Vibrational spectroscopy as a tool to investigate the effects of environmental contaminants in predatory birds

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Abstract

Predatory birds are vulnerable to contaminants in the environment due to their high trophic position and long lifespans. They are also important sentinel species so tools are needed to measure and monitor contaminants, not only to protect avian populations, but to confer protection to lower trophic species as well. Vibrational spectroscopy is an economic, high-throughput technique that can be used to determine biomolecular profiles and can also identify alterations induced by exposure to environmental contaminants. In this thesis, avian tissues and cells have been analysed for underlying biochemistry and for effects caused by exposure to common environmental pollutants, using attenuated total reflection Fourier-transform infrared (ATR-FTIR) and Raman spectroscopy techniques with multivariate analysis. By analysing untreated predatory bird tissues, vibrational spectroscopy was shown to reveal fundamental, underlying biochemistry. The ability to generate tissue-specific spectral profiles allows the identification of biomolecular compositional differences which may influence the effect of contaminant exposure. Brain tissue from wild, free-flying predatory birds was similarly analysed to assess the effects of 'real-world' contaminant exposure levels. Vibrational spectroscopy was demonstrated as a sensitive technique capable of distinguishing the effects of high and low contaminant exposures, protein secondary structures and elucidating sex- and age-dependant spectral differences. An avian cell line was also analysed using ATR-FTIR which was able to determine the biomolecular composition and identify significant differences between cell types. This not only verifies the suitability of such techniques for cell-based investigations but also shows they are sensitive enough to detect biochemical variations at the cellular level. ATR-FTIR was further demonstrated as a tool to identify alterations induced by single and mixtures of contaminants and as a screening tool to identify interactions in a mixture. Overall, vibrational spectroscopy was established as a sensitive tool to study the effects of environmental contaminants in avian tissues and cells, however, further research is necessary to fully validate the technique.

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Finally, I must thank my partner Matt who has never stopped encouraging me onwards and without whom none of this would have been possible.

Declaration

I declare that this thesis is my work and has not been submitted for the award of a higher degree or qualification at this university or elsewhere.

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List of abbreviations

AD: Alzheimer's Disease

AhR: Aryl Hydrocarbon Receptor

ANOVA: Analysis of variance

ATR: Attenuated Total Reflectance

B[*a*]P: Benzo[*a*]pyrene

BBB: Blood Brain Barrier

BDE: Brominated Diphenyl Ether

BHC(HCH): (β)Hexachlorocyclohexane

CCA: Canonical correspondence analysis

CCD: Charge-Coupled Device

CSF: Cerebrospinal fluid

CYP: Cytochrome P-450

DDE: p,p'-dichlorodiphenyldichloroethylene

DDT: Dichlorodiphenyltrichloroethane

DMEM: Dulbecco's Modified Essential Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic Acid

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-Linked Immunosorbent Assay

EROD: Ethoxyresorufin-O-Deethylase

FBS: Fetal bovine serum

FFS: Forward feature selection

FSH: Feature selection histogram

FTIR: Fourier-Transform Infrared

HEOD: Dieldrin

Hepox: Heptachlor epoxide

HI: Hazard Index

IR: Infrared

IRE: Internal Reflection Element

LDA: Linear Discriminant Analysis

Low-E: Low-Emissivity

MOET: Margin of Exposures

MSC: Multiplicative Scatter Correction

OC: Organochlorine

PAH: Polycyclic Aromatic Hydrocarbon

PBDE: Polybrominated Diphenyl Ether

PBPK: Physiologically Based Pharmacokinetic

PBS: Phosphate Buffered Saline

PC: Principal Components

PD: Parkinson's Disease

PC: Principal Component

PCA: Principal Component Analysis

PCB: Polychlorinated Biphenyl

PLS: Partial-Least Squares

PNEC: Predicted No Effect Concentrations

PODI: Point of Departure Index

POP: Persistent Organic Pollutant

RIPA: Radioimmunoprecipitation assay

RNA: Ribonucleic acid

ROS: Reactive Oxygen Species

RPF: Relative Potency Factor

SDS: Sodium Deoxycholate

SERS: Surface-Enhanced Raman Spectroscopy

SG: Savitzky-Golay

SGAR: Second Generation Anti-coagulant Rodenticide

SNR: Signal-to-Noise Ratio

SNV: Standard Normal Variate

SSD: Species Specific Distribution

TBS: Tris Buffered Saline

TBST: Tris Buffered Saline Tween-20

TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-diozin

TDE: Tetrachlorodiphenylethane

TEF: Toxic Equivalency Factor

UV: Ultraviolet

WoE: Weight of Evidence

Chapter 1. General Introduction

Contents

1. Introduction

Since the very first humans appeared on the globe, they have had an impact on the natural world around them. With the advent of modern farming practices and the industrial revolution, this impact has become more concerning. In the 21st century with an increasing population and therefore increasing anthropogenic activity, it is well recognised that contamination of environmental compartments with pollutants is not only detrimental to humans but also to wildlife as well. Table 1 details some of the most common chemical contaminants and their potential toxicities. All types of ecosystems are vulnerable to environmental contaminants and release can occur during the purposeful usage of chemicals such as pesticides, unintentionally due to human activities and through disposal processes, either of intentionally used chemicals or through treatment of waste such as sewage (Walker et al. 2012a). The nature of contaminants can vary from simple inorganics such as heavy metals to nanomaterials to more complex molecules such as those used as consumer product additives e.g. polybrominated diphenyl ethers (PBDEs), pesticides e.g. organochlorine (OC) insecticides or anticoagulant rodenticides. Awareness of the presence of pollutants in the environment and the potential danger they may pose has led to the development of biomonitoring methods and technologies. Biomonitoring allows the measurement of contaminant levels and the risk those chemicals pose to various species as well as facilitating any intervention that is required. An important part of biomonitoring is the use of bioindicators which are organisms or communities of organisms which are monitored for reactions to certain stimulus, in the case of environmental pollution this is the presence and concentration of chemical pollutants. Certain organisms are more suitable for use as bioindicators and factors such as distribution and practicality must be considered (Gerhardt 2002). Further to this, some bioindicators are also sentinel species which means that by monitoring those species, protection is conferred to other organisms in an ecosystem particularly those lower down in the food chain.

Contaminant	Sources	Toxic Effects
Dioxins and Furans	Car exhaust, incineration of fossil fuels, wood, peat and waste e.g. medical and municipal waste	Liver and skin damage, impaired immune function, carcinogenicity, reproductive toxicity
Heavy metals	Natural sources, anthropogenic sources from mining, smelting, burning of fossil fuels, inappropriate disposal of waste e.g. medical waste, batteries, pollution from gunshot and fishing tackle	Oxidative stress, damage to nervous, renal and pulmonary systems, acute poisoning from ingestion of contaminated material
OC pesticides	Pest management, run-off from treated land, products to protect humans from vector borne diseases e.g. anti-malarials	Neurotoxicity, endocrine disruption, reproductive toxicity
PAHs	Diesel exhaust, emissions from domestic cooking fires, cigarette smoke, other sources of incomplete combustion	Neoplasia, chronic non-cancerous effects seen in pulmonary, gastrointestinal, renal and dermatological systems
PBDEs	Release from flame retardants in furniture foams and electronics in landfill or when incinerated	Endocrine disruption including thyrotoxicity, neurotoxicity, oestrogenicity
PCBs	Accidental spillage, release from lubricants, coolants and plasticisers in electronic waste during landfill or incineration	Endocrine disruption including thyrotoxicity, neurotoxicity, oestrogenicity, developmental and reproductive toxicity, carcinogenicity
Pharmaceuticals	Leeching from landfill, agricultural run-off, effluent wastewater from hospitals, domestic buildings and drug manufacturing plants	Highly dependent on type of pharmaceutical contaminant; altered immune function, reproductive toxicity, nephrotoxicity, acute poisoning in non-target species

Table 1 – Sources of common chemical contaminants found in the environment with potential toxicity to wildlife and humans

As a group, birds have historically been vital in creating awareness of environmental pollution and its dangers. In fact, many consider the 1962 publication of 'Silent Spring' which focused on the use of DDT and the effect on songbirds (Carson 2002) as a key, first step in the

development of modern environmental monitoring work. This sparked the conception of the ecotoxicology discipline (Truhaut 1975) and set us on the path of controlling the release of substances into the environment which may harm wildlife and humans. Many species of bird are used as bioindicators but predatory birds are particularly vulnerable sentinel species. Birds of prey species accumulate contaminants as they are long-lived, apex predators thus making them sensitive monitors of environmental pollution. This thesis makes use of vibrational biospectroscopy, a powerful tool which is emerging in the field as a sensitive technique for studying environmental pollutants. Using biospectroscopy methods, both the native biochemistry of predatory bird tissues and alterations induced by exposure to environmentally relevant chemicals are identified in tissues from predatory bird sentinel species as well as in avian cells.

2. Environmental Contaminants

2.1. Organochlorine (OC) Pesticides

OC pesticides are a large and diverse class of compounds which are mainly used as insecticides. The group gets its name as each compound has at least one covalently bonded chlorine atom in its structure. The most widely used compounds in the group have included dicofol, lindane, aldrin, dieldrin and the most famous OC chemical, dichlorodiphenyltrichloroethane (DDT) along with its metabolites (Singh et al. 2016). OCs find their way into environmental compartments as they are deliberately released during their use; they are commonly applied to land and crops to prevent pests. OC pesticides were widely used during the 20th century and although usage is banned in many countries, they are still used in many developing nations such as Iran and Pakistan (Ali et al. 2014; Pirsaheb et al. 2015). DDT was first discovered to have insecticidal properties in 1939 and was used by the military in World War II to protect against vectors carrying diseases such as malaria and typhoid. After the war, it was widely used in agriculture as it was recognised as an effective and cheap insecticide (Turusov et al. 2002). However banning of the substance started later in

the century, first in Sweden in 1970 then in the United States in 1972, after scientific investigations found it to be responsible for harm to wildlife populations such as reduced reproductive success in birds (Hickey and Anderson 1968). Despite limited current usage, OCs are listed as Persistent Organic Pollutants (POPs) meaning that they resist degradation in the environment and can remain for long periods of time. Legacy POPs are also being released from melting Arctic ice (Ma et al. 2011) making OC pesticides a relevant and current concern even in the Western world.

OC pesticides work to control pest populations as they are nerve poisons. They can bind to sodium channels on nerve membranes and disturb the sodium and potassium ionic balance across membranes (Du et al. 2016). This can lead to hyper-excitation of the nervous system by causing an action potential to always be fired (O'Reilly et al. 2006). OCs can also interfere with oxidative phosphorylation processes by binding to the membranes of mitochondria and thus cause oxidative stress in cells (Kaushik and Kaushik 2007). The toxic effects of OC pesticides have been documented in many aquatic, marine and terrestrial organisms including humans. Exposure to these chemicals is not often caused by acute poisonings but chronic, cumulative exposure over time and long term exposure has been linked to multiple disease states. In humans, exposure to these compounds has been associated with dysfunction of various bodily systems and is implicated in the aetiology of multiple chronic diseases including asthma, diabetes, cancer and neurological diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD) (Mostafalou and Abdollahi 2013). Population studies have also linked a range of cancers including breast (Pestana et al. 2015), lung (Weichenthal et al. 2010), testicular (Hardell et al. 2003) and prostate (Koutros et al. 2015) cancer with OC exposure from the environment. It is thought that OC chemicals are able to cause neoplasms by interfering with the function of the endocrine system. Some OCs can mimic the structure of endogenous hormones and bind to receptors thus blocking normal hormone binding and may even alter the metabolism and synthesis of native hormones (Mostafalou and Abdollahi 2013). DDT for example has been established as an implicating factor in development of

some breast cancers. It can mimic oestrogens compounds and mediate effects by binding to oestrogen receptors (Xu et al. 2006). As OCs are also non-polar and so highly lipophilic, accumulation of these chemicals in adipose tissue particularly adipose breast tissue may be implicated. Being lipophilic, the brain is often a site of OC accumulation and possible damage as it has a high lipid content. Elevated serum levels of some OC pesticides such as lindane (Richardson et al. 2009) and dieldrin (Weisskopf et al. 2010) have been associated with PD risk. The involvement of OCs in AD risk has not been as well explored as in PD but it has been found that elevated concentrations of p,p'-dichlorodiphenyldichloroethylene (DDE), a metabolite of DDT, is a risk factor (Richardson et al. 2014). Although the etiology of these diseases is multifactorial and the role that OC pesticides may play in their development is not well understood, it is thought to concern their ability to induce oxidative stress. PD is characterised by accumulation of α-synuclein into Lewy bodies and deterioration of dopaminergic nerves in the substantia nigra, possibly due to oxidative stress and resulting loss of mitochondrial function. It has been shown in studies using dieldrin that OC-mediated generation of reactive oxygen species (ROS) can lead to α-synuclein aggregation by interfering with the function of the ubiquitin proteasome pathway (Hatcher et al. 2008). This causes α-synuclein deposition and damage in dopaminergic neurons, altering the action potential of the membranes within the neurons (Kitazawa et al. 2003; Uversky et al. 2001). Similarly, ROS induced by OC compounds is thought to stimulate aggregation of amyloid into the senile plaques which are characteristic of AD (Singh et al. 2014).

OC pesticide exposure can also affect many non-human organisms in the environment at all trophic levels but as POPs accumulate up food chains, the consequences are most pronounced in apex predatory birds and mammals. OCs can accumulate in the adipose of these creatures from relatively small exposures (Edwards 2013). OC compounds have been shown to alter reproductive (Wiig et al. 1998), immune, metabolism and endocrine (Bergman 2007) systems both in wild animals and lab subjects. The effects of releasing OC pesticides into the environment were first recognised in the 1950s when population declines of some bird species

in North America reached critical levels with predatory birds including fish eating species such as raptors (Lincer 1975) and pelicans (Blus et al. 1974) affected worst. Reproductive success was extremely low due to thinning of eggshells which was found to be due to DDE (Blus et al. 1972). It is thought that DDE can inhibit certain molecules in the muscosa of the eggshell gland which is essential for egg formation. Suggested causes are inhibition of prostaglandin synthesis and that of carbonic anhydrase which is responsible for transporting bicarbonate into the eggshell (Lundholm 1997a). Additionally, exposure to OCs has been associated with increasing embryo deformation and embryo mortality rates in birds (Tillitt and Giesy 2013). The effects of OCs have also been documented in mammals. For example, DDE also caused large population declines of Mexican free tailed bats (*Tadarida brasiliensis*) although it was thought to be due to genotoxic effects. DDE concentrations were found to be significantly increased in these bats and caused alterations in DNA (Thies et al. 1996). Accumulation of OCs in body fat in mammals adds another level of exposure as maternal transfer of pollutants can occur both *in utero* as well as through lactation. It is clear that in the past, the use of OC pesticides has caused significant detrimental effects to wildlife but to this day, OC residues are still being detected in fauna around the globe (Abbasi et al. 2016; Gundersen et al. 2013; Jürgens et al. 2015).

