at large.

PFOS has been shown to readily bioaccumulate in tissues of mammals (marine and terrestrial), birds, fish, and humans at levels far exceeding environmental concentrations (Perez et al. 2012; Wang et al. 2012a; Lin et al. 2014). Ankley et al. (2005) showed PFOS concentrations in the ovaries of flathead minnows of 21,600µg/kg when exposed to 30µg/L PFOS for a 21-day period. While typical surface/river water PFOS concentrations are <0.1µg/L, such research demonstrates the ability of PFCs to bioaccumulate at much higher and potentially biologically-significant levels (Du et al. 2009). Certain PFCs have been indicated as endocrine and developmental disruptors. PFCs are also suggested to affect the development of embryos and larvae of maternally exposed fish. Du et al. (2009) showed the F1 offspring of zebra fish exposed to concentrations of PFOS ranging from 10-250µg/L suffered malformation and mortality. Zebra fish exposed to PFOS at levels as low as 50µg/L

presented vitellogenin up regulation, inhibited gonadal growth, and liver lipid droplet accumulation in this 70-day exposure experiment (Du et al. 2009). With increasing molarities of PFOS and PFOA in combination, Ding et al. (2013) report changes from additive to synergistic then antagonistic before again synergistic interactive effects on zebra fish embryos. Combinations of PFOS and PFOA as surfactants are suggested to modify cell membrane properties at levels below reported NOECs and no effect concentrations (NECs). Rodea-Palomares et al. (2012) used flow cytrometry to reveal modifications in membrane integrity and potential of *Anabaena* CPB4337 exposed to 5mg/L PFOA and PFOS for 72hrs (well below calculated NOECs and NECs). Alterations to membranes were suggested to alter susceptibility to toxic herbicides in compound-specific and at times opposing ways (Rodea-Palomares et al. 2012).

Perfluroinated alcohols have been suggested to exert potential toxic effects. Fluorotelomer alcohols (FTOH) 6:2 and 8:2 have shown xenoestrogenic action, causing human MCF-7 breast cancer cells to re-enter S-phase of the cell cycle after 24hrs exposure at 30µM and 10µM respectively while PFOS did not (Maras et al. 2006). FTOH compounds have been found in both the atmosphere and precipitation. Typical concentrations of FTOH compounds are <5ng/L in surface water and <2ng/L in precipitation which are below levels typically associated with biological disruption/dysfunction (Mahmoud et al. 2009). While research defines the potential toxicity of trace-level PFC exposure, further work is needed to determine possible biological responses to PFCs at concentrations more typical of those observed in water or accumulated in organisms.

2.7 Plasticisers

Plasticisers are used to increase the fluidity/ plasticity of a material and are among the most commonly detected contaminants showing a nearly ubiquitous presence in all components of modern society. The use of plasticisers is vast and can be found in all media

from plastic bottles to dental sealants and in food via plastic packaging. Environmental toxicity of plasticisers is relatively well studied, at both chronic low-dose and acute high-dose exposure levels. The most well-known, and well-studied, of ecotoxic plasticisers is bisphenol-A (BPA). The suggested toxic effects of BPA, and other plasticisers, are numerous and often classified as endocrine disrupting compounds (see Section 2.5). Additionally, new plasticizer-mediated biological disruptions are continually being characterised, as replacements for regulatory limited compounds are brought to market. Plasticisers, and in particular BPA, are indicated as endocrine disrupting compounds (EDCs), reproductive disruptors, obesogens, genotoxins, developmental toxins, neurobehavioral disruptors (autism and attention deficit hyperactivity disorder), stimulants of hormonally mediated cancers, and are associated with various epidemiological endpoints (Kuruto-Niwa et al. 2005; Bredhult et al. 2007, 2009; Avissar-Whiting et al. 2010; Rubin 2011; Vom Saal et al. 2012; Feng et al. 2012; Whyatt et al. 2014). BPA in particular has been indicated as a possible disrupter of human biology (see Section 5) although such research is highly debated.

It should be noted as well that the toxic effects of plasticisers, such as BPA and BPS, are reported to show non-monotonic dose responses with effects at low doses not necessarily being observed at high contaminant concentrations (Rubin 2011; Vandenberg et al. 2012). However, the non-monotonicity of biological responses to plasticisers is debated (see Section 4.1) (Willhite et al. 2008). Exposure to BPA in particular is ubiquitous across species and location showing presence in water and food to even the atmosphere, with up to 100 tonnes released atmospherically every year (Rubin 2011). Proposed human exposure to plasticisers however occurs largely orally through sources such as food and other substances exposed to plasticiser or epoxy linings (such as canned food and packaged water/other foodstuffs) (Calafat et al. 2008; Liao and Kannan 2012).

Plasticisers have been suggested to cause biological disruption in humans which is often studied through epidemiological associations between urinary or serum concentrations of contaminants and occurrence of disease/disruption (See section 4.4). Disruption to humans is only briefly discussed in this section and further detail can be found in Section 5. He et al. (2009) found arithmetic mean and geometrical mean serum BPA levels of 2.84μg/L and 0.18μg/L respectively in 17% of 952 non-occupationally exposed Chinese adults. Interestingly, urinary BPA was found to be present in only 50% of the studied Chinese population (He et al. 2009) while urinary surveys of North American populations commonly report occurrence in 95-98% of subjects (Calafat et al 2005; CDC 2014; Lassen et al. 2014). Human serum BPA levels have been reported elsewhere with average levels generally not exceeding single-digit μg/L levels and have been suggested to be higher in human males than females possibly due to gender differences in BPA androgen-related metabolism (Takeuchi and Tsutsumi 2002; Hiro et al. 2004). Greater than 95% of urinary BPA is thought to be excreted and leaving <5% entering general circulation (Inoue et al. 2001, 2003; Tyl 2009). Table 3 shows concentrations of several contaminants (including BPA) commonly detected in human tissue.

Studies regarding the toxicity of plasticisers have considered both lower and higher level organisms. Specific effects are numerous and only a few are discussed in detail here. Several dedicated reviews of plasticiser toxicity can be found elsewhere with more detailed and extensive information (Richter et al. 2007; Patisaul and Adewale 2009; Vandenberg et al. 2009; Rubin 2011). Kang et al. (2002) showed gonadal intersex in Japanese Medaka fish at a BPA concentration of 837μg/L after a relatively short three-week exposure, indicating BPA as a weak estrogenic toxin. Although here indicated as a weak toxin, more recent evidence suggests BPA to be far more potent in the stimulation of other cellular and physiological responses (Rubin 2011). Exposing time-mated Wistar rats to 25μg BPA/kg bodyweight per

day (gavage feeding) from gestation day 7 to postnatal day 22, Christiansen et al. (2014) report significantly decreased female anogenital distance, a finding not reported in male rats below a daily exposure of 250µg/kg bodyweight. Such work indicates BPA may disrupt prenatal sexual development in the rat at levels far below the currently accepted tolerable daily BPA intake of 5000µg/kg bodyweight (Christiansen et al. 2014).

Similar to BPA, other plasticisers are indicated in biological disruption. Bisphenol-AF (BPAF), a trifluoromethylated biphenyl compound, was shown to inhibit testosterone production by altering gene and protein expression in the testosterone biosynthesis pathway of male Sprague-Dawley rats orally exposed to BPAF at high concentrations (both 50 and 200mg/kg bodyweight/day) for 14 days (Feng et al. 2012). In addition to BPAF, bisphenol-S (BPS), a common replacement for BPA, is indicated to proarrhythmically affect excised female fat hearts as Gao et al. (2015) report increased ventricular arrhythmia in response to 10-9M (equal to 2.28µg/L) BPS (exposure via acute perfusion). Proarrhythmic response to BPS followed an inverted-U dose-response relationship (non-monotonic) and was also associated with alteration in Ca2+ channels (including the spontaneous release of Ca2+ from the sarcoplasmic reticulum) via estrogen receptor β signaling as well as increases in the phosphorylation of Ca2+ handling proteins (ryanodine receptor and phospholamban) (Gao et al. 2015).

Due to widespread concern regarding the health effects of BPA, its use has begun to be restricted through regulatory authorities such as the European Commission (Grignard et al. 2012). The so-called ‘free’ or non-polymerised use of BPA in thermal receipt paper pushed the USEPA to produce a comprehensive report in early 2014 on toxicological hazards of 19 possible replacements to BPA (USEPA 2014). Despite presenting 19 chemical alternatives to BPA use in thermal printer paper, most evaluated compounds were more environmentally persistent than and similarly toxic to BPA (USEPA 2014). Similarly, BPS, which has

replaced BPA in many uses, such as plastic resins, has been suggested as more environmentally persistent and similarly estrogenic to BPA (Kuruto-Niwa et al. 2005; Grignard et al. 2012; USEPA 2014).

Other so-called 'BPA-free' plastic materials are suggested to leach estrogenic compounds (Bittner et al. 2014). Using MCF-7 cell proliferation and BG1Luc firefly luciferase induction assays, Bittner et al. (2014) demonstrate that many of the compounds used as replacements for BPA-containing polycarbonate resins leached chemicals with the ability to interact with estrogen receptors. Such estrogenic replacements included acrylic, polystyrene, Tritan™, and polyethersulfone while materials such as glycol-modified polyethylene, co-polymer resins, and cyclic olefin polymer showed no estrogenic activity (Bittner et al. 2014). It should be noted that the occurrence of such activity may also be dependent upon material additives (such as colour dye) rather than the material itself.

3.0 Emerging topics: Ultra-low dose effects, non-monotonicity, pre/perinatal developmental toxicity, and environmental epigenetics with brief discussion of respective mechanisms

It should be noted that work both demonstrating and reviewing toxic or otherwise biological disruption as a result of exposure to PPCPs/ECs at very low doses (in particular non-monotonicity of such dose responses) has been sharply and in some cases well-criticised in recent years (Sharpe 2010; Rhomberg and Goodman 2012). Despite what is an undeniable and growing weight of evidence such concepts are not without on-going debate and at times controversy resulting from conflicting evidence (Goodman 2009; Rhomberg and Goodman 2012).

The ability of, and extent to which PPCPs and ECs exert toxic effects on exposed organisms appear to be dependent upon several factors. Among these are the contaminant

exposure concentration, when exposure occurs during an organism's lifecycle, and the toxin's ability to disrupt the molecular mechanisms/ genetics of an organism. While specific mechanisms of action are relatively unclear, general mechanisms are reviewed in this section, where appropriate.

3.1 Receptor-mediated toxicology: ultra-low dose (low ng/L and below) effects

Many PPCPs and ECs present at trace or ultra-trace (low ng/L and below) concentrations may elicit biological responses via binding or blockage of cellular hormonal receptors rather than through traditional means of toxicity (e.g., cell/organism death) (Welshons et al. 2003; USEPA 2009). Transcriptional activity can increase agonistically when exogenous hormone mimics (such as BPA) over-saturate receptor binding sites (in lieu of endogenous hormones) and initiate mRNA production leading to a natural biological response (USEPA 2009). Similarly, under-expression or a complete lack of receptor-dependent activity can occur antagonistically by exogenous compounds simply blocking receptor binding sites to endogenous hormonal signals, preventing any transcriptional activity (USEPA 2009). In this regard, the physiological response to exogenous compounds interacting with hormone receptors can result in a natural biological event occurring within an inappropriate developmental context (e.g., prematurely, in the wrong gender, or to an inappropriate extent). It should be noted that such natural and normal biological functions occurring within an inappropriate biological context may still be considered as biological dysfunctions/disruptions. For example, vitellogenin expression and demasculinisation of dorsal fat pads and nuptial tubercles of adult male flathead minnow fish is described by Vajda et al. (2011). Here while described flathead minnows may appear healthy and still exhibit some relative reproductive success, respective reproductive systems are still physiologically disrupted (Vajda et al. 2011). Such manipulations are suggested to initiate at extremely low concentrations (suggested as low as parts per quadrillion) as hormonal receptors, such as the

highly conserved estrogen receptor (ER), function with extraordinary affinity (Welshons et al. 2003; Baumann et al. 2014; Prins et al. 2014; Reyhanian Caspillo et al. 2014).

It is important to note a difference between traditional toxic responses and those of so-called receptor-driven toxicology. Traditional endpoints of toxic response (such as cell/organism death) occur far above the point of complete hormonal receptor saturation, often from upper µg/L to lower mg/L contaminant concentrations (Welshons et al. 2003). Receptor-mediated toxicology is suggested to occur at contaminant concentrations from upper pg/L to very low µg/L (often less than 10 ng/L), much lower than regulatory testing limits (Welshons et al. 2003; Baumann et al. 2014; Reyhanian Caspillo et al. 2014). Reviewing study of ultra-low dose disruption/dysfunction, Welshons et al. (2003) suggest that physiological feedback is much more responsive when only a small amount (<10%) of ERs are bound. In the same review it was estimated that response saturation occurs when approximately half of the available ERs are bound to a hormone or exogenous hormone mimicking contaminant (Welshons et al. 2003). Of the total bound receptors inducing transcriptional activity, only a small proportion may be exogenous as the potential disruption/dysfunction occurs in an already physiologically active system (Welshons et al. 2003). Recent study suggests that receptor sensitivity may also be manipulated throughout life by developmental contaminant-receptor interaction within early stem and progenitor cells (Prins et al. 2014) (see Section 4.2).

Numerous examples of biological disruption/dysfunction have been reported and several are discussed in this section specifically regarding 17α-ethinylestradiol (EE2), a major component of oral contraceptive pills and an excellent example of effects at ultra-low doses. Other compounds have been suggested to exert disruption/dysfunction at low doses via hormonal receptors (see Section 2.5) and are reviewed elsewhere (Welshons et al. 2003; Patisaul and Adewale 2009; Fisher et al. 2014).

Exposing zebra fish (*Danio Rerio*) to 0.1-10ng/L EE2 from approximately 4hr to 60 days post-hatch (dph) followed by 40 days in a clean environment, Baumann et al. (2014) assess the occurrence and reversibility of disruption in gonad maturity, vitellogenin (VTG) expression, gender ratio, body size and weight. Here gonad immaturity was demonstrated at 3-10ng/L at 60dph in females and 1-10ng/L in males, a significant reduction in body length was shown at EE2 exposure levels between 3-10ng/L, and a female biased gender ratio (10% male where control was 61% male) at 3ng/L and an exclusively female population at 10ng/L sampled 60dph (Baumann et al. 2014). Exposing biologically disrupted fish to a 40 day EE2-free so-called 'clean recovery' period, all of the observed disruption significantly diminished or returned to levels similar to the control except reduced body length in fish exposed to 10ng/L EE2 and the body weight of fish exposed to only 1ng/L EE2 which was significantly increased (Baumann et al. 2014). Biological disruption in flathead minnows (*Pimephales promelas*) has been previously observed at lower EE2 concentrations and a longer duration of exposure. Exposing flathead minnows to EE2 from hatch through development and reproduction (150dph), Parrott and Blunt (2005) report decreased egg fertilisation success and a female-biased sex ration in fish exposed to 0.32ng/L EE2 and demasculinisation marked by a decreased male secondary sex characteristic index at EE2 exposure of 0.96ng/L.

Similar concentrations of EE2 have also been suggested to manipulate transcriptional activity of 4 genes linked to male sex determination of zebra fish: anti-mullerian hormone, double sex and mab-related protein, Sry-related HMG box-9a and nuclear receptor subfamily 5 group number 1b (Reyhanian Caspillo et al. 2014). After 14 days exposure to 25ng/L EE2, mRNA expression of all the above 4 genes were significantly decreased (Reyhanian Caspillo et al. 2014). Additionally, hepatic Estrogen Receptor (ER) alpha mRNA levels were significantly increased at an EE2 exposure as low as 5ng/L while hepatic ER-beta as well as

testicular ER-alpha and beta were unaffected by EE2 of 5-25ng/L showing tissue-specific ER sensitivity (Reyhanian Caspillo et al. 2014).

While much research has centered on ER agonism and antagonism by EDCs, this concept is inherently more extensive and not limited to the ER. As a general example, the differentiation and proliferation of bone resorbing osteoclasts from macrophages is largely dependent on the receptor for activation of nuclear factor kappa B ligand (RANKL) binding RANK (receptor for activation of nuclear factor kappa B) on pre-osteoclast surfaces (Griffen and Ojeda 2004). RANKL-RANK binding can be blocked by secretion of osteoprotegerin (OPG), thereby reducing or blocking osteoclast differentiation and proliferation (Griffen and Ojeda 2004). Absent or poor RANKL-RANK binding can result in osteoporosis while over-expression can result in osteopetrosis (Griffen and Ojeda 2004). In this example, the presence of OPG results in a similar outcome as environmental chemicals binding and blocking receptors, such as the ER, from responding to natural hormonal signals in an appropriate manner. When considering PPCPs and other ECs, research focus must regard any potentially ultra-sensitive receptors, in particular hormonal receptors. Contaminants interacting with receptors may potentially elicit toxic responses in non-target organisms by means, independent of traditional toxicological mechanisms and at concentrations far below standard regulatory testing limits (Welshons et al. 2003).

3.2 Non-monotonicity of dose responses to many PPCPs and other ECs

Conventional toxicological study holds that dose and effect move together with a positive slope in a largely linear fashion where very low drug doses result in very little to no biological response within exposed organisms. This basic foundation of contemporary toxicology (the dose makes the poison) has been challenged by the relatively recent proliferation of studies regarding endocrine disruption by chemicals at extremely low doses

(Fagin et al. 2012; Vandenberg et al. 2012). Such research has demonstrated the existence of so-called non-monotonic curves where biological responses to contaminants such as EDCs are observed and mapped at doses far below what was once thought to be too minuscule to be toxic. Such non-monotonic curves are characterised by multiple positive-to-negative slope changes of the response curve resulting in U or inverted U-type trends (Fagin et al. 2012; Vandenberg et al. 2012). Figure 1 shows several generalised representations of monotonic and non-monotonic dose response curves.