2.2. Polybrominated Diphenyl Ethers (PBDEs)

Polybrominated Diphenyl Ethers (PBDEs) are widely used as flame retardants in products such as plastics, electrical goods and furniture foams (WHO 1997). Increased usage in the past has led to rising environmental levels and in 2009, penta- and octa-BDEs were also listed as POPs as they can remain in the environment for long periods of time (Stockholm Convention 2012). This global rise in the concentration of PBDEs in both human tissues and the environment during the past few decades has led to safety concerns and although some congeners are no longer in use, their persistent nature has allowed them to remain a current research interest (Hites 2004). PBDEs are released into the environment both during their manufacture, transport, handling and also via their intended usage (EFSA 2011). During a

fire, they release a large volume of inflammable gas which acts to reduce heat, dilute any flammable gases present in the surrounding atmosphere and to scavenge free radical species in order to prevent a radical chain reaction (Segev et al. 2009). PBDE molecules are brominated hydrocarbons. They are composed of two phenyl rings connected by an oxygen bridge which creates the diphenyl ether moiety. There are multiple locations at which bromine atoms can attach to the rings and this variability has led to 209 possible congeners in the PBDE compound family (Rahman et al. 2001). Less brominated PBDE congeners (those with five or fewer bromine molecules) are considered more toxic and so the production and use of these PBDEs has been tightly regulated in many countries in the western world. The most available and used PBDE congeners have historically been penta-, octa- and deca- congeners but due to evidence that these chemicals may be harmful, penta- and octa- were phased out in Europe and the US before being banned by the European Union (Besis and Samara 2012; Kemmlein et al. 2009). PBDEs are released into the environment by two main mechanisms; incineration, as described above, and also via disposal of products that contain brominated flame retardants. Electronic waste makes up one of the largest components of disposed PBDE-containing waste and inappropriate disposal of such items causes PBDEs to be leeched into soils and sediments from landfill sites (Huang et al. 2011a). Exposure to PBDEs in humans is multifactorial but is thought to be largely due to the consumption of contaminated food or inhalation of dust (Jones-Otazo et al. 2005). The major exposure route can differ between geographical areas with exposure due to ingestion being key in countries such as Sweden where fatty fish are common in the adult diet (Domingo et al. 2008; Fromme et al. 2009; Tornkvist et al. 2011) whereas in the USA, inhalation of contaminated dust is potentially as important (Stapleton et al. 2005; Wu et al. 2007). Another key exposure route, affecting young children, is maternal transfer through breast milk (Carrizo and Grimalt 2007; Jakobsson et al. 2012).

Aquatic ecosystems are particularly vulnerable to PBDEs as discharge from sewage and waste water treatment plants can lead to accumulation of these compounds in the tissues of fish, particularly those close to the source of contamination or those with high fat content

(Darnerud 2003). Aquatic ecosystems also often contain mollusc species such as snails, mussels and shrimp which are filter feeders and so vulnerable to PBDEs (Wu et al. 2012). Consequently, birds and mammals which eat these are at high risk of accumulating brominated flame retardants (Voorspoels et al. 2007). Birds in particular have played an important role as global monitors of PBDEs (Elliott et al. 2005; Van den Steen et al. 2009) although much of the work has looked at piscivorous birds rather than terrestrial species which are exposed through different food sources. In terrestrial ecosystems soils are a major sink for PBDEs to accumulate in (Hale et al. 2002; Wang et al. 2011) as they are deposited from the atmosphere and by treatment with sediment from water treatment and due to their persistent nature, they are not degraded quickly (van der Veen and de Boer 2012). Plants, including edible species, that grow in contaminated soil can take up the brominated compounds (Ma et al. 2009) and then may be eaten by higher trophic species.

There is little known for certain about the toxicity of PBDE congeners in environmental settings which are usually characterised by chronic, cumulative, low level exposures, as the majority of toxicity research uses laboratory animal models and acute concentration ranges. In rats, acute exposures to penta-, octa- and deca-BDEs have been found to cause neurotoxicity, endocrine dysfunction (Hamers et al. 2006) and damage to liver and kidneys (Dunnick et al. 2012). Endocrine disruption, particularly disruption of thyroid hormones, is thought to be a key toxic mechanism of PBDE compounds. PBDEs may be able to mimic thyroid hormones and bind to the thyroxine transporter protein thus decreasing the circulating levels of thyroxine (Lema et al. 2008; Miller et al. 2009). It is also postulated that BDE mediated induction of phase II detoxification enzymes in cells may increase the clearance of thyroxine (Szabo et al. 2009). Thyroid dysfunction is of particular concern during developmental phases e.g. during foetal development for which thyroid hormones are essential (Kuriyama et al. 2007). During these stages, lower concentrations of PBDEs are needed to cause an effect (Darnerud 2003). Although many studies have measured the concentration levels of PBDEs in environmental organisms (Crosse et al. 2012b; Green and Larson 2016), scarce few studies

have looked at toxicity in environmentally relevant species and those that have often use fish or daphnia. In fish, exposure to PBDEs has been found to cause reduced spawning (Hornung et al. 1996) and in daphnia, studies often focus on mortality rates (Nakari and Huhtala 2008). It is clear that more work is needed to fully understand the effect of long-term PBDE accumulation in environmental species. Similarly in humans, measurement of PBDE levels in various body tissues (Kucharska et al. 2015; Orta-García et al. 2014) has been done but there is still much about potential toxic effects that is not understood. However, occupational exposures have been associated with nervous system and thyroid dysfunction (Darnerud 2003) so these are considered known risks.

2.3. Polychlorinated Biphenyls (PCBs)

PCBs are synthetic organic compounds with a chemical structure which allows 209 possible congeners. They were in many ways predecessors to PBDE chemicals and similarly have many industrial and commercial applications. PCBs have been commonly used as lubricants, coolants and plasticisers in electronic equipment due to their insulating properties and heat stability (Gioia et al. 2014). The use of PCBs was largely banned or restricted in the 1970s, after which their prevalence in the environment greatly reduced (Sweetman and Jones 2000), but with evidence of global recirculation (Hung et al. 2016) and as they are still used in some developing countries they are still a monitored environmental pollutant (Batterman et al. 2009). PCBs are also listed as POPs so they remain in the environment for many years. In the past, release was largely due to accidental spillages or incineration of PCB-containing materials but since the usage was restricted, inappropriate disposal of electronic waste is the most common source of release into atmospheric, terrestrial and aquatic ecosystems (Miller et al. 2015; Zhou et al. 2013). Exposure of organisms to PCBs is thought to be mostly caused by eating contaminated food, inhalation and dermal absorption. In food chains, lower trophic organisms are exposed to congers in soils and sediments and the PCBs then accumulate up the chain (Teuten et al. 2009). Being at the top of their food chain, ingestion is a major exposure

route in humans particularly through eating fatty animal-derived food products (Mamontova et al. 2007).

Structurally, PCBs can be planar or co-planar depending on the position of the chlorine molecules and the latter have been identified as dioxin-like. This is important to their toxicity as they have some ability to bind the aryl hydrocarbon receptor (AhR) and induce CYP1A1, a cytochrome P450 enzyme (Van den Berg et al. 1998). PCBs are associated with various harmful health effects including immune (Tryphonas et al. 1991), neurological (Seegal et al. 2005), developmental and reproductive dysfunction as well as potentially being carcinogenic (Smith et al. 2016). In the body, PCBs are converted into two main metabolites, by the addition of hydroxyl and methyl sulfone groups, as seen in Fig. 1, which are both reported to cause detrimental health effects. This transformation of PCB congeners gives rise to an even larger number of potentially toxic compounds (Quinete et al. 2014). PCBs with methyl sulfone groups have been detected in the environment in marine mammals (Larsson et al. 2004) and human and rat livers (Larsson et al. 2002) whereas hydroxylated PCBs have been detected in birds, humans and other mammals for decades (Jansson et al. 1975). Adverse health effects in humans and wildlife can be caused by both parent PCB compounds as well as by related metabolites. Some metabolites resemble endogenous hormones including thyroid hormones and can bind to associated receptors with very high affinity. As with PBDEs, endocrine disruption is a key toxic mechanism of these chemicals (Quinete et al. 2014). Hydroxylated PCBs with appropriately situated hydroxyl groups can bind to the thyroxine transporter protein with higher affinity that thyroxine itself (Iwasaki et al. 2002; Lans et al. 1994) and as the transporter can cross the blood brain barrier (BBB) and maternal membranes, these metabolites are thought to cause neurological problems and *in utero* developmental defects (Meerts et al. 2002). In humans, PCB-mediated endocrine disruption has been implicated in neonatal development issues such as lower birthweight, immune dysfunction and impaired development (Baibergenova et al. 2003; Wu et al. 2011b). PCB metabolites are

also reported to have strong binding affinities for other receptors such as oestrogen receptors and the AhR which can result in a wide range of downstream effects (Selvakumar et al. 2011).

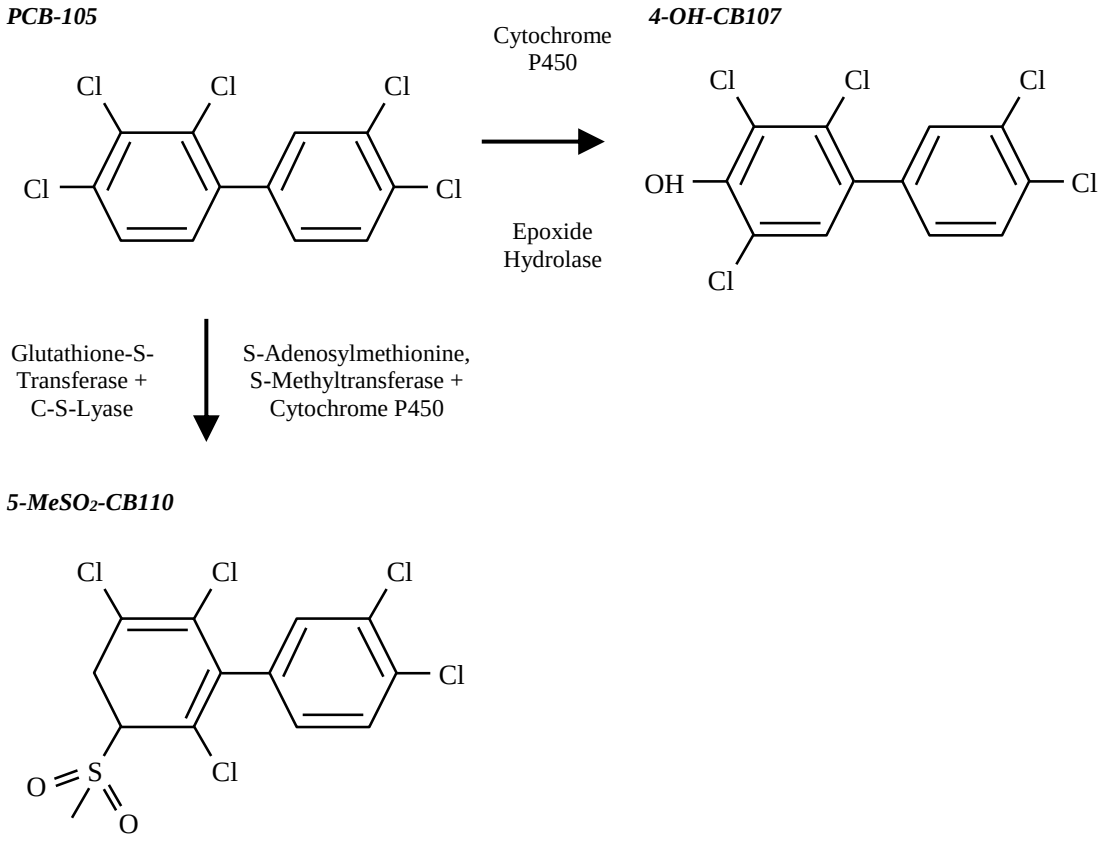


Figure 1 – Conversion of PCB molecules into hydroxylated and sulfonated reactive metabolites. Adapted from Quinete et al, 2014.

The full effects of PCB-induced endocrine disruption in environmentally important species is not completely understood but it is clear that exposure to PCBs is not only a risk to humans. In wild birds, PCBs have been reported in eggs and livers (Pereira et al. 2014) and exposure may impact reproductive success by increasing mortality and slowing the growth of chicks (Hoffman et al. 1998). Various congeners have been detected in eggs (Quinn et al. 2013) and laboratory experiments have shown that presence of PCBs in eggs can lead to feminisation of male gonads due to xenoestrogenic effects thus also affecting reproduction (Dawson 2000). Apex mammalian predators are also vulnerable. For example, one of the first cases demonstrating the toxicity of PCBs was seen in mink that failed to breed after being fed

contaminated fish (Aulerich et al. 1971; Basu et al. 2007). Very high PCB body burdens have also been detected in polar bears (*Ursus maritimus*) (Verreault et al. 2005) and experimental evidence suggests that metabolites of PCBs can fully saturate binding sites on the thyroxine transporter protein and reduce circulating levels of the hormone in these mammals (Gutleb et al. 2010). Similar observations in other marine mammals such as seals (Chiba et al. 2001) and sea lions (Debier et al. 2005) have been made. Furthermore in otters, PCB exposure is reported to cause a decrease in bone mineral density due to disruption of hormone homeostasis (Roos et al. 2010). At the other end of the food chain, studies have shown that even earthworms can accumulate PCBs from contaminated soils (Shang et al. 2013) suggesting that these chemicals can have implications for many environmental species.

2.4. Benzo(*a*)pyrene (B[*a*]P)

B[*a*]P is a five ring polycyclic aromatic hydrocarbon (PAH) which is released into the environment through incomplete combustion from wood burning, vehicle engine exhaust and cigarette smoke. Then in the atmosphere, it is adsorbed onto fine particulate matter. Unlike the other chemicals discussed here, there is no commercial usage for B[*a*]P, it is only produced for experimental purposes and environmental release comes from both anthropogenic and natural sources. B[*a*]P is a genotoxic agent and reported carcinogen although strictly speaking, in its parent form it is only a pro-carcinogen as it requires metabolic activation. When B[*a*]P enters into cells, it is bound by cytosolic AhR which activates cytochrome P450 enzymes such as CYP1A1 and CYP1B1. These enzymes along with epoxide hydrolase convert B[*a*]P into the active, diol epoxide form (see Fig. 2). In this form, it is able to intercalate into DNA by covalently bonding to guanine and can cause mutations if not repaired. The bioactivation of B[*a*]P also generates ROS which is thought to be, in part, responsible for its toxicity (Umannová et al. 2011).