Non-monotonic responses have also been reported in natural (non-contaminant driven) biological contexts. For example, Vandenberg et al. (2006) suggest the estrogen 17β-estradiol can act agonistically and antagonistically on multiple targets or tissues in both monotonic and non-monotonic responsive manners. Exposing ovariectomised immature mice to 10 days of 17β-estradiol at eight doses (0, 0.25, 0.5, 1, 5, 10 or 50μg/kg body weight/day), Vandenberg et al. (2006) show a clear monotonic response in the development of the uterus and gene expression within the mammary gland but a non-monotonic response to the estrogen for mammary gland morphometric parameters. Here, low- and moderate-dose 17β-estradiol resulted in induction of terminal end bud formation and ductal elongation within the mammary gland while the high doses inhibited such alterations (an inverted-U non-monotonic response) (Vandenberg et al. 2006). This finding demonstrates that the same compound can naturally act on different biological end points (gene expression and morphogenesis) within the same tissue in both monotonic and non-monotonic ways. Implications on the study of responses to PPCPs and other ECs should be considered as these compounds may also act in similar manners on other hormonal receptors.

The existence of non-monotonic dose responses has been debated and the concept's relevance as a significant concern for toxicological study continues to draw critics (Tyl et al. 2008; Willhite et al. 2008; Ryan et al. 2010; Fagin et al. 2012). Study has been conducted and

reviewed which present monotonic dose responses to compounds such as BPA and induced gene expression, contradicting the existence of non-monotonicity (Willhite et al. 2008). However, non-monotonicity has previously been shown to be tissue and target specific, sometimes showing both monotonic and non-monotonic responses to the same compound in different tissues or biological contexts (Vandenberg et al. 2006).

Over relatively recent years, acceptance of non-monotonicity in regard to exposure to PPCPs and other ECs has become more widely accepted, most notably with policy changes by 8 well-respected organisations such as the US Endocrine Society, the American Society of Human Genetics, the Society for Developmental Biology, among five others in a 2011 letter published in *Science* (American Society of Human Genetics et al. 2011). In one of the subject's most comprehensive reviews, Vandenberg et al. (2012) cites over 600 studies demonstrating the existence of non-monotonic dose responses to contaminants at extremely low exposure concentrations. Much research now focuses on characterising specific mechanisms causing non-monotonic dose responses rather than demonstrating their existence (Do et al. 2012; Prins et al. 2014).

Specific examples of both non-monotonic responses and contradicting evidence is presented in greater detail elsewhere (Tyl et al. 2008; Fagin et al. 2012; Vandenberg et al. 2012). However, as a general example, Welshons et al. (2003) demonstrate inverted-U non-monotonicity when examining proliferation of MCF-7 human breast cancer cells in response to estradiol. Here MCF-7 cell proliferation begins to increase above a non-exposed control group at estradiol exposures of upper pg/L levels, reach a maximum response at low ng/L (receptor saturation and physiological dose range of estradiol) and returns to proliferation levels of the control when estradiol becomes cytotoxic at low mg/L exposure levels (Welshons et al. 2003). Estradiol, a large component of oral contraceptive medication, has been quantified in surface/river waters up to 200ng/L (Kolpin et al. 2002; Petrie et al. 2015).

3.2.1 General mechanisms causing non-monotonicity

It should be noted that mechanisms causing non-monotonicity are currently not well understood. However, biological responses elicited at extremely low concentrations are suggested as a result of the extraordinarily high affinity of hormonal receptors to both endogenous hormones and exogenous hormone-mimicking compounds (see Section 2.1) (Welshons et al. 2003; Vandenberg et al. 2012). The observed peak and plateau in inverted U-type non-monotonic dose response curves is likely due to receptor saturation. After all available receptors are occupied, no further increase in hormone or contaminant concentrations can elicit an increased response (Welshons et al. 2003). Furthermore, Welshons et al. (2003) assert that such a plateau will persist until contaminant or hormone concentrations reach cytotoxic levels, where compound response then diminishes and more traditional measures of toxicity manifest (e.g., cell death).

EDCs interacting with cellular hormone receptors during critical periods of development (see Section 4.2), may result in blockage, activation, or altered sensitivity of such receptors at stages potentially much later in life (Fagin 2012; Prins 2014). Prins (2014) shows that bisphenol-A, at concentrations typical to human exposure, binding to estrogen receptors in human prostate stem cells during developmental and on-going life stages is capable of reprogramming certain hormonal receptors to exhibit heightened sensitivity later in life. Such a mechanism is potentially a key factor in causing non-monotonic dose responses at extremely low drug/contaminant concentrations throughout an organism's respective life cycle.

3.3 Environmental toxins and pre/perinatal development

During pre/perinatal development, organisms may interpret environmental signals in such a way that phenotypes are adapted to better suit the likely demands of an environment

within which the organism will live (Moore et al. 2012). Biologically disruptive and toxic chemicals may function as such environmental signals (Kopras et al. 2014). Routes of exposure are specific to organism habitats and can include contaminated water, food, and through the atmosphere (Calafat et al. 2008; Liao and Kannan 2012). Numerous developmental effects of pre/perinatal PPCPs and other EC exposure have been suggested and several key examples are discussed in this section. Detailed reviews of environmental chemicals and developmental toxicity can be found elsewhere (Rubin and Soto 2009; Hu et al. 2012; Kopras et al. 2014). Trace-level contaminants may be particularly influential during certain stages of pre and perinatal development where foetal chemical sensitivity is heightened. During discrete developmental windows, mainly between gestation and neonatal life, hormones, at extremely low levels shape the brain in such a way that sex-specific physiology and behaviour later emerge (Patisaul and Adewale 2009). During such sensitive periods of development environmental EDCs may be interpreted *in utero* by non-target organisms as meaningful hormonal signals outside of an appropriate biological context (Kopras et al. 2014).

Several physiological barriers exist which impede the transport of potential contaminants into particularly sensitive tissue of many organisms such as that of the brain and reproductive organs of a developing foetus. However, it has been suggested that such barriers are not entirely effective at excluding certain fat-soluble or amphipathic (such as surfactants) compounds (Rodricks 2006). In this regard, many PPCPs and other emerging contaminants have the ability to cross the placental barrier and thus affect prenatal development during ultra-sensitive periods. Inoue et al. (2004) showed PFOS blood concentrations in pregnant humans of 4.9-17.6ng/ml and 1.6-5.3ng/ml in the developing foetus, indicating the placental barrier may not be entirely effective at removing certain PFCs.

Non-target developmental exposure of PPCP and EC compounds have been associated with altered birth weight. Several studies exposing pregnant mice to BPA report increased birth weights of offspring (Rubin et al. 2001, 2009; Hugo et al. 2008; Angle et al. 2013). For example, exposing pregnant CD-1 mice to doses of BPA ranging from 5-50,000μg/kg/day (10-fold below to 10-fold above the current NOAEL) Angle et al. (2013) report average unconjugated BPA fetal serum levels of 2-200pg/ml and an increase in male offspring body and liver weight at the low (but not high) exposure levels (indicating a non-monotonic dose-response relationship). In the aquatic environment, Du et al. (2009) found that zebra fish embryos and larvae (F1) from 70-day maternal (F0) exposure to PFOS concentrations ranging from 10-250μg/L exhibited malformation and mortality. Rubin and Soto (2009) review commonly disrupted endpoints in various organisms (typically rodents) affected by pre/perinatal exposure to BPA including permanent alteration to estrogen sensitivity, enhancement of adipocyte differentiation and lipid accumulation, disruption of thyroid hormone pathways (via antagonistic inhibition of thyroid hormone receptor stimulated transcriptional activity), regulation circuits for food intake and metabolism that develop perinatally in the brain, and pancreatic function of glucose regulation. Figure 2 shows several possible biological effects of PPCP and EC pre/perinatal exposure.

3.3.1 General mechanisms of toxicity during early-life-exposure

While recent research indicates developmental exposure at certain key stages may exert biological disruption throughout an organism's life, mechanisms and pathways of action are not always well, if at all characterised. There is no argument that pre/perinatal development is among, if not the most sensitive stage of development and thus PPCP and EC contamination possibly here has the greatest impact on an exposed organism's biology.

In order to affect neonatal health, PPCPs and ECs must be able to cross physiological barriers. Certain contaminants, including PFCs and synthetic musks, have demonstrated the

ability to manipulate activity (*via* inhibition) of transmembrane ATP-dependent efflux pumps, thus potentially increasing contaminant exposure where such efflux transporters contribute to physiological barriers (Lindeman et al. 2012). Perhaps the most well-studied of these efflux transporters is the p-glycoprotein (p-gp) which is present in human adrenal, blood-brain and blood-testis barrier, and kidney tissues (Stevenson et al. 2006). Study suggests that contaminant inhibition of p-gp action occurs indirectly by a relatively uncharacterised mechanism, rather than direct p-gp binding site saturation (Stevenson et al. 2006). Proposed mechanisms include interaction with trans-/integral-membrane proteins and disruption of cellular membrane fluidity leading to increased contaminant permeability (Hu et al. 2002; Stevenson et al. 2006).

Once through physiological barriers, contaminants manipulating the cellular receptors of stem or early progenitor cells in such a way that responses to hormones are disrupted later in life is one proposed mechanism by which this occurs (Prins et al. 2014). The concept of developmental exposure to certain PPCPs and other ECs reprogramming signalling pathways that result in life-long alterations in the organism is an emerging research focus and continues to draw criticism (Hu et al. 2012; Prins et al. 2014). Hu et al. (2012) review how exposure to EDCs during development, or developmental estrogenisation, is suggested to be capable of reprogramming the prostate gland in such a way that both initiates and promotes prostatic carcinogenesis later in life. This suggestion is corroborated by Prins et al. (2014) in the analysis of BPA developmental exposure on human prostate stem/progenitor cells. Here mice with human prostate stem/progenitor cell renal grafts fed 100 and 250µg BPA/kg body weight/day for two weeks showed statistically significant increases in prostatic intraepithelial neoplasia and adenocarcinoma (Prins et al. 2014). Rubin and Soto (2009) assert that the contaminant concentration, levels of circulating endogenous hormones, and the specific time

of contaminant exposure all greatly affect the mechanisms and pathways of pre/perinatal developmental disruption.

Delayed manifestation of biological disruption may have a genetic component. Some PPCPs and other ECs, including plasticisers and PFCs, have shown the ability to affect DNA both directly and through indirect, epigenetic manipulations (Yao et al. 2005; Skinner et al. 2011; Lindeman et al. 2012). Yao and Zhong (2005) showed PFOA (at 100-400µM concentrations) caused DNA strand breaks in human hepatic HepG2 cells in a dose-dependent manner, a finding that has also been confirmed in lower organisms (Fernandez Freire et al. 2008). Here, oxidative HepG2 DNA strand breakage was shown to be a result of increased intracellular reactive oxygen species after exposure to PFOA (Yao and Zhong 2005). In terms of toxicity during neonatal development, the ability of PPCPs and other ECs to affect DNA integrity in stem and early progenitor cells remains unclear. Mechanisms of epigenotoxicity of environmental pollutants are discussed in Section 4.3.1.

3.4 Environmental epigenetic action of PPCP and other ECs and multi/ transgenerational disruption

In recent years, environmentally present contaminants, such as certain PPCPs and other ECs, have been shown to interfere with epigenetic genome regulation, a possible mechanism by which human exposure to such contaminants can elicit biological disruption (Schug et al. 2011; Skinner et al. 2011; Guerrero-Bosagna et al. 2012; Nilsson and Skinner 2014). In general terms, epigenetics has become known as the study of alterations to an organism's genome independent of DNA sequence that causes the potential for modification of gene expression with the ability to be inherited across generations (Jaenisch and Bird 2003; Skinner et al. 2011). Such alterations do not include re-coding of DNA sequences but rather subtle alterations in the expression (*via* up or down regulation) of the existing genes (Schug et al. 2011; Skinner et al. 2011; Guerrero-Bosagna et al. 2012; Aiken et al. 2014;

Nilsson and Skinner 2014). In an environmental toxicological perspective, study focuses on the phenotypic manifestations resulting from epimutated genes and their inheritance across generations. Such epimutations result in altered gene expression and can elicit physiological disruption which may persist through life and even along multiple generations (Singh and Li 2012).

Contaminants present in the environment have been shown to elicit epigenetic genome alteration. Here, exposure to an epigenetic toxin, such as several known PPCPs and other ECs, in a gestating female causes either multi or transgenerational phenotypic alterations. Exposure (in the gestating F_0 generation) can affect epigenetic gene regulation of the developing embryo (F_1 generation) and organisms created by the developing germ cells within the exposed embryo (F_2 generation). This pathway is now referred to as multigenerational epigenetics (Schug et al. 2011; Skinner et al. 2011). Transgenerational epigenetics involves phenotypic disruption in the F_3 generation and generations beyond *via* the inheritance of epimutated genes as a result of ancestral (F_0 generation) exposure. Such transgenerational effects begin in progeny (F_3 generation) of the F_2 generation organisms created with germ cells that developed within the F_1 embryo exposed to an epigenetic toxin within the gestating F_0 mother (Schug et al. 2011; Skinner et al. 2011). These transgenerationally-disrupted organisms are considered exposure-free but still phenotypically altered. Figure 3 shows a schematic pathway by which organisms can be multi and transgenerationally altered by means of a DNA methylation alteration *via* PPCP or other EC F_0 generation exposure.

Early development appears to be a critically sensitive period for epigenetic manipulation by environmental toxins. Around the period of sexual differentiation of the mammalian embryo, primordial germ cells move down the genital ridge towards the newly developed gonad (Pereda et al. 2006). During this and subsequent periods of sexual tissue

differentiation/proliferation, alterations in the methylation patterns of genes by environmental toxins can result in multi and transgenerational biological disruption (Skinner et al. 2011; Guerrero-Bosagna et al. 2012). Furthermore, shortly after fertilisation, a series of demethylation events wipe the two newly joined haploid gamete genomes clean of epigenetic marks, except for several imprinted genes that are not demethylated (Schmidt 2013; Nilsson and Skinner 2014). Subsequently, all demethylated genes are ready for remethylation in line with distinct developmental stages (Schug et al. 2011; Schmidt 2013; Nilsson and Skinner 2014). Methylation configurations programmed during these periods are later largely responsible for directing differentiation of stem cells into respective tissues (Schug et al. 2011). Thus such critical periods of development are key windows where exposure to environmental epigenetic toxins can have lasting effects on organisms and their progeny.

Numerous examples of multi and transgenerational epigenetic toxicity *via* common PPCPs and other ECs are reported in humans and lower organisms. Compounds including BPA, pesticides, phytoestrogens, recreational drugs such as cocaine, and many other EDCs have shown both multi and transgenerational epigenetic toxicity (Schug et al. 2011; Skinner et al. 2011; Doyle et al. 2013). Doyle et al. (2013) showed that pregnant mice exposed to 500mg/kg body weight/day di-(2-ethylhexyl) phthalate (DEHP), a common phthalate used as a plasticiser, showed decreased sperm counts and mobility as far as the F_4 generation after exposure. It should be noted however that such an exposure is far above typical environmental levels.

Both presentation and type of biological disruption appear to be distinct between individual generation, gender and exposure concentrations (Skinner et al. 2011). For example, Anway et al. (2008) showed differing phenotypic disruption profiles at different exposure concentrations for specific compounds. Here pregnant mice were intraperitoneally exposed to either 100mg/kg body weight/day vinoclozolin (a fungicide), 5mg/kg body

weight/day flutamide (anti-androgen pharmaceutical), or 20mg/kg body weight/day flutamide. Mice exposed to vinoclozolin showed a transgenerational decrease in sperm number and increase in sperm apoptosis while flutamide exposed mice showed similar disruption in the F_1 generation only. Interestingly, spinal agenesis and limb polymelia were shown only in F_2 generation mice of the low-dose flutamide exposure group, indicating the profile of biological disruption may be exposure concentration based and generation specific (Anway et al. 2008). However, it should be noted that others have not observed such transgenerational effects of these specific compounds (Schneider et al. 2013). Despite relatively high exposure concentrations, such research is likely to inform mechanisms by which human PPCP and other EC mediated biological disruption could occur.

While most epigenetic research involves mammalian models, evidence of aquatic epigenetic toxicity does exist. Mammalian model dominance is likely due to the predominant oviparous nature of organisms in the aquatic environment. Du et al. (2009) show multigenerational toxicity of the perfluorinated compound PFOS in zebrafish. Here, after a 70-day PFOS exposure period at concentrations ranging between 10 and 250μg/L female zebrafish were allowed to breed under clean conditions where F_1 fry exhibited malformation and mortality (Du et al. 2009). However, effects beyond the F_1 generation were not analysed.

Further research is needed in this emerging field. Specifically, the degree to which synergistic interactions between epigenetic toxins can affect phenotypic disruption is particularly unclear. Furthermore, most multi and transgenerational epigenetic studies simply associate exposure with observed phenotypic (typically physiological) disruption with little, if any, investigation into specific molecular mechanisms leading to heritable epimutations. Such research will prove vital in predicting risk and development of possible treatment/ corrective measures (Skinner et al. 2011).