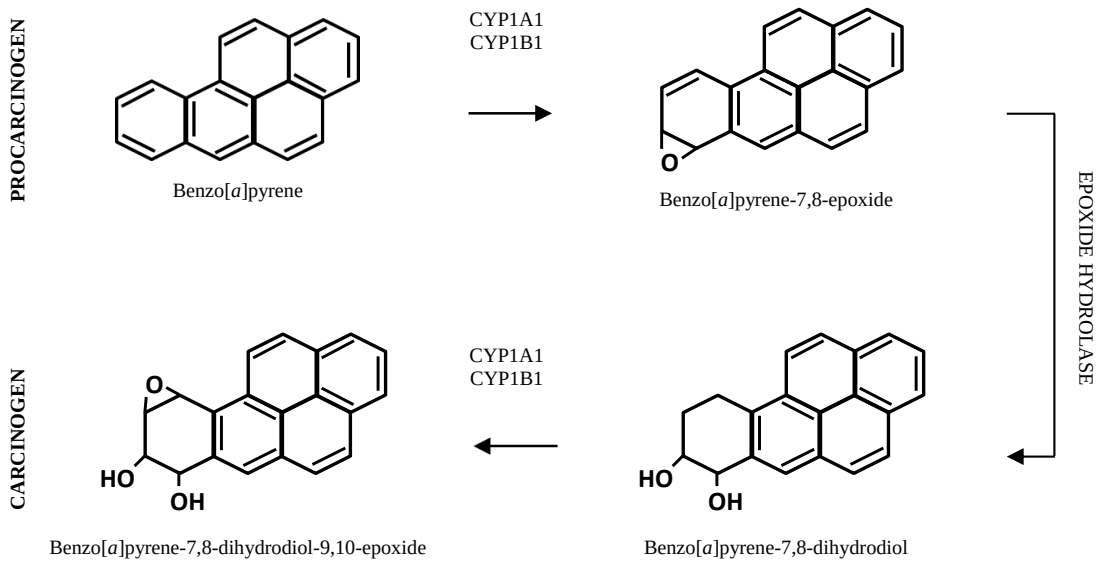


Figure 2 – Metabolic activation of parent benzo[*a*]pyrene molecule to the carcinogenic diol epoxide form.

The main route of exposure to B[*a*]P is from inhalation of contaminated air particularly from breathing in cigarette smoke and smoke from fires (Waldman et al. 1991). Ingestion of food which is burned or cooked at high temperatures is also considered an important route, particularly in non-smokers (Chen and Chen 2001). In humans, B[*a*]P is classed as an International Agency for Research on Cancer (IARC) type I carcinogen and is associated with a number of cancers including lung (Alexandrov et al. 2010), skin (Knafla et al. 2006) and liver cancers (Ba et al. 2015). B[*a*]P has been very well studied in lab animals but its role as a pollutant to ecosystems is more unclear. It has been shown to accumulate in contaminated soils where it can remain for many years and interact with microbial populations (Hernández-Castellanos et al. 2013). B[*a*]P has also been found to be slightly toxic to some terrestrial plant species, causing a reduction in growth (Sverdrup et al. 2007). PAHs have also been speculated to cause neoplasms in various wild species and it is possible that B[*a*]P causes intestinal cancer in Beluga whales (McAloose and Newton 2009). Multiple studies looking at fish have also found it to cause cancers especially liver neoplasms as well as being a powerful

inducer of ethoxyresorufin-o-deethylase (EROD) which is a marker of cytochrome P450 induction (Geeraerts and Belpaire 2010; Pacheco and Santos 2001). With a large global population of humans, production and release of B[*a*]P into the atmosphere is inevitable and so understanding its toxicity to environmental species is a vital step in understanding the risk it poses.

3. Predatory Birds

3.1. Routes of Exposure to Environmental Pollutants

Exposure of all species in the environment, including birds, to chemical pollutants is dependent on a range of biotic and abiotic factors such as dose, exposure frequency, physiological species characteristics and habitat usage. For avian species, exposure occurs through a number of major exposure routes, namely through ingestion of food, dermal absorption of contaminants or inhalation of aerosolised chemicals (Smith et al. 2007) (see Fig. 3). Ingestion of contaminated food is considered to be the most important exposure route. For some bird species, exposure can occur directly through ingestion of contaminated plant material such as seeds whereas predatory birds experience secondary exposure through consumption of prey (Hughes et al. 2013). Bioaccumulation of contaminants up food chains means that apex predatory species are particularly vulnerable to exposure by ingestion. Exposure can also occur through intake of water containing pollutants in all bird species, particularly wastewater from mining processes which is not only contaminated with harmful chemicals but is often also acidic (Eisler and Wiemeyer 2004). In terrestrial ecosystems, accidental or purposeful consumption of contaminated soil can be an important exposure factor (Mateo et al. 2006) and although predatory birds are not necessarily known for such behaviours, some species such as buzzards (*Buteo buteo*) forage for earthworms and insects and may ingest soils in this way (Dietrich et al. 1995).

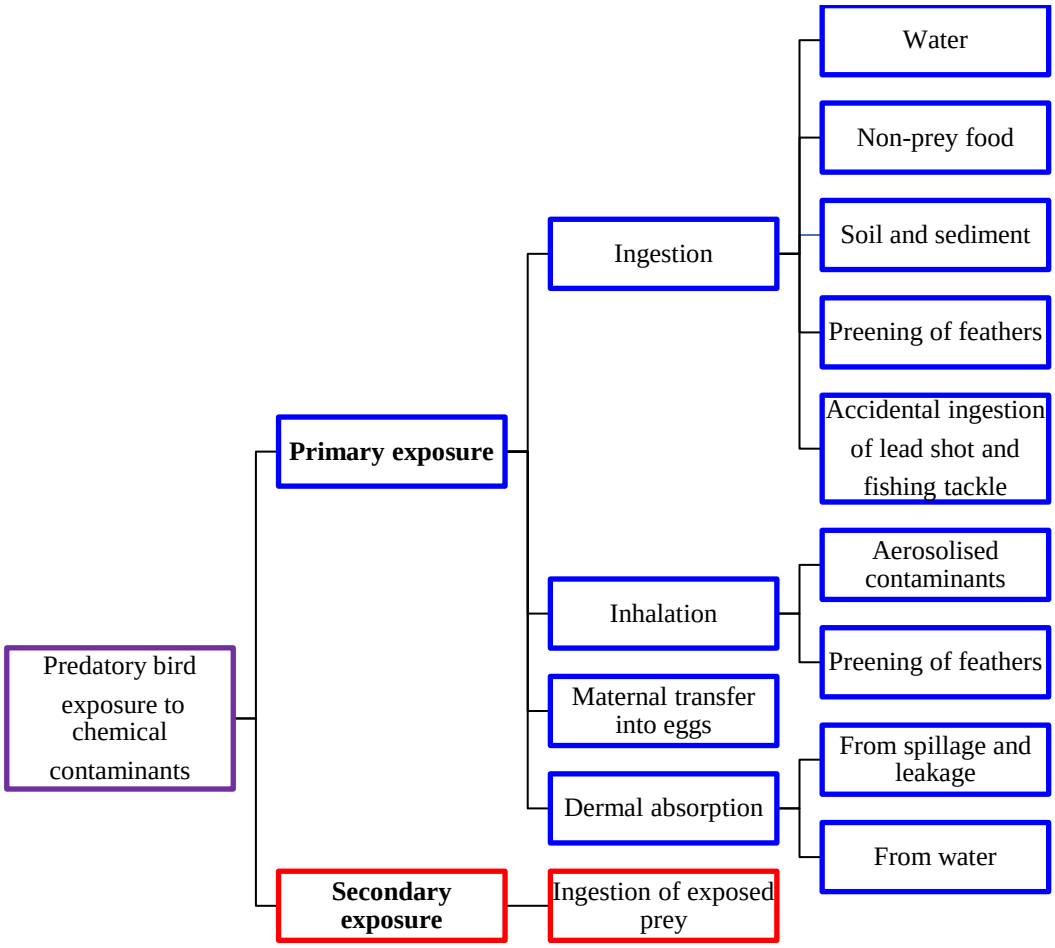


Figure 3 – Primary and secondary routes of exposure to environmental contaminants seen in predatory birds

Primary exposure routes in predatory birds are largely through intake of chemicals when preening and through contact with pollutants either through leakages or due to intentional poisonings (Berny et al. 2015; Dauwe et al. 2003; Pastor et al. 2001). However, direct exposure can also occur through accidental ingestion of lead shot or lead-containing fishing tackle in scavenged prey (Haig et al. 2014). Dermal absorption of chemicals is a less common event as the feathers can act as a skin barrier but it may be particularly important in aquatic species which enter water regularly (Smith et al. 2007). As with mammal species, maternal transfer of contaminants to offspring can also occur. Transfer of pollutants from the bodies of females into eggs, in some cases, can cause up to a third of a female's body burden to be relocated into lipid-rich egg yolks (Bargar et al. 2001; Newton et al. 1981). Furthermore, exposure can occur in young chicks due to provision of contaminated food in the nest site

from adult birds (Reynolds et al. 2001). Finally, it is important to note that exposure routes may differ for migratory bird species. Migratory birds may be exposed to a wider range of contaminants than residential birds due to changes in diet and behaviours during migration and due to differences in the destination countries (Rainio et al. 2012).

3.2. The Effects of Pollutants on Predatory Birds

Predatory birds are a diverse group which not only make ideal sentinels of environmental pollution but are also ecologically important. They act as trophic process linkers, influencing vertebrate and invertebrate prey populations, and some species also provide a regulatory function in ecosystems by scavenging carcasses (Sekercioglu 2006). Unfortunately, predatory bird populations can be severely affected by exposure to environmental contaminants due to their position at the top of their food chains and long lifespans. They are particularly vulnerable to the effects of pollutants which bioaccumulate such as POPs. Due to their ecological significance, alterations in predatory bird numbers can have substantial effects on ecosystems and food webs.

One of the first indicators that predatory birds could be significantly affected by contaminants was the population declines caused by DDT and related metabolites. Reproductive failure caused by chemicals such as DDT is still considered one of the most serious ecotoxicology events observed since the advent of the discipline. The cause of DDT-mediated predatory bird population declines is thought to be due to eggshell-thinning which led to eggs breaking during essential brooding (Ratcliffe 1967). It is also thought that DDT exposure can interfere with vitamin E synthesis in developing embryos which is a key antioxidant species (Miljeteig et al. 2012). OC contaminants have been associated with decline in numbers of many environmentally important predatory birds including ospreys (Odsjö and Sondell 2014), bald eagles (Grier 1982), gannets (Chapdelaine et al. 1987), eagle owls (Gómez-Ramírez et al. 2012) and pelicans (Blus 1982). A number of other contaminants such as PCBs, heavy metals and brominated flame retardants have also been associated with eggshell thinning of predatory

birds (Fernie et al. 2009; Wiemeyer et al. 1984). In general, OC contaminant residues are declining in bird tissues and reproductive rates have recovered for most species. However recently, elevated DDE concentrations in wild Latvian Black Stork (*Ciconia nigra*) eggs have been associated with observed decreases in egg volumes (Strazds et al. 2015) indicating that in some locations, OC pollutants may still be concern to predatory birds.

PCBs and brominated flame retardants including PBDEs are also environmental contaminants which have been pegged as potential risks to the health of wild predatory birds. Although these compounds are thought to have been partly responsible for population declines in some species, their roles have not been fully elucidated as the toxicity at environmental concentrations is poorly understood. At high enough concentrations, they are suspected to have reproductive and embryonic toxicity in birds due to endocrine disruption but environmental levels are often far below those used in laboratory studies (Quinn et al. 2013). In wild birds, PCB levels in eggs have been associated with decreased hatching success (Brunström and Halldin 2000) and thought to cause reproductive failure in piscivorous waterbirds such as cormorants and herons (Antoniadou et al. 2007). Additionally, PCB blood concentrations in adult glaucous gulls (*Larus hyperboreus*) has been linked with reduced hatching success due to decreased incubation behaviours from parents (Bustnes et al. 2001). The bioaccumulative and persistent properties of PCBs and PBDEs have enabled almost ubiquitous presence of these compounds in the environment. In particular, they have been detected in eggs and body tissues from numerous predatory bird species around the world (Chen et al. 2010; Crosse et al. 2012a; Jaspers et al. 2006; Liu et al. 2010). Given the constant exposure of wild predatory birds to these contaminants and the potential for reproductive effects, there is clearly a case for continued environmental monitoring and further laboratory-based experimentation to understand low-level effects.

As well as long-term accumulation of contaminants, acute poisonings can also have a drastic impact on wild predatory bird populations. Pharmaceuticals have received much attention in

recent years after diclofenac was found to be responsible for rapid declines in vulture numbers. Diclofenac is a non-steroidal anti-inflammatory which was used widely in Asian countries to treat cattle (Green et al. 2004). Unintentional exposure of vultures to diclofenac during scavenging behaviours was shown to cause visceral gout and renal failure leading to death. It is estimated that due to diclofenac toxicity, 99% of vultures in India, Pakistan and Nepal were lost (Cuthbert et al. 2014). Cases such as this highlight that it is not only chronic exposures that can affect predatory birds and acute exposures to some contaminants can also have devastating effects on population numbers. Poisonings from exposure to second generation anti-coagulant rodenticides (SGARs) are also a significant risk to wild predatory birds and are regarded by many as one of the most pressing environmental concerns facing these birds today. An increase in anthropogenic activity has led to an increase in rodent numbers and so rodenticides have been developed to control pest populations and protect public health. First generation rodenticides include compounds such as warfarin and chlorophacinone but resistance to these agents in rodent populations necessitated the development of SGARs such as brodifacoum and bromadiolone (Hadler and Buckle 1992). Anti-coagulant rodenticides block the vitamin K cycle in the liver and inhibit the synthesis of clotting factors leading to death by haemorrhage (Weitzel et al. 1990). Although they are very effective at controlling numbers of rats and mice, toxicity to non-target species including predatory birds has been well documented (Lima and Salmon 2010). SGARs are also more persistent in animal tissues than their predecessors and although they can be fatal to birds in as little as one feeding, they can also remain partially active in tissues and sensitise wildlife to further exposures (Mosterd and Thijssen 1991). Rodent-eating avian species such as owls are particularly at risk (Albert et al. 2010) as well as birds such as kites which may scavenge rodent carcasses (Walker et al. 2008). SGARs provide an ideal case study to demonstrate how environmental contaminants can affect non-target species such as predatory birds even when exposure risk is theoretically low. It has been suggested that death in predatory birds due to SGAR exposure results from a relatively small proportion of exposures (Murray 2011). However, population effects in species that are at critical points, like some birds of prey

species, may be significant. Additionally, for species that are long-lived and may produce only a few offspring per year, the death of only a few mature birds can significantly affect local populations (Rattner et al. 2014).

3.3. Predatory Birds as Biomonitors

As apex predators with large territories and long lifespans which allow accumulation of contaminants, predatory birds are ideal bioindicators in which to monitor environmental contaminants. These characteristics which make them apt biomonitors are also somewhat similar to humans so monitoring activities may confer contaminant information which is relevant to humans as well as wildlife. In some cases, common harmful contaminant-induced effects have been observed in both predatory birds and humans e.g. heavy metals causing immune dysfunction in kite chicks and children (Gómez-Ramírez et al. 2014). Although measuring contaminant levels in abiotic media such as soil or sediment can provide information on the concentrations of chemicals, it does not indicate exposure levels in organisms or provide measures of bioaccumulation or biomagnification which are particularly pertinent to predatory birds. Monitoring contaminant levels in predatory birds does not only measure biologically relevant exposure concentrations but may also be used to determine if exposures are having a harmful effect. The potential of predatory birds as environmental sentinels has inspired national initiatives such as the Predatory Bird Monitoring Scheme (PBMS) in the UK, which routinely measure contaminants in these birds (Walker et al. 2008). Predatory birds have been used to monitor levels of many compounds including OC pesticides, heavy metals (Newton et al. 1993), PCBs (Helander et al. 2002), PBDEs (Crosse et al. 2012b) and rodenticides (Walker et al. 2013) among others. Contaminants can be measured in soft tissues from predatory bird carcasses such as liver and brain (Kenntner et al. 2003) but measurements can also be taken from non-destructive samples such as feathers (Eulaers et al. 2011a), blood samples (Sonne et al. 2010), addled eggs (Guerra et al. 2012) and oil from the preen gland (Jaspers et al. 2011). Both the use of birds found dead and non-destructive samples are most practical for conservation purposes as many species are protected.