3.4.1 General mechanisms of epigenotoxic contaminants

Three mechanisms by which epigenetic modification occurs have been suggested: direct methylation of DNA, methylation or acetylation of histones surrounding DNA, and alteration of microRNA (miRNA) expression (Singh and Li 2012; Aiken et al. 2014). Direct methylation of DNA is indicated as the most significant and well-understood mechanism of epigenetic regulation (Aiken et al. 2014). Methylation of DNA occurs by the action of DNA methyltransferase adding a methyl group to cytosine at the carbon-5 position (Klose and Bird 2006). While DNA methylation silences gene expression by means of blocking transcriptional components, hypomethylation can promote gene expression by making these sequences more accessible to transcription, both with potential phenotypic alterations (Schug et al. 2011). Histone modification is currently not well understood (Aiken et al. 2014). Zentner and Henikoff (2013) suggest histone modifications such as methylation, phosphorylation and acetylation can alter DNA access in such a way that that affects transcription, replication and repair. It has been indicated that BPA can act as an epigenetic toxin on steroid receptor expression via this mechanism (Walker and Gore 2011). Small non-coding RNA molecules known as miRNA (microRNA) can also exert epigenetic regulation of gene expression by binding complementary regions at the 3’ end of mRNA transcripts, thus preventing translation and silencing the gene (Aiken et al. 2014; Dai et al. 2014). Dai et al. (2014) suggest that epigenetic modification and miRNA molecules can exert various effects on each other in such a way that miRNA expression can regulate epigenetic modifications as epigenetic modifications can so too regulate miRNA expression. The specific ability and extent to which PPCP and other ECs initiate the above described mechanisms remains unclear with a notable lack of research.

4.0 Overview of proposed effects in humans

Currently, insufficient direct evidence exists to positively conclude that humans are biologically or genetically affected by personal care products and emerging contaminants in the environment to any significant degree. It should be noted that there is an inherent speculative nature to the discussion of PCP/EC (personal care product/emerging contaminant) toxicity to humans as direct exposure trials with human populations are extremely unethical. Lower organisms must be used as models and a careful scientist must reasonably and intelligently draw connections between disruption in higher and lower organisms using study of highly conserved biological mechanisms when primary research is impractical or impossible. Epidemiological study can provide useful links between contaminant exposure and disease in the human model and focus future research. An incredibly large amount of epidemiological evidence exists showing links (but rarely, if ever, causation) between exposure to environmental chemicals and disease etiology. Despite numerous statistical links between the occurrence of PCPs/ECs in humans and disease, humans are undeniably exposed to other, perhaps more significant, stimuli which may also influence the development of disease. Heindel et al. (2015) suggest that biological disruption in humans may not be exclusively a result of exposure to chemicals in the environment but also by poor nutrition and stress.

The time-period of exposure to humans is also of significant importance. For example, identical exposures to an adult human and a human embryo undergoing a critical period of development (e.g., such as genomic remethylation events) are unlikely to result in identical biological disruption, if any. Uzumcu et al. (2012) suggest such exposure during development may result in permanent biological disruption/dysfunction while any exposure during adulthood may result in more transient or reversible effects. Not all proposed disruption in humans are classic toxic outcomes (e.g., such as cancer). Many studied exposure effects could be considered completely natural (such as increased adipose tissue

collection) but may simply occur at an augmented rate or within an inappropriate biological context. A natural biological response, which may not always be particularly harmful, occurring as a response to an exogenous chemical may still be considered biological disruption/dysfunction.

4.1 Routes of human exposure

Water is unlikely to be a significant source of PPCPs or ECs for human exposure as such compounds are rarely, if ever, detected in drinking water. When PPCP and other ECs are detected in drinking water their concentrations are almost exclusively at the lower levels of current analytical sensitivities (Jelic et al. 2012). Humans however are undeniably exposed to PCPs and ECs through modern lifestyles and the environment as many (non-pharmaceutical) compounds have been detected in human urine, plasma, and tissue (see Table 2 for detailed occurrence in various human tissues/liquids). In an Endocrine Society scientific statement Diamanti-Kandarakis et al. (2009) suggest human exposure to PPCPs/ECs can occur through drinking contaminated water, interacting with contaminated soil, breathing contaminated air, and ingesting food. The most significant source of exposure to humans is considered to be through diet *via* packaging (e.g., plastic containers and canned food linings) (Calafat et al. 2008; Liao and Kannan 2012).

4.2 Occurrence and possible effects of PCPs/ECs in humans

Chemicals found in the environment are also commonly found in nearly every human tested (Diamanti-Kandarakis et al. 2009). For example, the plasticiser bisphenol-A has been detected in over 90% of people sampled in the United States (Calafat et al. 2008; Rubin and Soto 2009) while the perfluorinated acid PFOA was found in 98% of US adults (Calafat et al. 2007). Perez et al. (2012) quantified the presence of eight perfluorinated acids in the hair of

24 adults including PFOA up to 6.1µg/kg of hair, PFOS up to 7.2µg/kg, PFNA up to 1.3µg/kg and PFBA up to 39.3µg/kg using turbulent flow liquid chromatography-tandem mass spectrometry. Perfluoroalkyl compounds are thought to be particularly bioaccumulated due to their high affinity for proteins (Perez et al. 2012). Inoue et al. (2004) showed PFOS blood concentrations in pregnant humans of 4.9-17.6ng/ml and 1.6-5.3ng/ml in the developing foetus, indicating the placental barrier is not entirely effective at removing certain amphipathic PFCs. Zimmers et al. (2014) quantified the presence of non-glucuronated BPA (free BPA) in the breast milk of 62% of studied humans at concentrations ranging from 0.22–10.8ng/mL. Concentrations of perfluoroalkyl compounds (including PFOA, PFOS and PFNA) as well as brominated flame retardants (such as tetrabromobiphenol A, or TBBPA) have also been detected in human breast milk (Lankova et al. 2013). Here, eight perfluoroalkyl compounds were detected in the breast milk of 50 humans including PFOA (0.012-0.128ng/ml), PFOS (0.007-0.114pg/ml) and PFNA (<0.006-0.015ng/ml) as well as TBBPA at levels of <0.060-16.2ng/ml (Lankova et al. 2013).

Typically the so-called free form of a PCP or EC is metabolized in the liver to a less toxic and more easily excreted conjugate. For example, a glucuronic acid residue is added to BPA via a glycosidic linkage to increase water solubility and hence ease of elimination from the body via urine or faeces (Liao and Kannan 2012). Nachman et al. (2015) found BPA-glucuronide (no free BPA) at concentrations ranging from <0.1µg/L to 11.21µg/L in the urine of 71% of healthy full-term urban neonates tested. It is commonly understood that glucuronated conjugates are less toxic or biologically inert which may be used as evidence that such contaminants do not exert toxic effects on humans (once glucuronated). However, recent evidence suggests in some cases this assumption may not be completely accurate. BPA, for example, can be deglucuronated back to its more toxic form by β-glucuronidase, highly present in the placenta, liver, kidney and intestines (Ginsberg and Rice 2009; Liao and

Kannan 2012). Furthermore, Boucher et al. (2015) showed treatment of human and 3T3L1 murine preadipocytes with 0.01-10μM BPA-glucuronide (BPA-G) for 8-days exhibited significant lipid accumulation and induction of adipocyte differentiation at the 10μM exposure level using Nile Red staining and quantification. Further investigation revealed statistically significant increases in mRNA expression of adipogenic markers SREBF1 and LPL after 6-days post-treatment with 10μM BPA-G (Boucher et al. 2015). BPA-G showed no estrogen receptor transcriptional activity indicating action occurs through an unknown pathway (Boucher et al. 2015). Thus, glucuorination of BPA, for example, may only change the resulting biological disruption rather than the general disruptive capabilities of the compound itself. The current safety standard for human exposure to BPA, calculated by the USEPA, is 50μg/kg body weight/day and has remained so since 1988 despite evidence that numerous biological disruptions can occur at much lower levels (Rubin 2011).

Recently, BPA has been implicated as a developmental toxin capable of promoting the self-renewal of human prostate stem/progenitor cells, thus increasing susceptibility to human prostatic carcinogenesis (Prins et al. 2014). Here statistically significant increases in prostatic intraepithelial neoplasia and adenocarcinoma were demonstrated in mice with human prostate stem/progenitor cell renal grafts fed 100 and 250μg BPA/kg body weight for two weeks (Prins et al. 2014).

4.3 Environmental epigenetics and humans

Currently there is not sufficient evidence to definitively determine if chemicals in the environment can affect the human epigenome. However, environmental epigenetics and possible effects on humans is of increasing research interest and likely a subject that will dominate future toxicological study. Several reviews can be found incorporating discussion of potential links to humans in greater detail than here including with regard to endocrine

disease (Fleisch et al. 2012), gonadal effects and female reproduction (Uzumcu et al. 2012), and DNA methylation in adults (Ruiz-Hernandez et al. 2015).

Endocrine disrupting compounds have been hypothesised to affect human gene regulation via epigenetic mechanisms (Fleisch et al. 2012; Rissman and Adli 2014; LaRocca et al. 2015; Ruiz-Hernandez et al. 2015). Despite limited epigenetics study regarding DNA methylation, Ruiz-Hernandez et al. (2015) conclude that DNA will generally tend towards hypomethylation (potentially opening promoter regions to transcription machinery) with increasing contaminant exposure levels. However, exceptions to this trend exist. For example, measuring serum levels of PFOS twice (4-5years apart) in 685 adults, an increase of 12ng/ml was associated with a 20% increase in LINE-1 (a proposed marker for cardiovascular risk) methylation (Watkins et al. 2014). No association was found with PFOA or PFNA in the same study (Watkins et al. 2014).