Bird body tissues are one of the most widely used matrices for the measurement of contaminants which is considered to be the most accurate measure of exposure. Use of tissues and organs is particularly good for analysis of long term accumulations of contaminants such as POPs. Such methodologies require access to wild predatory bird carcasses which have died in the field but carcasses can also provide other necessary information such as body condition and in some cases age and sex (Espín et al. 2016). The tissue of choice is often dependant on the type of contaminant to be measured and may also be influenced by the condition of bird or cause of death but the liver is most commonly used. Soft tissue decomposition in the carcass may affect measurements as microbes can metabolise some contaminants (Butzbach 2010) but overall, concentrations in body tissues are directly indicative of environmental exposure. Measurements can also be made from whole blood, serum or plasma from live birds as after exposure, contaminants are transported around the body in the blood (Ehresman et al. 2007; Elliott and Shutt 1993). Blood measurements are considered most suitable for short term exposures as many contaminants have a shorter half-life when in the blood. It is also more difficult to accurately measure many POPs in blood as these tend to sequester into lipid-rich body compartments such as tissue and egg yolks (Norstrom et al. 2007). A larger volume of blood may be needed for such measurements which may not be possible particularly in smaller predatory bird species (Volz et al. 2001).

After body tissues, feathers and eggs are the most common samples for biomonitoring. Due to maternal transfer of contaminants from the liver into eggs, addled or abandoned eggs collected by a licenced specialist can provide valuable measurements. Biomonitoring using eggs is particularly useful for measuring lipophilic compounds as liver lipids are transferred into eggs to form the yolk and contaminants move with them (Russell et al. 1999; Verreault et al. 2006). Therefore, eggs provide a measurement which is directly related to body burdens in adult, female birds and is connected to reproductive success. It does not give direct information on levels in non-breeding or male birds though (Espín et al. 2016). Additionally, eggshell measurements can be made and it is postulated that pigmentation on eggshells may be a sign

of exposure to contaminants such as OCs (Jagannath et al. 2008). There is a potential element of bias to egg measurements as they are addled or abandoned and therefore represent failed attempts to breed and in some cases, contaminant exposure may be implicated in this.

Multiple studies have shown that contaminant levels in feathers are directly correlated with levels in body tissues (Jaspers et al. 2013; Rajaei et al. 2011). Feathers can be easily and safely collected from carcasses, plucked from live birds or taken when moulted by wild birds. As well as contaminant information, they can also provide other measurements of interest such as levels of stress hormones (Strong et al. 2015). In adults, contaminants are deposited into feathers when they are laid down during moulting and so differences between feather and tissue levels can occur due to when and where feathers were developed (García-Fernández et al. 2013). However in young pre-fledge birds, feathers are still connected to a blood supply and so many provide a more accurate representation of body burdens (Eulaers et al. 2011a). Further, pollutants can also be measured in preen oil (Jaspers et al. 2011). Preen oil is a lipid-rich secretion for the uropygial preen gland at the base of the tail and bird distribute it through feathers when preening to make them waterproof and protected (Jaspers et al. 2008). The levels of contaminants in the oil are thought to correlate to those found in internal body tissues and it can be collected from birds by a minimally invasive procedure (Yamashita et al. 2007).

3.4. Current Assessment Methods

Once predatory bird samples have been collected, they usually require further analysis to determine contaminant levels and/or measurement of biological effects of exposure. Analytical chemistry techniques such as gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) are currently the most commonly used methods to provide quantitative data regarding contaminant concentrations in the samples of interest (Muir and Sverko 2006). These methods can be used to analyse many sample types including tissue, egg contents and feathers but involve extraction of analytes from the sample matrices using gas extraction or soxhlet/column extraction procedures (El-Shahawi et al.

2010). Analytical chemistry techniques are extremely useful as they can provide absolute contaminant values with a high degree of accuracy. However, they require expensive laboratory equipment and reagents and development of extraction methods can be very time-consuming (Sosa-Ferrera et al. 2013). A number of enzyme-linked immunosorbent assays (ELISAs) have been developed to detect environmental contaminants such as PCBs (Lin et al. 2008) and OC pesticides (Sherry 1997) in samples. ELISA methods can provide semi-quantitative estimations of chemical concentrations and may be used alone or as a complimentary technique to analytic chemistry. They are usually inexpensive to perform and require little sample preparation but may not be suitable for all samples, for example feathers may be difficult to homogenise to an appropriate degree. Also, ELISAs may not be suitable for some contaminants which are too small or lack certain atomic groups (Morozova et al. 2005).

Other methods measure the effects of environmental contaminants in samples. These tests are often used in conjunction with quantitative methods and usually focus on a specific biological endpoint or effect biomarker such as circulating levels of thyroid hormones. There are a wide range of biochemical, toxicodynamic and physiological alterations which can occur in response to exposure to environmental contaminants and can be used to study that exposure. Liver enzymes (Sonne et al. 2012), cytochrome P450 monooxygenase enzymes (Letcher et al. 2014), thyroxine hormone levels (Marteinson et al. 2011) and reactive oxygen species (Koivula and Eeva 2010) are just a few endpoints used to measure the effect of contaminant exposure in predatory birds. Over the past decade, vibrational spectroscopy techniques have emerged as valuable tools for analysis of environmental samples including those from predatory birds (Llabjani et al. 2012). Although these techniques do not result in absolute contaminant values, they provide information regarding the biochemical bonds and biomolecular structure in samples which can be altered by exposure. Thus a range of potential endpoints can be investigated. They are particularly useful for studies where two or more sites of contamination are studied or where comparison to control samples is possible. Vibrational

spectroscopy can also be used alongside analytic chemistry to determine concentration dependant effects in environmental samples and may be useful as a screening tool before further chemical analysis. These techniques have garnered considerable interest as the instrumentation is relatively inexpensive, little sample preparation is needed and a large amount of samples can be analysed rapidly especially with automation technologies. The analysis is also non-destructive so samples can be retained and re-analysed if desired (Kelly et al. 2011). The two types of vibrational spectroscopy that are most commonly used to analyse environmental samples are Fourier-Transform Infrared (FTIR) and Raman spectroscopy.

4. Vibrational Spectroscopy

4.1. Fourier-Transform Infrared (FTIR) Spectroscopy

FTIR is one of the most popular applications of vibrational spectroscopy and has been particularly well used in biomedicine with a focus on cervical cytology (Walsh et al. 2008). It measures the absorbance of infrared (IR) light by biochemical bonds in a sample. It can be used to analyse samples of all phases, gas, liquid or solid provided that the molecular bonds within are IR active and have an electric dipole moment. All molecules will exhibit some degree of movement which can be small motions of the two atoms in a diatomic molecule (coupling) or more complicated movements such as bending, stretching, wagging and twisting (Kelly et al. 2011). A molecule with n atoms will have $3n$ degrees of motional freedom which allows for many vibrational modes (Griffiths and De Haseth 2007). IR spectroscopy exploits the concept that a molecule is promoted to a state of excitation if a dipole moment changes during vibrations or movements. IR light does not cause electronic transitions like ultraviolet (UV) light as it does not have enough energy. Instead, it is most effective when used with IR active molecules which only have a small energy difference between vibrational states as when molecular movements cause a net change in the dipole moment, absorption of IR radiation can occur. If the frequency of fluctuations in dipole moment and the frequency of the alternating electric field of the IR radiation match, absorption of radiation can occur and the

size of movements is altered. Each molecule has slightly different vibrational modes so IR absorption is unique and can be used to identify biochemical bonds and biomolecules within a sample. For biological samples, the mid IR region of 400-4000 cm^{-1} is most commonly used. Within this range, there are a few areas of importance as the molecules which absorb IR in those regions are relevant to biological investigations. The first is the biochemical fingerprint region of 900-1800 cm^{-1} which contains vibrational modes for most biological molecules including lipids, Amide moieties from proteins, carbohydrates, DNA and RNA. There is also a higher frequency region of 2500-3500 cm^{-1} which is associated with S-H, C-H, N-H and O-H bond vibrations, mostly from lipids, fatty acids, triglycerides and proteins (Baker et al. 2014a).

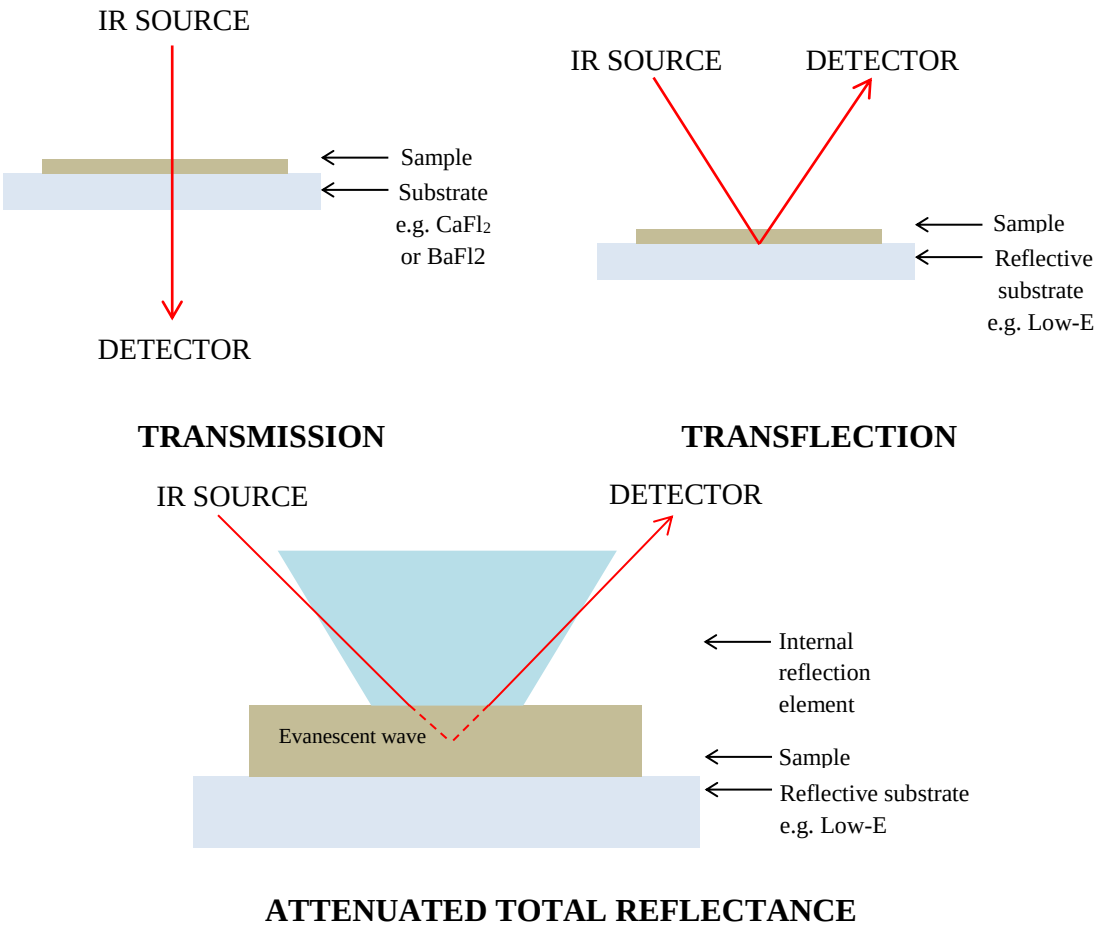


Figure 4 – Transmission, transflection and attenuated total reflectance (ATR) sampling modes used in FTIR spectroscopy

There are three main sampling modes for FTIR spectroscopy; transmission, transflection and attenuated total reflectance (ATR) (see Fig. 4). The choice of which mode to use will be influenced by the sample being studied and the substrates available to the researcher. For transmission measurements, the IR beam passes through both the sample and the substrate and is measured at a detector on the other side. It is therefore imperative that the substrate is not composed of a material which will absorb the IR radiation in the spectral region of interest or this will interfere with the results (Davis et al. 2010). Commonly used substrates for transmission measurements are calcium fluoride or barium fluoride slides. Substrate selection is also important when using transflection modes as the IR beam needs to pass through the sample and reflect off the substrate to return to the detector. A substrate with an IR-reflective coating, such low-emissivity (low-E) slides, is needed (Reich 2005). During ATR-FTIR, the IR light is first passed through an internal reflection element (IRE) composed of a material with a high refractive index such as diamond, germanium or zinc selenide. The beam is reflected off the internal surfaces of the IRE and an evanescent wave is created which can penetrate beyond the crystal and into the sample by a few microns (Martin et al. 2010).

The main advantage of FTIR spectroscopy over other techniques is that it is very quick to make measurements. Traditional spectroscopy techniques were slow and inefficient as each IR frequency had to be individually measured. FTIR spectroscopy uses an optical device called an interferometer which contains a beamsplitter. The beamsplitter splits the IR beam into two separate beams which take different paths; one beam is reflected off a stationary mirror, taking a constant pathlength whereas the other beam is reflected off a moveable mirror meaning that the pathlength is always changing. When these two beams are combined to produce an interferogram, it contains all of the IR frequencies from the source within it, thus allowing fast and simultaneous measurement across the whole spectrum (Smith 2011). Interferograms cannot be deciphered directly so Fourier-transformation is employed to interpret them and this results in a spectrum with absorbance plotted at each wavenumber (Faix 1992). By applying Beer-Lambert's law which describes the linear relationship between

concentration of an analyte and its absorbance, the presence of and amounts of biomolecules within a sample can be determined (Griffiths and De Haseth 2007). This function has enabled FTIR spectroscopy to be used for many applications such as detection of drug residues (Chan and Kazarian 2006), distinguishing between bacterial species (Helm et al. 1991) and discrimination of cancer grades (Bird et al. 2008). FTIR spectroscopy provides chemical data with spatial specificity and by pairing with optical microscopy, it can also be used for rapid, label-free imaging purposes (Bhargava 2012). Recently, there has been some focus on the use of vibrational spectroscopy techniques to analyse environmental samples including identifying the effects of contaminant exposures (Obinaju et al. 2015; Strong et al. 2016a). However, this application of FTIR spectroscopy is somewhat in its infancy and further work is needed to explore the use of such technology in the context of environmental research.

4.2. Raman Spectroscopy

Raman spectroscopy is a complimentary technique to FTIR spectroscopy and although they are similar in some respects, it measures a very different phenomenon. Unlike FTIR spectroscopy which uses a polychromatic light source, for Raman techniques, a monochromatic source such as a laser is direct at the sample under investigation and the scattering of photons is measured. An occurrence known as inelastic or Raman scattering is measured which is relatively weak and very rare, arising from less than 1% of scattering (Kelly et al. 2011). Ordinary scattering, also known as Rayleigh scattering, occurs when an absorbed photon is promoted to a higher virtual energy state and then instantly scattered back at the original energy level so that no overall energy change has taken place. Thus, this is known as elastic scattering where the scattered light has the same frequency as the photons from the laser source (McCreery 2005). There are two types of inelastic (Raman) scattering of light; Stokes and anti-Stokes. During Stokes Raman scattering, the emitted photons have lower frequency and energy than the photons in the incident radiation (i.e. from the source) whereas during anti-Stokes scattering, emitted photons have a higher energy state and frequency than the absorbed photons. Anti-Stokes scattering does not frequently occur at

room temperature as electrons favour staying at their ground energy state but whether the inelastic scattering is Stokes or anti-Stokes, an overall change in the energy of the system occurs (Butler et al. 2016). As with FTIR spectroscopy, the movement and vibrational modes of molecules in a sample are important, as the shifts in the energy of photons occurs due to interactions between the electromagnetic waves from the incidence beam and the vibrational energy levels of the molecules. Molecules cause characteristic energy shifts and so by plotting the intensity of the energy shift against wavelength, a Raman spectrum is created from which molecular composition and structure can be inferred (Schrader 2008). The main regions of the spectrum which pertain to vibrations of biological molecules such as proteins, carbohydrates and DNA are found between 400-2000 cm^{-1}. The higher frequency region of 2700-3500 cm^{-1} is associated with stretching of C-H, N-H and O-H molecules from lipids and proteins (Movasaghi et al. 2007). However, the measured spectrum can go far below 400 cm^{-1} if desired, making Raman useful for inorganic analyses as well (Kendix et al. 2008).

One of the largest advantages of Raman spectroscopy over FTIR techniques is superior spatial resolution. The extent of the resolution is limited mainly by the diffraction limit of light so it is reliant on the wavelength of the laser being used and the numerical aperture of the objective. In principle, resolutions of down to 0.2-1 μm may be possible but due to imperfect optics, it is larger than this in practice (Butler et al. 2016; Everall et al. 2007). Other than improved resolution, a key benefit is that water is a weak Raman scattering agent and so Raman can be used to study aqueous samples or those with high water content. Therefore, analysis of fresh, fixed or live cells and tissues is possible (Ellis and Goodacre 2006). Carbon dioxide is also a weak Raman scatter so atmospheric interferences are lessened. Raman spectroscopy allows rapid generation of information-rich spectral datasets which can be used with computational analysis techniques to distinguish specific spectra thus relaying important biological data. This functionality has caused Raman methods to become increasingly popular in clinical and biomedical fields. In particular, Raman has been widely used in cancer diagnosis and prognosis notably when paired with imaging and mapping techniques (Draux et

al. 2009; Smith et al. 2003). Used in this way, it has been shown to differentiate brain (Fullwood et al. 2014), lung (Oshima et al. 2010) and breast cancer (Rehman et al. 2007) types and grades. Furthermore, these classification capabilities have been applied to other disciplines including plant science (Schulz and Baranska 2007) and also environmental studies (Alvarez-Puebla et al. 2007). Previously, it has been used to monitor air emissions and water quality and Surface-Enhanced Raman Spectroscopy (SERS), in particular has proved to be a powerful investigatory tool for environmental samples. SERS overcomes fluorescence within samples which can hide spectral features by enhancing the Raman signal from Raman-active molecules (Halvorson and Vikesland 2010). This is achieved by adsorbing such molecules from the sample onto roughened surfaces often in the form of metal nanoparticles. Used in this way, Raman can detect contaminants in the femtomolar range with a high degree of specificity (Kneipp et al. 1999). Thus although it has traditionally used in clinical settings, Raman spectroscopy techniques including SERS modifications have great potential as a means to monitor environmental contamination in many sample types.

5. Spectral Data Analysis

One of the largest challenges of using vibrational spectroscopy techniques is extracting meaningful information from spectral datasets. These practices result in large, complex datasets which are difficult to handle and points of interest within the spectra may be very small, so computational and multivariate analysis is employed. This is particularly important when analysing biological samples which are, by nature, very heterogeneous and contain a diverse array of molecules. The data handling is a multi-step process including pre-processing of the spectra to prepare them for the final computational analysis. There are numerous methods which can be employed at each step of the pre-processing and analysis but ultimately, the choice is determined by the nature of the spectral data and the purpose of the experiment. Table 2 shows the pros and cons of common pre-processing methods.

5.1. Pre-Processing

Before pre-processing, the spectral area of interest should be selected and the spectra cut down to reflect this. This prevents the introduction of background noise, from spectral areas of little interest or absorption, into the analysis. Once this has been done, the spectra must be pre-processed to remove variation which is not related to chemical or molecular information and so would interfere with the results. As the particles of biomolecules and the wavelength of the near-IR electromagnetic radiation are similar in size, scattering effects can occur which cause shifts in spectral baselines and other non-linearity phenomenon (Rinnan et al. 2009). The scattering effects can be enhanced by many factors present at the time of spectral acquisition including sample thickness, temperature and atmospheric changes or contamination. Therefore, pre-processing is important not only to progress the spectra into a state fit for computational analysis but also to improve the reproducibility of the results. Improving the robustness and accuracy of results also allowed better comparison between experiments. Pre-processing is an umbrella term for a number of procedures, primarily comprising of baseline correction, normalisation and de-noising (Baker et al. 2014a) (see Fig. 5).

5.1.1. Baseline Correction

In an ideal situation, a spectral baseline would be flat and in the absence of a sample, it may approach such a state. However once a sample is introduced into the beam path, scattering can cause baselines to be shifted or skewed. It is essential that these aberrations are corrected as they distort Beer-Lambert's law; the absorbance is altered and thus does not reflect the concentration of the analyte. The simplest forms of baseline shifts can be corrected manually. For example, baseline offsets occur when the sample attenuates the radiation equally at all wavelengths thus the absorbance is raised relative to the baseline. This can be simply corrected by subtracting the absorbance minima from all the other absorbances. Similarly, sloping baselines can be manually adjusted using a two-point correction and subtracting a ramp or slope function from the spectra to return the baseline absorbance to zero. In practice,

such simple correction methods are rarely used as baseline irregularities are not often linear and so more complex techniques are needed. This is particularly true for biological analyses as in the fingerprint region (below 1500 cm^{-1}), skewed baselines commonly occur due to overlapping bands and increased biomolecule concentrations in samples (Griffiths and De Haseth 2007; Siesler et al. 2008).

More complex baseline corrections often employ polynomial functions. These methods do not use a joined line of points defined by the experimenter to subtract from the absorbance spectra but instead an *n*th order polynomial function is fitted to the spectra. Although technically any order of polynomial can be used, low orders are preferred to prevent artefacts being introduced (Lasch 2012). Rubberband baseline correction is another commonly used technique which first divides the spectra into equally sized regions and then determines the lowest absorbance in each range. By connecting these minima together, a new baseline is constructed and all points in the spectrum are pulled down by the difference between the lowest point in the current range and the lowest point in the baseline (Wartewig 2003). This approach is considered particularly useful if polynomial correction would require a high order function. Finally, differentiation of spectra may also be used to correct skewed baselines and is also useful for resolving overlapping peaks. Derivatives of absorbance with respect to the wavenumber are created, usually at the first or second order. In first order derivatives, peak maxima become zero and this point is accompanied by a negative and a positive peak at either side at the same wavenumber as the original inference points. In second order derivative, negative bands appear with the maxima at the same wavenumber as the initial peak. Derivative spectra are free of baseline slope and offset (Smith 2011).

5.1.2. Normalisation

Spectra must be normalised in order to mitigate the effect of confounding factors, such as differences in sample thickness and varying optical pathlength, which alter the apparent absorbance of molecules within the sample. Normalisation reduces inter-spectra variation by

removing the differences in peak heights between spectra due to differences present at the time of spectral collection (Trevisan et al. 2012). Thus, the overall aim is to numerically perform that which was not possible at the time of collection and to ensure that the spectra are replicates in the absence of biological differences. This is commonly done by dividing all absorbances or intensities across the spectrum by a specific numerical criterion so that they are scaled relative to that factor (Randolph 2006).

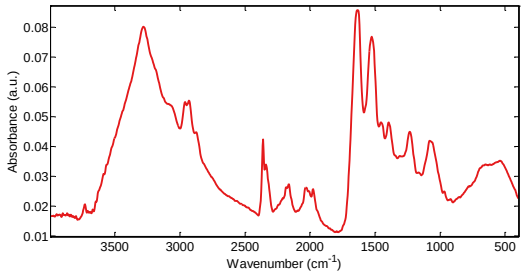
The most common methods are min-max methods such as peak normalisation and vector normalisation. As the name suggests, in min-max normalisation, the minimum and maximum absorbance values must be calculated first. Then the minimum is subtracted from the spectrum before it is divided by the range (Gautam et al. 2015). Peak normalisation methods use a stable peak which is consistently present in all the spectra collected; often Amide I, Amide II or the CH_2 asymmetric stretching peak. All peaks across the spectrum are divided by the absorbance or intensity at the chosen peak so that the new absorbance of the reference peak becomes one and the values at other peaks are scaled relative to it (Baranska 2013). Researchers must exercise caution when using peak normalisation as use of certain peaks should be avoided if it is likely that the reference peak may shift in position due to experimental conditions. For example, Amide I normalisation is not recommended for studying some protein conformational changes as Amide I and III are known to exhibit a band shift between native and denatured proteins (Ozaki et al. 1993). Vector normalisation is not limited by this as it does not rely on specific peaks to scale the spectra. Instead spectra are divided by their Euclidean norm, that is they are divided by the square root of the sum of squared absorbances or intensities (Gautam et al. 2015). This normalisation method is particularly useful after derivative baseline correction which alters peak appearances so that stable, positive peaks may not be present (Severcan and Haris 2012).

Stage	Purpose	Method	Advantages	Disadvantages
Cut	Reduces spectra down to region(s) containing vibrational modes of molecules of interest	For biological investigations, often cut somewhere between 700-2000 cm^{-1}	Prevents introduction of noise from superfluous areas of spectrum	May lose information on molecules with higher or lower frequency movements
Baseline correction	Corrects sloped, skewed or raised baselines	Manual 1-point or 2-point correction	Quick and simple to perform	Not often used as baseline aberrations are rarely linear
		Rubberband	Particularly useful is polynomial techniques would require a high order function	Creates loose baselines if the number of ranges is not appropriate and can cause amplified baselines when there is lots of random
		Polynomial	Can be used for complex, non-linear baseline irregularities	High order polynomials may distort baseline
		Differentiation	Resolves overlapping peaks	Amplifies noise and alters spectral shape
Normalise	Remove interferences of confounding factors such as sample thickness in order to standardise spectra in the absence of biological variation	Peak	Simple to use and multiple peaks may be constant giving more flexibility	If chosen peak shifts during experiment then baseline will be inaccurate
		Vector	Useful when stable peak is not present, particularly after differentiation	
		Standard normal variate (SNV)	Corrects Mie scattering, does not require a common reference signal	Can be sensitive to noise
		Multiplicative scatter correction (MSC)	Corrects Mie scattering. Conserves spectral features well and can be used to correct baseline as well.	Requirement for common reference signal may not be practical
De-noise	To improve to SNR of spectra	SG smoothing	Allows effective smoothing of whole spectrum, can be used simultaneously in conjunction with differentiation methods	May result in loss of information particularly at either side of smoothing window. Parameters defined by experimenter and so may distortion spectra
		Wavelet de-noising	Only the 'noise' part of the spectrum is removed leaving spectral features intact, can remove high frequency noise but keep sharp peaks	Can be computationally intensive

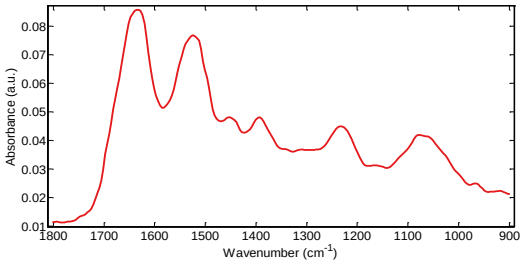
Table 2 – Summary of commonly used pre-processing methods with advantages and disadvantages which may direct usage by the experimenter

5.1.3. De-noising

In order to gain the most accurate chemical information from spectra, they must have a good signal-to-noise ratio (SNR). However, noise can enter the spectrum from a number of sources including from a charge-coupled device (CCD) or from temperature effects and lower the SNR (Mark and Griffiths 2002). When the SNR is lower than desired, rather than re-taking spectra from a sample and increasing the number of scans which may not be practical, de-noising or smoothing techniques may be used. De-noising aims to remove noise whilst leaving features related to biochemical structure intact. Nevertheless, it usually does have some degree of effect on the spectral resolution such as band widening but the benefits often outweigh any alteration (Griffiths and De Haseth 2007). Savitzky-Golay (SG) smoothing and wavelet de-noising are two commonly employed methods used to de-noise FTIR and Raman spectra. SG smoothing is a moving window averaging method which involves numeric derivation of a vector with a smoothing effect. A window of the spectrum is selected for processing then the data is fitted by a polynomial and used to estimate the peak centre (Savitzky and Golay 1964). Although this method can be very effective, there are a number of disadvantages to smoothing in this manner. The first is that there must be equal smoothing points on either side of the spectral window so that some spectral point and potentially some features are left out (Rinnan et al. 2009). The other limitation is that the smoothing is highly dependent on a number of experimenter defined parameters such as the order of the polynomial and number of smoothing points, which can lead to under or over smoothing and distortion of the spectra (Zimmermann and Kohler 2013). Due to these problems, many researchers prefer to use wavelet de-noising, a decomposition technique which uses non-linear filtering to remove the noise. Wavelet (a wave-like oscillation) coefficients are ‘threshholded’ so that only the noisy part of spectra is removed meaning that the underlying spectrum is better kept intact (Cohen 2012; Ehrentreich and Sümmchen 2001). This method is particularly good for de-noising of Raman spectra as it can remove high frequency noise whilst maintaining large, sharp peaks.