MicroRNA (miRNA) expression can also be associated with exposure to environmental chemicals. Occurrence of serum phthalates and phenols are associated with manipulation of miRNA expression in the placenta of pregnant humans targeting several biological pathways including serine/threonine kinase activity (LaRocca et al. 2015). Analysing first trimester urinary concentrations of 8 phenols and 11 phthalates in 179 woman in the United States, LaRocca et al. (2015) found positive correlations with expression of 3 out of 29 miRNAs tested (miR-142-3p, miR-15a-5p and miR-185). In silico prediction of mRNA targets of the three expressed miRNAs revealed inverse correlations between miRNA levels and expression of 10 genes with miR-142-3p, 20 genes with miR-185 and none with miR-15a-5p (LaRocca et al. 2014). No associations were found between placental miRNA expression and gestational age, birth weight or birth length.

Demethylation and remethylation events are among the first embryonic developmental steps and represent times of high vulnerability to epigenetic mutations within

a developing embryo. Depending on the time of exposure to epigenotoxic chemicals, mutations (particularly in imprinted genes) may result in phenotypic alterations in several subsequent generations. Uzumcu et al. (2012) describe two major epigenetic programming steps. First is a complete removal of epigenetic marks via genome-wide demethylation in the preimplantation embryo (except imprinted genes) after which remethylation can occur in a cell lineage-specific manor (Uzumcu et al. 2012). As previously mentioned, demethylation of the imprinted primordial germ cell genes does not occur until migration of the cells to the newly developed genital ridge (from the extragonadal sites). The second major epigenetic programming step involves the remethylation of imprinted genes within the primordial germ cells (Uzumuc et al. 2012). Here, remethylation occurs in a sex-specific manner: males between embryonic day 14 and 16-17 while in females, remethylation of the primordial germ cells occurs between postnatal day 1-5 through oocyte growth to the pre-antral follicle stage (Ueda et al. 2000; Obata and Kono 2002; Uzumuc et al. 2012). As remethylation occurs in sex-specific time periods, mutations due to exposure during each respective period could result in sex-specific ultimate effects. Mutations during remethylation of imprinted genes in the primordial germ line can affect future generations while mutations in the preimplantation embryo (first step) can affect both the current (developing embryo) and future generations (Uzumuc et al. 2012).

4.4 Epidemiological evidence of human effects

An incredibly large amount of epidemiological evidence has and continues to associate exposure to environmental chemicals with disease in human populations (particularly surrounding exposure to BPA, alkylphenols and other well-known endocrine disrupters). While epidemiological study may provide a statistical link between exposure to

emerging contaminants in the environment and the development of human disease, a causal link is rarely, if ever, established.

Epidemiological research has established significant links between exposure to EDCs and reduced IQ (Factor-Litvak et al. 2014; Bellanger et al. 2015). Measuring the urinary metabolite concentrations of di-n-butyl phthalate (DnBP) and di-isobutyl phthalate (DiBP) in 328 inner-city pregnant woman (measured in late pregnancy), Factor-Litvak et al. (2014) found significant inverse associations between metabolite concentrations and the children's IQ measured at age 7 years. Children of mothers within the highest and lowest quartiles of urinary metabolites of DnBP (19.4ng/ml and 79.8ng/ml respectively) and DiBP (5.0ng/ml and 19.0ng/ml respectively) concentrations showed 6.7 and 7.6 IQ-point reductions respectively when measured by the Wechsler Intelligence Scale for Children at 7 years of age and after control for cognition was completed at age 3 years (Factor-Litvak et al. 2014). Using a weight-of-evidence approach to reviewing available literature, Bellanger et al. (2015) report a 70-100% probability that polybrominated diphenyl ether and organophosphate exposure contribute to IQ loss in studied European populations. Recent research has also suggested a positive association between exposure to phthalates and the incidence of asthma in prenatally exposed inner-city children (Whyatt et al. 2014). Furthermore, analyzing pooled data of urinary BPA concentrations from adults aged 18-74 in cohorts from 2003/4 (n=1455) and 2005/6 (n=1493) significant associations were found between increased BPA concentrations and the occurrence of disease (Melzer et al. 2010). Here, a statistically significant odds ratio (OR) increase per standard deviation of urinary BPA (geometric mean 2.49ng/mL in the 2003/4 cohort and 1.79ng/mL in 2005/6) was found in association with myocardial infraction (OR=1.32, p=0.003), angina (OR=1.24, p=0.005), coronary heart disease (OR=1.42, p=0.001) and diabetes (OR=1.24, p=0.001) in the pooled cohorts (Melzer et al. 2010).

Numerous reviews have examined primary study of exposure to chemicals in the environment and the incidence of obesity (Hatch et al. 2010; La Merril and Brinbaum 2011; vom Saal et al. 2012; Thayer et al. 2012; Jeon et al. 2015), type 2 diabetes mellitus (T2DM) (Thayer et al. 2012; Jeon et al. 2015), breast cancer (Soto and Sonnenschein 2015), DNA methylation alterations in adults (Ruiz-Hernandez et al. 2015), advancement of puberty (Howdeshell et al. 1999; Fisher and Eugster 2014), poor semen quality/viability (Li et al. 2011), and various disruptions to childhood development (Meeker 2012). Conducting a comprehensive preliminary review of primary research in humans linking prenatal or early childhood exposure (up to 8 years of age) to environmental chemicals (other than tobacco smoke, alcohol, recreational drugs and pharmaceutical compounds) and susceptibility to disease/dysfunction across the human lifespan, Heindel et al. (2015) found 283 publications reporting a significant correlation between exposure and disease/dysfunction and 60 reporting non-significant associations. Of the 343 total publications identified, 169 studied links to neurological/cognitive outcomes, 45 studied links to cancer, 33 to respiratory dysfunction, 25 to reproductive health, 23 to immune disorders, 23 to obesity and other metabolic outcomes, 15 to cardiovascular dysfunctions, and less than 10 studied links to skin, thyroid, visual, and liver dysfunction. While epidemiological evidence does not provide a causal link between exposure to contaminants and biological disruption, numerous statistical associations have been noted and such research is likely to inform more detailed and future study.

4.5 Significant criticism

Perhaps the most significant question posed by such research is whether the level these chemicals are found in humans is significant enough to cause biological dysfunction/disruption. It should be noted that conflicting findings have been reported regarding the very occurrence of low-dose effects caused by exposure to plasticisers (Willhite

et al 2008; Patisaul and Adewale 2009). Critics of the low-dose toxicity of plasticisers such as BPA have cited that >95% of oral BPA is degraded in the rat prior to entering general circulation and therefore the compound cannot be responsible for low-dose effects (Inoue et al. 2001, 2003; Tyl 2009). Much criticism of plasticiser toxicity is based on viewing environmentally relevant exposure concentrations as insufficient to cause biological disruption in humans (Willhite et al 2008). Upon exposing 20 adult humans to an estimated 0.27μg BPA/kg bodyweight over a 24hour period, Teeguarden et al. (2011) found serum BPA levels ≤ limit of detection (0.297μg/L) in all collected samples. However, conflicting results have been presented elsewhere (e.g., Liao and Kannan 2012).

Research indicating effects in the human model (positive findings) has been met with criticism specifically around exposure to BPA (e.g., Goodman et al. 2009; Teeguarden et al. 2013). Reviewing reproductive and developmental effects of (mainly lower organism) exposure to BPA, Goodman et al. (2009) conclude the weight of evidence does not support the hypothesis that BPA can affect reproductive or developmental endpoints in exposed humans. Five ultimate, and potentially controversial, conclusions were drawn regarding positive findings: 1) positive findings are countered by null finding in more numerous trials, 2) are rarely if ever replicated, 3) are not coherent nor plausible, 4) are not consistent between time points, doses or species and 5) are typically from work using non-oral contaminant exposure pathways (Goodman et al. 2009). Conflicting findings have been reported between exposure to the same compound using similar methods. For example, using similar methods, Robledo et al. (2013) and Lassen et al. (2014) found conflicting ED capabilities of the same compound, BPA, but on different biological endpoints and within groups of differing contaminant exposure profiles. Examining free BPA in the urine of pregnant women (mean concentration 0.52-0.83ng/mL, group dependent), Robledo et al. (2013) found no evidence to suggest BPA had ED capabilities in terms of affecting glucose homeostasis during

pregnancy. Also examining free BPA in adult human urine (median concentration 3.25ng/mL), Lassen et al. (2014) however suggest a correlation between BPA and male reproductive health. Thus caution must be used as completely valid and well-executed studies can easily be taken out of context and applied to the study of endocrine disruption as a whole.