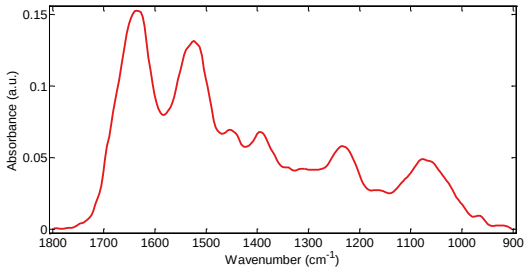
RAW DATA



CUT

To region of interest

Often to 900-1800 cm-1, the biological fingerprint region

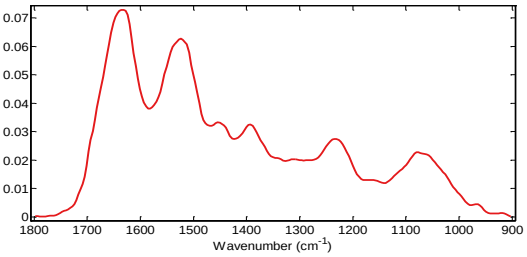


NORMALISE

Min-max

Peak e.g. Amide I

Vector

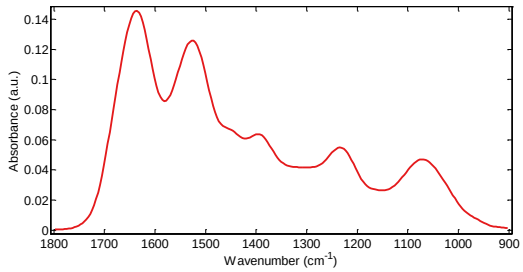


BASELINE CORRECT

Rubberband

Polynomial

Differentiation



SMOOTH

Optional step

Savitzky-Golay

Wavelet de-noise

Figure 5 – Workflow of pre-processing techniques and their effects on spectral data

5.2. Computational Multivariate Analysis

Spectral data has many layers of dimensionality; as well as absorbance values at each wavenumber, there is also the presence of biomolecular vibrations encoded within those as well as a large overlap of biological molecule vibrations in the fingerprint region. Experimentally, there are usually multiple spectra taken from each sample, various experimental conditions and multiple experimental repeats. Thus, multivariate computational analysis methods are required to allow data reduction, classification and interpretation of spectral results. Through this, meaningful biological and chemometric information can be gained such as differences in biomolecules, differences between samples and possibly identification of biomarkers (Severcan and Haris 2012). There are two important parts of the computational analysis stage known as feature extraction and feature construction. In this context, features are input variables and refer to information to be input into the subsequent analyses. In some cases, absorbances may be used directly as features but more commonly, feature extraction is used to reduce the dimensionality of the data. Feature construction is a phase of feature extraction which refers to building a new set of linear variables out of the wavenumber absorbances. Partial-least squares (PLS) as well as principal component analysis (PCA) and linear discriminant analysis (LDA) either alone or together are often used for feature extraction. Visualisation of the spectral data after such techniques can be seen in Fig. 6.

5.2.1. Principal Component Analysis (PCA)

PCA is an unsupervised statistical technique which is widely used in computational analysis of spectral datasets and has also been used in fields such as image compression and facial recognition. It is a multidimensional factorial method used to determine patterns in data with high dimensionality and allows expression of those patterns as trends of similarity or dissimilarity. PCA can also reduce the dimensionality of the data, making complex spectral datasets easier to analyse. The ultimate aim of PCA is to identify small uncorrelated variables, called principal components (PCs) from a large dataset (Smith 2002). PCs essentially describe

the directions in data where the most variation can be found i.e. where it is most spread out. Data points are deconstructed into eigenvectors and corresponding eigenvalues where the vector indicates the direction through the data and the value is the amount of variance this direction captures. The eigenvector with the highest eigenvalue becomes PC1 and the following PCs explain the maximum variance possible in descending order (Smith et al. 1985). Eigenvectors are input as PC loading vectors (i.e. PC1, PC2, PC3 etc.) into a PCA loadings matrix. Each loading vector is a linear combination of coefficients from the data which are used to generate a new set of variables called PCA factors. For spectral data, this results in a linear dataset of wavenumber absorbances with each PC accounting for as much variance as possible (Severcan and Haris 2012). The newly created PC factors are orthogonal to each other (at 90˚) so they are uncorrelated to one another. It is due to this that PCA can be used as a data reduction technique. Variables within the data that have a strong correlation to one another may fall into the same line or plane in lower dimensions and so can be effectively ignored. In this manner, data is reduced into only the important components and eliminates those which are not useful in discriminating between data classes (Kemsley 1996).

PCA is particularly useful for pattern recognition and data reduction as it is an unsupervised technique which requires no input from the researcher regarding the data classes etc. Therefore it is considered less bias that other techniques as patterns of maximum variance are calculated indiscriminate of experimental details (Kelly et al. 2011). Once PCA has been performed on pre-processed spectra, the results can be visualised as scores, points in space along a PC axis which each represent a spectrum, or loadings, which highlight the absorbances at each wavenumber that are responsible for the variation seen in PC scores. For some datasets, PCA alone may be enough to separate the data into meaningful trends and patterns. However, for many complex spectral datasets this is not the case and the use of additional techniques such as LDA may be used.

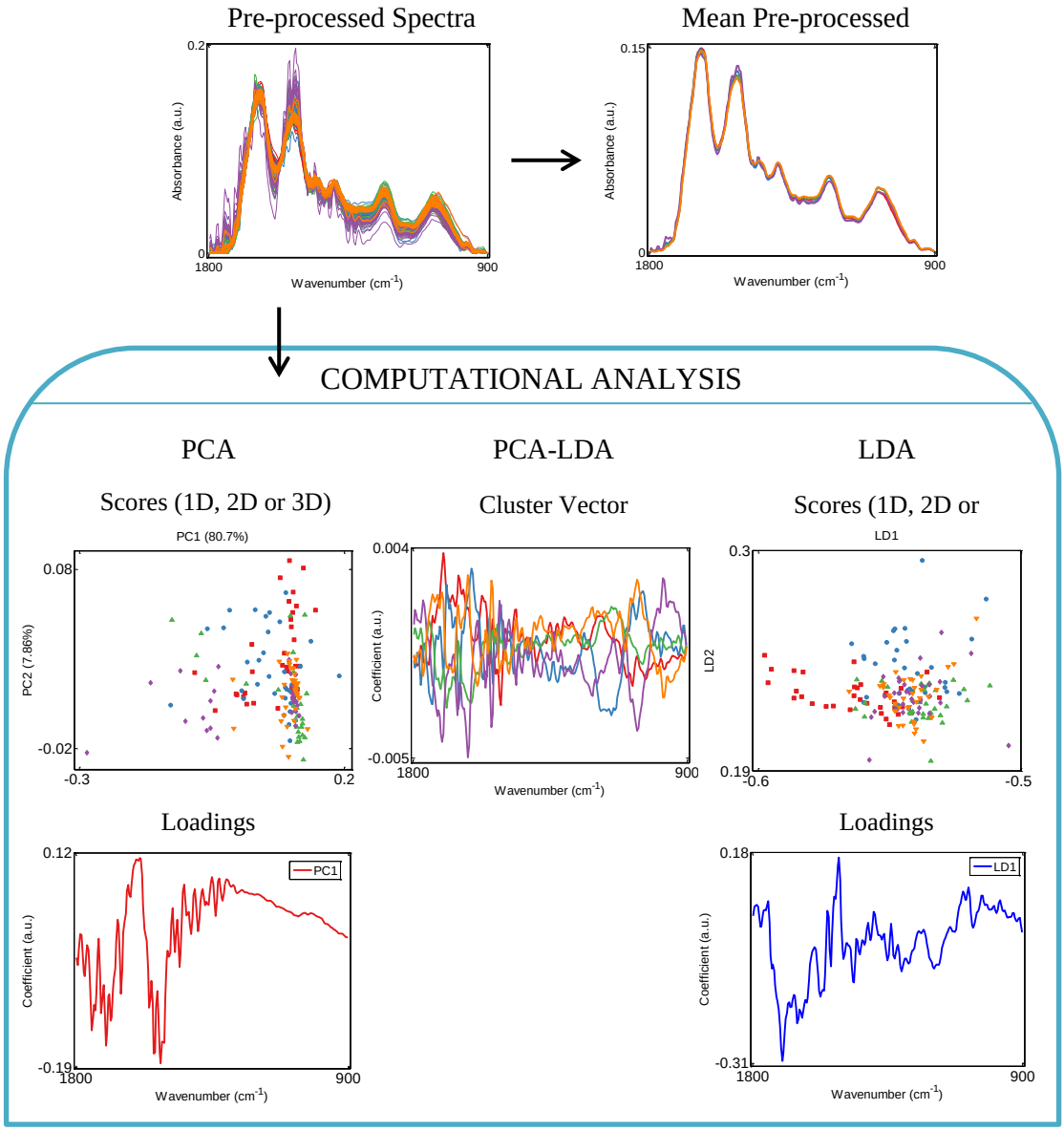


Figure 6 – Ways in which spectral data can be visualisation after computational analysis

5.2.2. Linear Discriminant Analysis (LDA)

LDA is a multivariate classification technique which is often used to analyse spectral datasets in order to achieve class separation. It is a supervised technique as it requires knowledge of input data membership into classes or groups in order to analyse the structure of data. LDA is largely used to optimise between class variation and reduce within class variation by forming linear combinations of variables, the combinations of which are dependent on the differences between data groups (Rencher 2003). As the aim is to attain segregation and it is a supervised

technique, care must be taken not to cause overfitting of data. Therefore it is recommended that the spectra must number at least 5-10 times more than the number of variables and that an unsupervised method such as PCA is used beforehand. Overfitting may still occur so the use of cross validation may also be of benefit (Trevisan et al. 2012).

In theory, during LDA the data is projected onto an imaginary hyperplane or line. The linear combinations of data and the positions at which they are projected are calculated so that optimal separation of data groups occurs as determined by maximum ratio of inter-class to intra-class variation. Finding the linear combinations which separate the data optimally means finding the eigenvectors which maximise this variance ratio (Silva et al. 2013). The largest positive eigenvector is determined and the original data is multiplied by the corresponding eigenvalue to produce LD1. LD2 is created by multiplication by the second largest eigenvalue, LD3 by the third largest and so on. As linear combinations are created, the original variables are weighted according to a coefficient that determines the relative importance of the variable in separating groups (Rencher 2003). This results in LDs which are not correlated and which encapsulate the variation in the data. As with PCA, LDA scores can be viewed for each LD dimension as well as the loadings along each LD. Used in this manner, LDA is an extremely powerful data segregation tool. It can also be used as a classification or prediction model as by using a subset of the data as a training dataset, the model can be used to classify new observations (these are spectra in the case of FTIR or Raman spectroscopy) into the most probable data class or group (Gajjar et al. 2013a).

6. Aims and Objectives

This thesis is composed of four first author research projects which aim to explore the application of FTIR and Raman spectroscopy with multivariate computational analysis to studying avian, particular predatory bird, tissues and cells exposed to environmental contaminants. In the environment, predatory birds are simultaneously exposed to multiple

contaminants so with this in mind, another aim was to study the effects of exposure to single agents and mixtures of contaminants. The behaviour of contaminants in mixtures and the risk of mixture toxicity to wildlife has been explored in the review presented in chapter 2. The research using vibration spectroscopy to study exposure to contaminants has been done through a number of experimental projects which aim to:

Understand the fundamental biochemistry and spectral signatures of predatory bird tissues using FTIR and Raman spectroscopy (Chapter 3)

Investigate the effects of high and low levels of OC pesticide exposures in sparrowhawk brains, including the effect on cerebral amyloid aggregation, using FTIR and Raman spectroscopy techniques (Chapter 4)

Compare, using ATR-FTIR spectroscopy, the biochemistry of an avian fibroblast cell line, which is more environmentally relevant, with the commonly used MCF-7 human breast cancer cell line and to identify biochemical alterations in both cell types caused by exposure to environmentally reported PBDE and PCB congeners (Chapter 5)

Determine cellular biomolecular alterations induced by single agent and binary mixture exposure to B[*a*]P, PBDEs and PCBs, agents with different toxic mechanism, as well as aiming to establish if potentially hazardous additive interactions occur in these binary mixtures and if so, can they be predicted using ATR-FTIR techniques (Chapter 6)

A road map of experimental questions and how the rationales for each chapter are related can also be found in Chapter 7, Fig. 1.

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Chapter 2. Risk assessment of environmental mixture effects

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Risk assessment of environmental mixture effects

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Abstract

In the environment, organisms are exposed to a diverse array of chemicals in complex mixtures. The majority of approaches that aim to assess the risk of environmental chemical mixtures, including those used by regulatory bodies, use toxicity data generated from the individual component chemicals to predict the overall mixture toxicity. It is assumed that the behaviour of chemicals in a mixture can be predicted using the concepts of concentration or dose addition for chemicals with similar mechanisms of action or response addition for dissimilarly acting chemicals. Based on empirical evidence, most traditional risk assessment methods, such as Toxic Equivalency Factors and the Hazard Index, make the assumption that the components of a mixture adhere to the concentration addition model. Thus, mixture toxicity can be predicted by the summation of the individual component toxicities. However in some mixtures, interactions can occur between chemicals or at target sites that alter the toxicity so that it is more or less than expected from the constituents. Many regulatory and experimental methods for predicting mixture toxicity rely on the use of a concentration addition model so that if interactions occur in mixtures, the risk posed may have been significantly underestimated. This is particularly concerning when considering environmental mixtures which are often highly complex and composed of indeterminate chemicals. Failure to accurately predict the effects chemicals will have if released into the environment, where they can form mixtures, can lead to unexpected detrimental effects on wildlife and ecosystems. The number of confounding factors that may alter the ecotoxicity of a mixture and the accuracy of predictive methods makes risk assessment of environmental mixtures a complex and intimidating task. With this in mind, this review aims show why accurate risk assessment of mixtures is vital by demonstrating the effect they can have on organisms in the environment. Furthermore, it also aims to look at the current challenges facing the assessment of mixture effects and examines future areas of focus that seek to develop methodologies more suitable for environmental mixtures.

Introduction

Most studies only consider the toxicity of chemicals in isolation whereas in the environment, organisms are exposed to a large number of different chemicals at the same time. The assumption of mixture toxicity based on the individual component data can lead to a significant under- or over-estimation of the potential risk that a mixture may present. This is particularly pertinent where regulatory bodies must make assessments on the usage of chemicals that may be used in the environment or have the potential for release into it. It has long since been acknowledged that the presence of a chemical in a mixture and its known toxicity in isolation is no guarantee of the effects when combined with other agents. Although considerable focus has shifted towards looking at mixtures as a whole, there are still gaps in our knowledge and our ability to assess them as such. It is not necessarily possible to know which combinations of chemicals will arise in the environment or to test for the innumerable agents and mixtures which may occur. Although environmental mixtures are often highly complex and vast in composition, a recent review of mixture studies performed by Kortenkamp et al found that of the experiments considered, less than 25% looked at mixtures with seven or more agents (Kortenkamp et al. 2012). With this in mind, the aim of this review is to look at how components in a mixture may interact and what effect this can have on organisms in the environment when they are exposed, simultaneously, to different pollutants. It will also consider current approaches used to evaluate the effect of such mixtures in order to understand how this impacts the assessment of risk that pollutants can pose to the environment.

Reasons for ecotoxicity risk assessment of mixtures

Around the world, environmental pollutants are found in various matrices and can affect organisms at any trophic levels in ecosystems of all different kinds. Research has shown that anthropogenic pollution can cause morbidity and mortality in organisms by affecting processes such as reproduction and development (Ahmed et al. 2012; Lundholm 1997b).

Although some exist transiently and will degrade, other chemicals are very stable and will remain in the environment for a long period of time. Such chemicals are of particular concern for top level predators that accumulate pollutants and are considered particularly important to ecotoxic risk assessment due to their longevity and resultant possibility for exposure to combination mixtures (Lohmann et al. 2007). It is clear that environmental pollution can have a significant effect on wildlife even before the consideration of mixture effects which adds a further level of complexity.