Drawing conclusions specifically focusing on human exposure, Teeguarden et al. (2013) assert BPA contributes little to no estrogenic disruption in humans. Reviewing 93 studies of more than 30,000 individuals across all life stages Teeguarden et al. (2013) suggest typical free BPA serum levels in humans may be several orders of magnitude below current testing limits and below levels required to occupy 0.0009% of type 2 estrogen binding receptors. It should be noted however that free and conjugated BPA levels have been reported at quantifiable levels in human serum (Liao and Kannan 2012). Furthermore, exposure to a single PCP/EC is extremely unlikely and possible synergistic interactions between contaminants must be considered.

4.6 Direction for future research

As direct exposure trials in human populations are highly unethical, directions of future research will likely remain dominated by in vitro laboratory-based and epidemiological study. Currently, few studies investigate the possible effects of synergistic mixtures of common environmental chemicals. Such study is likely to be more representative of typical exposure profiles. The study of PCP/EC exposure and possible effects on the human epigenome is of emerging focus. Such research has the potential to be explored on both epidemiological and *in vitro* scales which may shed light on possible multi- and transgenerational disruptions to organisms (biological disruption in generations not directly exposed to contaminant). Dose-response relationships must also be characterised for exposure to environmental chemicals and epigenetic outcomes.

5.0 Conclusions

PPCPs and other ECs are ubiquitous contaminants in the environment. To some degree most, if not all, organisms are exposed throughout their respective life cycles. Whether specific exposure levels are significant enough to cause biological disruption/dysfunction is debatable. Human exposure to so-called emerging contaminants is not limited to environmental sources and is dominated by exposure through day-to-day products including food, food packaging, and use of personal care products. The ability of these contaminants to persist through sewage treatment and into aquatic environments has been well established. The scientific community has at large acknowledged that such contaminants are able to exert disruptive effects across multi trophic levels in the environment. Numerous biological complications have been studied in both aquatic and terrestrial organisms as a result of trace and ultra-trace level PPCP and EC exposure (e.g., Parrott and Blunt 2005; Wooding et al. 2006; Baquero et al. 2008; Vajda et al. 2011; Hu et al. 2012; vom Saal 2012; Baumann et al. 2014; Reyhanian Caspillo et al. 2014). It is suggested that humans are no exception to this understanding. Evidence exists suggesting numerous disruptive effects of these contaminants on human biology on both laboratory and epidemiological scales (e.g., Maras et al. 2006; Patisaul et al. 2009; Fisher et al. 2014; Prins et al. 2014; Whyatt et al. 2014). Furthermore, as in lower organisms, some ECs have been suggested to affect the human epigenome via mechanisms that currently remain relatively unclear (Guerrero-Bosagna et al. 2012; Nilsson and Skinner 2014).

As the environmental presence of PPCPs and other ECs is largely unavoidable, research regarding where and under what conditions their presence is likely to elicit the greatest ecological and human harm is of upmost importance. To this regard, some studies have been conducted demonstrating clear toxic potential of many PPCPs and other ECs.

However studies rarely, if ever, consider the toxicology of synergistic activity, metabolites or biotransformation products, and full life cycle or generational exposure into account. Furthermore, research often focuses on PPCP and other EC capabilities to disrupt the endocrine systems of exposed non-target organisms (e.g., Patisaul and Adewale 2009; Vajda et al. 2011; Fisher et al. 2014). There is great scope for further research regarding possible disruption to other biological systems (e.g., nervous and immune) particularly in terms of system development during pre/perinatal exposure periods. There is a notable lack (although studies are now becoming more frequent) of evidence regarding specific epigenetic mechanisms leading to disruption/dysfunction and such research is likely to dominate the field in years to come.

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8. References

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7.0 Tables and Figures

Table 1: Concentration range of 27 commonly detected PPCP/ECs in surface water (ng/L)

Contaminant Class	Contaminant	Surface water (ng/L)
Analgesic	Ibuprofen	1-2370[a]
	Diclofenac	<0.5-154[a]
	Paracetamol	110-10000[b,c]
	Codine	12-1000[b,c]
Antibiotic	Amoxicillin	<2.5-245[a]
	Erythromycin	<0.5-159[a]
	Triclosan	140-2300[b,c]
Antidepressant	Amitriptyline	66-207[a]
	Fluoxetine	5.8-120[a,b]
	Venlafaxine	1.1-35[a]
Antineoplastic	Ifosfamide	0.05-.14[d]
	Cyclophosphamide	0.05-0.17[d]
Alkylphenols	4-nonylphenol	165.8-1187.6[e]
	4-t-octylphenol	2.4-14.5[e]
Beta Blocker	Metoprolol	<0.5-10[a]
	Atenolol	<1-487[a]
Hormones/Steroids	17α-ethynylestradiol	73-831[a,b]

	17β-estradiol	0.1-200[a,b]
	19-norethisterone	48-872[a,b]
Lipid regulator	Bezafibrate	<10-60[a]
	Gemfibrozil	48-790[b,c]
Perfluoroalkyls	PFBS	2.4-125[f]
	PFNA	0.03-52[f]
	PFOA	0.16-68[f]
	PFOS	0.4-2709[f]
Plasticiser	Bisphenol-A	140-12000[a,b,c]
	Diethyphthalate	200-420[b,c]

a Petrie et al. 2015 b Kolpin et al. 2002 c Boyd et al. 2004
d Buerge et al. 2006 e Wang et al. 2012 f Llorca et al. 2012

Table 2: Generalised biological disruption associated with specific contaminant classes: A summary of Section 2

Compound Class	**Example Biological Disruption**
Analgesics	• Renal failure and visceral gout in vultures feeding on diclofenac-exposed deceased livestock (Oaks et al. 2004) • Renal lesions and gill alterations in trout (Schwaiger et al. 2004) • Increased protein and lipid oxidation and oxidative DNA damage in the amphipod crustacean *H. Azteca* (Lucero et al. 2015) • Increase in the prevalence of micronuclei in *Oreochromis niloticus* (tilapia) exposed to 0.3µg/L for 10 days (Ragugnetti et al. 2011)
Antibiotics	• Development of antibiotic-resistant strains of both environmental and pathogenic bacteria (Baquero et al. 2008; Martinez 2009; Hong et al. 2013) • Toxicity to cyanobacteria and green alga (González-Pleiter et al. 2013)
Antineoplastic Compounds (ACs)	• Largely unknown at environmentally-relevant concentrations • Possible genotoxic effects on aquatic organisms (Toolaram et al. 2014) • Toxicity to algae and cyanobacteria (Brezovsek et al. 2014)

Beta-Blockers	• Interference with β_2-receptors in various fish (Haider and Baqri 2000; Fent et al. 2006) • Reproductive dysfunction in amphipod crustaceans (Huggett et al. 2002) • Decreased egg release in Japanese rice fish (Huggett et al. 2002)
Endocrine Disrupting Compounds (EDCs)	• Disruption of estrogenic hormonal pathways in humans and lower organisms (Patisaul and Adewale 2009; Vajda et al. 2011; Fisher et al. 2014) • Alterations of reproductive physiology and behaviour (Patisaul and Adewale 2009; Wooding et al. 2006; Vajda et al. 2011; Fisher et al. 2014) • Creation of female-biased sex rations in exposed white suckerfish populations (Wooding et al. 2006) • Delayed follicular maturation in white suckerfish (Wooding et al. 2006) • Demasculinisation of dorsal fat pads and nuptial tubercles of adult flathead minnow fish (Vajda et al. 2011) • Elevated levels of plasma vitellogenin in adult flathead minnows (Vajda et al. 2011) • Suggested as possible role in the advancement of pubertal maturation in humans (Fisher et al. 2014) • Possible targeting of stem cells during development of various organisms resulting in life-long reprogramming and alterations in structure and gene expression (Hu et al. 2012)
Perfluorinated Compounds (PFCs)	• Bioaccumulation in tissues of marine mammals, fish and birds (particularly in livers) as well as occurrence in human blood and milk (Wang et al. 2012b; Lin et al. 2014) • Induction of vitellogenesis in zebrafish (Du et al. 2009) • Malformation and mortality in the F1 generation of exposed zebrafish (Du et al. 2009) • Liver droplet accumulation and inhibited gonadal growth in exposed zebrafish (Du et al. 2009) • Stimulation of MCF-7 breast cancer cells to re-enter S-phase of the cell cycle after exposure to FTOHs (Maras et al. 2006)

Plasticisers	• Proposed action as EDCs (Kang et al. 2002; Rubin 2011; Christiansen et al. 2014) • Gonadal intersex in Jamanese Medaka fish exposed to 837µg/L BPA for 3-weeks (Kang et al. 2002) • Decreased female anogenital distance in rats exposed to 25µg BPA/kg bodyweight/day from gestation day 7 to postnatal day 22 (from 250µg BPA/kg bodyweight/day in males) (Christiansen et al. 2014) • Increased ventricular arrhythmia of excised rat hearts in response to 10^{-9}M (2.28µg/L) BPS (Gao et al. 2015) • Alteration of Ca2+ channels of excised rat hearts including spontaneous release of Ca2+ from the sarcoplasmic reticulum in response to 10^{-9}M (2.28µg/L) BPS (Gao et al. 2015) • Possible role in the advancement of puberty (Howdeshell et al. 1999; Fisher and Eugster 2014) • Epidemiological association with humans and diabetes (Thayer et al. 2012), cardiovascular disease (Melzer et al. 2010), obesity (Hatch et al. 2010; vomSaal et al. 2012; Jeon et al. 2015), deterioration of semen quality (Li et al. 2011), and development of asthma in prenatally BPA-exposed inner-city children (Whyatt et al. 2014) • Decreased human endometrial endothelial cell (HEEC) proliferation and alteration of miRNA expression in placental cells by exposure to BPA (Bredhult et al. 2007, 2009; Avissar-Whiting et al. 2010) • Promotion (BPA) of human prostate progenitor cell self-renewal and increased susceptibility to human prostatic carcinogenesis (Prins et al. 2014)

Table 3: Range of commonly detected PCPs/ECs in human tissue/liquids

Compound	Urine (ng/ml)	Serum (ng/ml)	Milk (ng/ml)	Hair (ng/g)
BPA-free	<0.01-2.82[a],<0.01-12.80*[b]	<0.01-0.59[b]	0.22-10.80[h], <0.30-19.40[i]	
BPA-Conjugated	0.11-136.15[a], 0.05-38.30*[b]	<0.05-11.90[b]		
BPA-Total	<0.50-15.90[c]		0.40-18.80[i]	
4-Nonylphenol	1.69-27.80[d]			
PFOA	2.60-1.04[e], <LOQ-2.89[f]	2.40-5.30[g]	0.21-0.49[g]	0.10-6.10[f]
PFOS	0.002-0.18[e]	0.82-48.00[g]	0.06-0.47[g]	3.70-7.20[f]

PFNA	<LOD-0.02[e]	0.43-2.50[g]	0.01-0.02[g]	0.50-1.30[f]
PFBA	52.72-1478.45[f]			8.20-39.30[f]

Reference	Notes (DF= detection frequency)
a Arbuckle et al. 2015	1,890 pregnant women (first trimester), DF BPA=43%, BPA-G=95%
b Liao and Kannan 2012	31 healthy adults, DF BPA=96.2%, BPA-G=87.1%, DF= 50% for all serum samples
c Calafat et al. 2008	2,517 participants of all age groups, total BPA detection frequency (DF)= 92.7%
d Jing et al. 2011	Urine from 60 healthy adults, nonylphenol DF= 48.3%
e Zhang et al. 2013	86 paired blood and morning urine samples, DF PFOA and PFOS= 100%, PFNA=92%
f Perez et al. 2012	30 urine samples, 24 hair samples, hair DF= 33.3% PFOA, 45.8% PFOS, 12.5% PFNA, 20.8% PFBA, urine DF= 100% PFBA, 56.7% PFOA
g Karramn et al. 2007	12 matched milk and serum samples from primiparous women, DF all serum=100%, milk PFOS/PFOS=100%, PFNA=16.6%
h Zimmers et al. 2014	21 breast milk samples from nursing mothers (USA), BPA DF=62%
i Mandonca et al. 2014	Milk from 27 postpartum women, urine from 31 of their infants, BPA-free DF milk=20%, total-BPA=75%, BPA-free DF infant urine=28%

*Creatinine adjusted

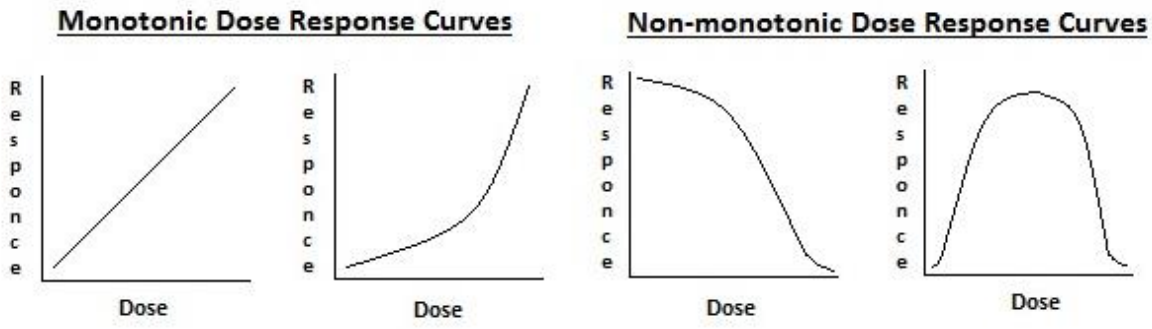

Figure 1: Representations of common monotonic and non-monotonic dose response curves plotting contaminant concentration/dose to biological response.

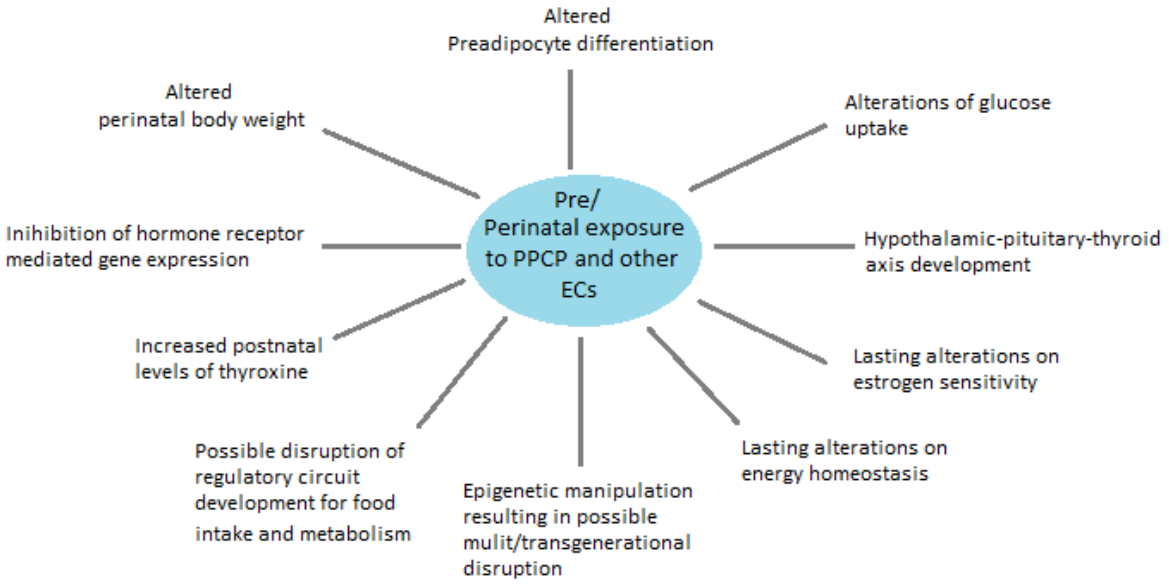

Figure 2: Possible effects of pre/perinatal PPCP and EC exposure

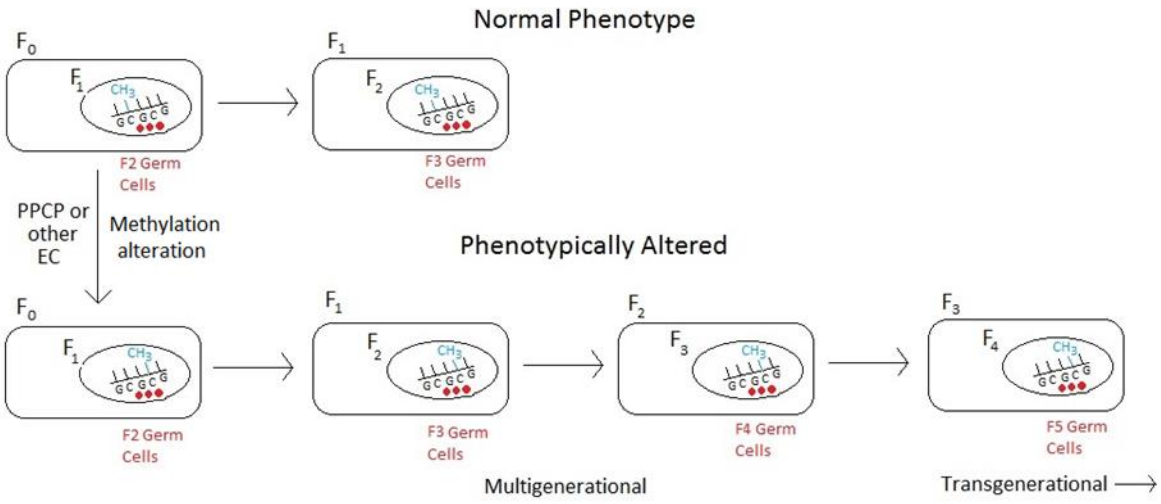

Figure 3: Generalised example of multi and transgenerational epigenetic manipulation by PPCP or other EC via DNA methylation alteration