Environmental mixtures typically exist at fairly low levels, the exact composition is often not known and there may be other external, non-chemical stressors that play a role in the combined effects too. Although not all mixtures will have ecotoxic effects, those that do can be significantly damaging to wildlife. There are a huge number of potential chemical combinations and it is not practical nor always possible to test them all for ecotoxic effects, so there is an obvious need for robust approaches to assess toxicity (Cedergreen 2014b). A common school of practice, often used for regulatory framework, is that if individual components of a mixture fall below certain toxicity or concentration thresholds then the overall mixture will be 'safe'. It is easy to see from the available literature, that this does not always match the real world situation and if components of a mixture interact the overall risk to environmental organisms can be much greater or smaller than expected. There are many classes of pollutants that can form mixtures in the environment and to consider just three of these groups, heavy metals, pharmaceutical drugs and pesticides, serves to illustrate the importance of accurate assessment of environmental mixtures.

Heavy metals

Heavy metals are a major source of environmental pollution caused by anthropogenic activities and are well known as a considerable risk to the health of wildlife. Both single toxicant studies (Daus et al. 2010; Gagné et al. 2008; Lock and Janssen 2001), and those looking at mixtures have shown that due to wide industrial use, heavy metal pollution spans

many ecosystems and many aquatic, marine and terrestrial organisms are constantly exposed to complex metal mixtures and the associated toxicities (Biesinger et al. 1986; Spehar and Fiandt 1986). There are numerous studies showing that metals in mixtures that are found in the environment or mixtures that simulate them are toxic to environmental organisms. The studies show that the mixtures affect organisms at all tropic levels of the ecosystem. Due to the nature of heavy metal pollution, aquatic ecosystems are particularly at risk. Binary mixture studies of nickel, cadmium and lead as well as arsenic and cadmium increase mortality rates and cause immobilisation in *Daphnia magnia*, a sentinel aquatic organism (Le et al. 2013; Vandenbrouck et al. 2009). Combinations of cadmium, copper and zinc alter the filtration rate of *Dreissena polymorpha*, a freshwater zebra mussel (Kraak et al. 1999; Kraak et al. 1994). In frogs, co-exposure to cadmium and chromium can cause increased accumulation of heavy metals in the kidneys (Loumbourdis et al. 2007). In fat head minnows, a mixture of six different heavy metals that were each at individual water criterion levels was found to impair growth (Spehar and Fiandt 1986). It is clear even from a small sample of the available data, that mixtures of heavy metals represent a risk of considerable concern.

Such studies have also highlighted that it is not always straightforward to predict the toxicity of heavy metal mixtures from tests that use single chemicals and the toxicity of a mixture can be greater or less than expected. Some mixtures of heavy metals are relatively simple to asses, for example *Daphnia* exposure to copper, cadmium, lead and zinc mixtures have been accurately predicted a number of times (Enserink et al. 1991; Yim et al. 2006) but toxicity predictions of other heavy metal mixtures have been incorrect due to effects on additional pathways that are not involved in single treatments (Le et al. 2013; Vandenbrouck et al. 2009). Due to their chemistry, many heavy metal species will interact with each other as well as with other chemicals and biological structures rather than simply exerting their own toxic effects. A comprehensive analysis looking at all possible combinations of some of the most common heavy metal contaminants (copper, lead, zinc and cadmium) in a sea urchin assay found that in the majority of mixture combinations, the metals interacted (Xu et al. 2011). In

many cases, heavy metal interactions increase the toxic effects such mortality rate in larvae (Zhu et al. 2011), renal damage (Palaniappan and Karthikeyan 2009), embryonic toxicity and spermiotoxicity (Xu et al. 2011) but some also yield a decrease in expected toxicity (Vellinger et al. 2013; Vellinger et al. 2012). There are also many other factors that influence the overall toxicity of a heavy metal mixture such as whether exposure is acute or chronic (Spehar and Fiandt 1986). Derivations from the expected toxicity cause concern that current prediction and assessment methods are not adequate and may lead to toxic mixtures that will harm biota in the natural environment.

Heavy metal mixtures are not limited to aquatic environments, their effects are also seen in terrestrial ecosystems. Heavy metals have exhibited interactions that alter the expected mixture toxicity in a number of terrestrial species such as isopods (Odendaal and Reinecke 2004), earthworms (Qiu et al. 2011) and nematodes (Martin et al. 2009). There is little experimental data from higher vertebrates on which to base ecotoxic risk assessment due to the difficulty of working with species that have more complex biological systems, longer life spans and the push to reduce animal testing. Given our knowledge that single agent heavy metal exposure causes detrimental health effects in birds (Gangoso et al. 2009; Heinz and Hoffman 2003; Pain et al. 2007) and mammals (Sánchez-Chardi et al. 2007), we can assume that at least some heavy metal mixtures will also have deleterious effects, potentially greater than would be expected from individual exposure data. To add a further level of concern, the co-exposure of metals with other types of pollutants can lead unique combination effects. Studies that have focused on the effect of heavy metals in combination with other chemicals have found that they can interact with other agents, particularly pesticides, which can also lead to altered mixture toxicity (Dondero et al. 2011; Ivanković et al. 2010; Maria and Bebianno 2011).

Pharmaceutical drugs

Pharmaceutical chemicals are designed with their effect on humans and animals in mind and specific care is taken to evaluate the risks of mixing with other chemicals or drugs. The risk of unpredicted, unregulated mixture effects is low during their intended use but the potential for release into the environment is significant and thus there is a great risk of such agents becoming components of toxic mixtures in the environmental (Heberer 2002). Due to frequent usage and the nature of pharmaceutical excretion into bodily fluids, which enter waste water treatment plants, mixtures of pharmaceuticals in water bodies are often highly complex with multiple interactions occurring between chemicals (Öllers et al. 2001; Roberts and Thomas 2006; Santos et al. 2010). The vast range of pharmaceutical compounds that make their way into the environment makes predicting such interaction difficult: non-steroidal anti-inflammatory drugs (NSAIDs), hormones used as contraceptives, medications, antimicrobials and more are all frequently detected (Kümmerer 2009).

Experimental evidence demonstrates that often mixtures of such drugs have unexpected toxicity compared to individual chemicals (Pomati et al. 2008). In a study using *D. magnia,* the toxicity of a mixture of NSAIDs, including ibuprofen and acetylsalicylic acid, was significant even at levels where the individual drugs showed little toxicity (Cleuvers 2004). Similar results have also been found with other types of drugs. For example, one study found that clofibrinic acid, a cholesterol lowering drug, alone caused 1% of *Daphnia* to become immobilised and carbamazepine, an anticonvulsant, alone immobilised 16% but in a mixture, they caused the immobilisation of 95% of the organisms (Cleuvers 2003). This represents a significantly greater toxicity than expected which would have been underestimated using traditional models. Another study showed that clofibrinic acid and fluoxetine, an antidepressant, cause higher rates of death and deformity than predicted by the single agents (Flaherty and Dodson 2005). Antimicrobial mixtures have also been a source of concern as interactions between agents is a fairly common occurrence. In fact, some antibiotics are purposely used in combination in order to enhance their efficacy thus increasing the likelihood

that antimicrobial mixtures will be more toxic than expected (Yeh et al. 2006). The intended usage of such agents is to inhibit or kill bacteria but when released into the environment, antimicrobial mixtures are potentially toxic to many non-target bacterial species as well other microorganisms that are critical to ecosystems (Backhaus et al. 2000b; Christensen et al. 2006; González-Pleiter et al. 2013).

Although much of the work looking at pharmaceutical mixtures focuses on lower trophic organisms, in the environment, this often confers risk throughout the ecosystem (Brain et al. 2004; Richards et al. 2004). Evidence shows that mixtures of these agents also endanger higher species such as fish and birds (Galus et al. 2013; Markman et al. 2011; Shore et al. 2014a).

Pesticides

The term 'pesticide' covers a wide range of compounds and chemicals and refers to one of the most commonly used category of pollutants, representing a multibillion dollar industry. Pesticides are designed to be toxic to at least one species which has meant they have been fairly well studied in order to define risk assessment legislature related to their use. Different types of pesticides are often purposefully used in mixtures in order to target 'pests' or to increase efficiency so co-exposures may occur to non-target species right from the time of release. Due to this, a substantial amount of work has gone into investigating the effects of pesticide mixtures on wildlife. It has been shown that mixtures of commonly used pesticides can increase mortality in bees (Blacquiere et al. 2012) and even pesticide solvents can be toxic when combined with insecticide agents (Zhu et al. 2014). They can have adverse effects on many fish species including damaging their olfactory tissues which are essential for processes such as migration and predator detection (Belden et al. 2007; Deneer 2000; Tierney et al. 2008). It has also been suggested that aquatic pesticide mixtures can lower the immune responses of bivalves thus putting them at risk of bacterial infections (Gagnaire et al. 2007) and are highly toxic to estuarine amphipods (Anderson et al. 2014). Pesticide mixtures have

been an area of particular worry in amphibian research due to the exposure risk inherent in having both amphibious and terrestrial life cycle stages. Insecticide mixtures have been found to dramatically increase the mortality rate of frog species (Relyea 2009) and slow larval growth (Hayes et al. 2006; Relyea 2004).

As with many types of chemical mixtures, there is an increasing body of data showing that some pesticides will be more toxic when in mixtures than expected based on the component chemical toxicities and so traditional assessment methods may miscalculate the risk to the environment (Coors and Frische 2011). For example a study looking at salmon, a species considered under ecological threat, found that sublethal combinations of organophosphate and carbamate pesticides exhibit considerable synergy of acetylcholinesterase inhibition which is essential for salmon survival (Laetz et al. 2009). Another found that commonly used fungicides that are often used simultaneously or in close proximity, can cause up to a twelve fold increase in immobilization of *D. magnia* that predicted by the single chemicals (Anderson et al. 2014). The usage of many pesticides that are damaging to the environment is now restricted or banned and so regulatory mixture assessments only apply to current-use chemicals. However, many pesticides are persistent in the environment and recent research has shown that legacy pesticides can still be found at levels that are capable of causing ecotoxic effects (Rasmussen et al. 2015). It is an issue of significant concern that there may be environmental organisms that are exposed to mixtures containing ecotoxic pesticides that are not captured in current evaluations of mixture safety.

Mode of effects of chemical mixtures

Early work in the field, from the 1930s onwards, led to the development of the three main models of mixture effects (see Fig. 1). The first two were termed concentration addition and independent action(Bliss 1939; Plackett and Hewlett 1948) and these apply where there is no interaction between the components in a mixture. The third category is applied to mixtures where there is interaction between the component chemicals leading to potentiation or

antagonism of toxicity (Plackett and Hewlett 1952). In the environment, mixtures are not always made up of simply similarly or dissimilarly acting chemicals and during risk assessment, all three models may need to be considered particularly for more complex mixtures.

Concentration addition and independent action

Both concentration addition and independent action use the model of non-interaction so mixture toxicity is predicted based on the assumption that components within a mixture will not interact or interfere with each other. In theory, a concentration addition model also known as an additive model applies to chemicals in a mixture where the summation of the individual component toxicities is equal to the toxicity of the mixture as a whole (Walker et al. 2012c). The model assumes that the different agents in the mixture share the same mechanism of toxicity and the same target site but are diluted by each other and so do not influence each other's toxicity. Therefore, all components of the mixture contribute to the total toxicity depending on their concentration and potency so that even if all components are at levels below the toxicity threshold, the overall mixture would have toxicity due to the additive effect (Escher and Hermens 2002). Many approaches towards mixture risk assessment, often used by regulatory bodies, work on the assumption that a mixture of chemicals will have an concentration additive toxic effect (Backhaus et al. 2013). A good example of a class of chemicals that generally adhere to the assumptions of concentration addition are xenoestrogens which can have significantly detrimental effects on the health of humans (Massart et al. 2005) and wildlife (Brian et al. 2005; Silva et al. 2012). Studies have shown that even if the individual estrogenic compounds are at levels where no effect is observed (when applied alone), the overall mixture has toxic effects due to concentration addition (Rajapakse et al. 2002; Vom Saal et al. 1997).

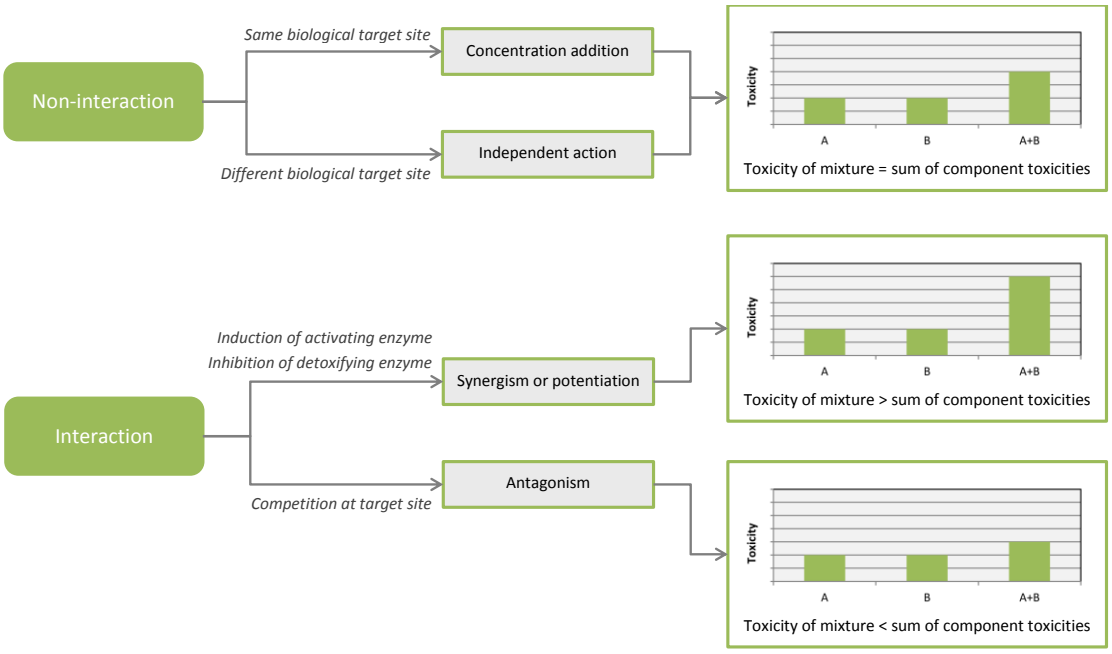

Figure 1 - Schematic demonstrating the theoretical models of chemical mixture effects.

Independent action, sometimes called response addition, refers to chemicals that have different modes of toxicity and so affect different biological target sites. As the name suggests, the components of the mixture are acting independently and so they do not impact the toxicity of each other (Cedergreen et al. 2008). Thus, in a mixture where the components exhibit independent action, it is expected that the overall mixture would have no toxicity providing that all the individual agents are at subtoxic levels. In terms of risk assessment, a mixture is often assumed to exhibit independent action if there is evidence to show the toxicity is not additive (Kortenkamp et al. 2009b). Unlike concentration addition, which has been well studied in a range of different organisms and with multiple chemicals, there is considerably less literature on the potential of independent action and its accuracy (Backhaus et al. 2004). The studies that have been done, mainly focus on microorganisms, bacteria (Backhaus et al. 2000a; Liu 2011) and algae (Faust et al. 2003), have found that independent action is a reliable way to predict the effect of dissimilarly acting chemicals in a mixture and is more accurate for such chemicals than concentration addition. More recently, studies looking at higher organisms have also found similar results (Ermler et al. 2013).

Interactions

In some cases, relatively few yet environmentally significant, the toxicity of a mixture differs from that expected using the assumptions of concentration addition or independent action. In these cases, mixture components influence each other to result in the overall toxicity being stronger or weaker than predicted. This is due to interactions. When the mixture toxicity exceeds that of the individual chemicals together, it is known as synergy or potentiation. Multiple studies have shown that heavy metals, in particular, often have potentiated toxicity due to interaction with each other in mixtures (Chaperon and Sauve 2007; Utgikar et al. 2004; Wah Chu and Chow 2002). The term potentiation is sometimes used interchangeably with the word synergism but they each refer to distinct occurrences. Mixture toxicity is described as synergistic if only one compound is present at a toxic level and the other components in a mixture are present at subtoxic levels (Paul 2011; Walker et al. 2012c). Therefore, the chemical present at a subtoxic level would have no effect if applied in isolation and only has toxicity in the context of the mixture. This component would be known as the synergist (Altenburger et al. 2013). Potentiation or synergism occurs when a chemical in a mixture alters the way another is metabolised. In complex mixture it is possible that there may be more than one interaction where metabolism is changed making the overall outcome even harder to predict. There are two main ways in which a chemical can affect the metabolism of the other: the first is if it causes a second chemical to be activated quicker. This usually happens as a result of the first chemical inducing the expression of enzymes that are involved in the activation of a second chemical. The second way that metabolism can be altered is when a chemical prevents another from being degraded by inhibiting an enzyme, or its expression, that is involved in detoxification (Metcalf 1967; Walker et al. 2012c).

Carbon disulphide is a well-studied hepatotoxic pollutant that exhibits synergistic toxicity in certain mixtures. It can cause considerably greater level of toxicity than predicted by concentration or response addition due to its influence on detoxifying enzymes called mixed function oxidases (MFOs), specifically Cytochrome P450 (CYP) enzymes (Dalvi et al. 2002).

CYP2E1 enzymes metabolise and activate carbon sulphide (Dalvi et al. 1975) and in the presence of chemicals that also induce the expression of CYP enzymes, potentiation of carbon disulphide toxicity occurs as it is activated significantly quicker than if applied alone. This effect happens in both simultaneous and sequential exposures (Dalvi et al. 2013). Another example of altered metabolism leading to potentiation is that of malathion, an extensively used pesticide that is often contaminated during manufacture with an impurity called isomalathion. In combination, a mixture of the two has greater than expected cytotoxicity and genotoxicity (Josse et al. 2014) due to inhibition of carboxylesterases, another family of detoxification enzymes, by isomalathion. As detoxification of malathion is significantly slower, mixture toxicity is enhanced (Hernández et al. 2013).

The second type of interaction is known as antagonism. This occurs when toxicity of a mixture is less than expected using a concentration or response addition model, i.e. less than that of its individual components. This can happen due to direct interaction between chemicals, competition at receptors or altered metabolism (James et al. 2000). A well-studied, environmental example of antagonism is the interaction between mercury and selenium. The presence of selenium is thought to reduce the assimilation of methylmercury in the body thus reducing its toxicity (Luque-Garcia et al. 2013). The selenium is able to sequester the mercury and so decreases the bioavailability and ability to cause toxic effects. Sequestration prevents the action of selenium-dependant enzymes which contain sulphur molecules essential for a significant part of the toxicity of mercury (Dang and Wang 2011; Sørmo et al. 2011). In ecotoxic risk assessment, antagonistic interactions are not as concerning as synergistic or potentiated interactions as the toxicity of the mixture is underestimated rather than being more dangerous than expected.

Current approaches to mixture risk assessment

Environmental risk assessment is something of a complicated task; consideration of multiple chemicals, species, mechanisms of action, exposure ranges, etc. all have their part to play and

may be needed for the risk to be accurately predicted. In a laboratory situation, it is impractical if not impossible to experimentally recreate and test each combination of chemicals which might be found in the environment. For this reason, robust predictive models are critical to our understanding of how pollutants interact so that they can be appropriately evaluated. There are two ways to approach the risk assessment of a mixture; looking at the whole mixture or using data on the component chemicals. Fig. 2 demonstrates the data used in both whole mixture and component based approaches and provides example risk assessment methods appropriate for each.

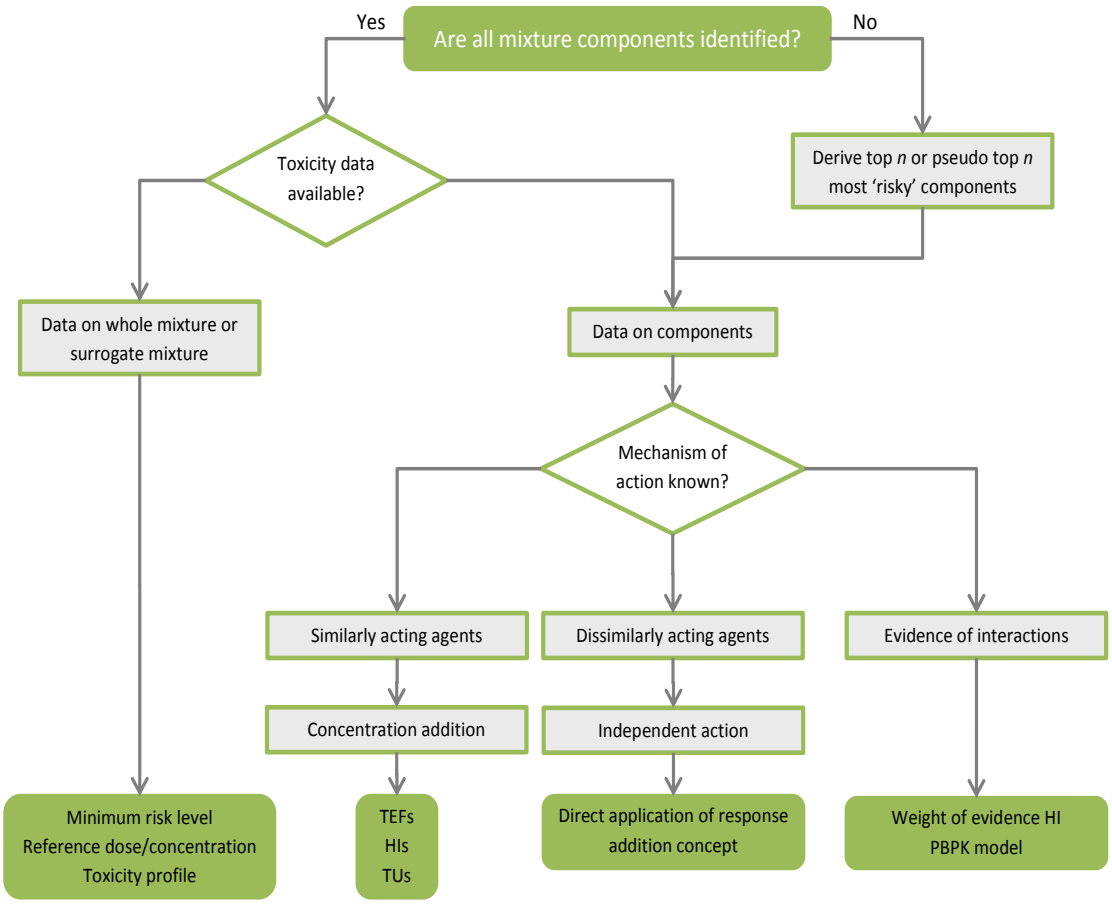

Figure 2 - Flow chart of general guidance on the depth of toxicity data available for chemical mixtures and appropriate risk assessment methods.

Whole mixture based approaches

Whole mixture approaches, sometimes called top-down approaches, use toxicity data in the form of the biological response to an entire mixture or from fractions of it in the case of hydrocarbons mixtures (Landrum et al. 2012). It is also possible to perform a risk assessment on a surrogate mixture if it is considered similar enough to the mixture under investigation. On first consideration, whole mixture approaches may seem the most logical as they appear to best represent the simultaneous exposure that organisms in the environment encounter. A key advantage of this type of ecotoxic risk assessment is that by using the whole mixture any interactions between the component chemicals, that may have been missed in a component based approach, are accounted for (Kortenkamp et al. 2009b). In an environmental setting, it is sometimes more appropriate to look at whole mixture data if the mix in question is poorly characterised such as in sludge, sediment or effluent water (Antunes et al. 2007), etc. as if there are any unknown or unidentified constituents, they are also captured in the assessment.

However, there are some important limitations; the information resulting from a whole mixture assessment is highly specific to that particular mixture and cannot be extrapolated to other mixtures or situations. It is only applicable to mixtures that are very stable in the environment as there it does not account for any change in composition that is typical of complex mixtures. This type of approach does not result in any information on the mechanism of action of components within the mixture (Groten et al. 2001). It is only possible to determine if there is an additive response (from some assessments) but nothing further can be concluded about potential interactions. Even if a specific toxic endpoint such as genotoxicity is identified and measured, it is not possible to identify the toxicant responsible for this outcome. For ecotoxicity risk assessment, whole mixture approaches are often not achievable as it is not possible to extract or recreate an entire environmental mixture due to the sheer scale of potential components (Boobis et al. 2011).

Component based approaches

When it is possible to identify all of the components in a mixture, risk assessment is often carried out using toxicity data on the constituent parts. Quantitative toxicity data is paired with risk and/or hazard classification to provide a full risk assessment. Usually, the type of mixture effect is determined using knowledge of the mode of action and this, along with the type of exposure and toxicity, directs the selection of the most appropriate risk assessment method (Kortenkamp and Faust 2010). Due to availability of data and practicality restrictions, the mode of action might not be known so for environmental toxicity assessments, many methods assume a concentration addition model. Although component based approaches solve many of the limitations of whole mixture assessments, the assumption of non-interaction in lieu of mode of action data may result in potentially ecotoxic mixture interactions being missed (Sexton and Hattis 2007). However, evidence suggests that for the majority of cases, concentration addition is adequate to predict mixture toxicity. Commonly used component based approaches that assume additivity include Relative Potency Factor (RPF) methods such as Toxic Equivalency Factors (TEFs), the Toxic Unit (TU) Summation approach and the Hazard index (HI) (Teuschler 2007). Here we will discuss a few of the most used methods but Table 1 provides a more comprehensive list of approaches.

Component Based Approaches		
Concentration Addition	**Independent Action**	**Interactions**
Direct application	Direct application	Weight of Evidence HI
Toxic Equivalency Factors (TEFs)		Physiologically-based pharmacokinetic (PBPK) model
Hazard Index (HI)		Interaction Profiles
Relative Potency Factors (RPFs)		
Combined Margin of Exposures (MOETs)		
Point of Departure Index (PODI)		

Table 1 - Component based methods of chemical mixture risk assessment approaches categorised by the assumed mechanism of action.

The summation of TUs is the most direct application of the concentration addition model and it is extensively used in ecotoxicology risk assessment (Ginebreda et al. 2014; López-Doval et al. 2012). The TU of a chemical is derived as a fraction where the concentration of the individual mixture component is divided by a toxic endpoint definer such as the EC50 (the dose at which a 50% effect is induced when that chemical is in isolation). For long term exposures, the no observed effect level (NOEL) can also be used. The overall toxic unit of the mixture is calculated by the summation of the individual TUs (Backhaus and Faust 2012; Kortenkamp et al. 2009b).

Risk assessment methods that use RPFs are generally used for groups of chemicals where the toxicity and dose-response of one particular individual chemical in the class is well characterised and studied (Barron et al. 2004; Budinsky et al. 2006). This chemical is known as the index compound. Using the assumption that others in the same class have the same mechanism of action, the toxicity of the chemical under question is expressed relative to that of the index compound. RPFs can be considered scaling factors that allow toxicity to be defined relative to how much of the index compound that would be needed in order to generate the same toxicity as the investigated chemical (Simon et al. 2007). For evaluating mixture effects, RPFs assume a concentration addition model so the toxicity of a mixture can be calculated by adding up the equivalent index compound doses. The most commonly used type of RPF method is the TEF but a similar RPF known as the potency equivalency factor (PEC) has also been developed for use with more diverse groups of chemicals including polyaromatic hydrocarbons (Barron et al. 2004).

Toxic Equivalency Factors (TEFs) are a specific type of RPF that are extensively used in environmental regulatory circumstances to assess the toxicity of dioxins, furans and other dioxin-like compounds, such as polychlorinated biphenyls (PCBs), that bind to the aryl hydrocarbon receptor (Bhavsar et al. 2008; Van den Berg et al. 2006). These chemicals have diverse ecotoxic properties and are highly persistent in the environment leading to

accumulation up food chains. TEFs express the equivalent toxicity of these compounds, between 0.00001 and 1, relative to the most potent dioxin; 2,3,7,8-tetrachlorodibenzo-*p*-diozin (TCDD) (Van den Berg et al. 1998). For cumulative assessment, the sum of equivalent TCDD doses for each dioxin-like compound is used which is generated by multiplying the dose by the TEF for that particular compound. Although highly useful, the TEF method makes some central assumptions that restrict the mixtures it can be applied to. The first is that the components of the mixture must exert toxicity via the aryl hydrocarbon receptor as the requirement for a similar mode of action validates the use of an additive approach (Safe 1998). As TEFs can be used for human and wildlife mixture assessments, it also assumes that the chemicals behave the same or similarly in different species (Haws et al. 2006). Finally, it also assumes that equivalent toxicity between the chemicals and TCDD remains the same at all doses. Although these rules represent potential limitations, TEFs are regarded as easy to use and easier to standardise across compound classes as they are based on potency rather than exposure which can have many factors. There have been multiple studies that have shown that the TEF approach works well to accurately predict the toxicity of mixtures of dioxin-like substances (Hamm et al. 2003; Walker et al. 2005).

The Hazard Index (HI) is more often used in human health risk assessment but it is increasingly being used to investigate environmental mixtures, particularly those composed of pharmaceutical agents (Cristale et al. 2013; García-Galán et al. 2011; Ginebreda et al. 2010; Yan et al. 2014). A HI is generated by the addition of hazard quotients for each component chemical in a mixture. It is often used when the mechanism of action is not known as unlike the TEF approach, the HI does not use a RPF scaling factor built from exposure data but instead uses a minimum risk reference level, usually derived from benchmark doses or the no observed adverse effect level (NOAEL) (EPA 1986). Hazard quotients are derived by comparing the exposure of each chemical to a reference level that is specific for that compound (Sarigiannis and Hansen 2012). The HI is used for assessing mixtures of similarly acting compounds so an overall HI is generated by summing the hazard quotients.

Calculating a HI gives a qualitative estimate of mixture risk and is stated in terms of whether it exceeds unity or not. A HI of a mixture that is more than one is considered to exceed acceptable levels of toxicity and is not 'safe' in the environment (Sanderson et al. 2004). The HI is considered to be more flexible than using TEFs as the exact mechanism of action does not need to be known. Also, different types of data can be used interchangeably as exposure data and acceptable limits as long as they are expressed in the same limits. The comparison to a reference level strengthens the HI approach as it is a well characterised acceptable risk level and providing they are already derived, calculating the HI is relatively fast. However, this can also be a downside to the method as the way that the reference value is calculated may differ, due to the use of uncertainty factors, for each mixture component resulting in inconsistencies in the hazard quotients. Ideally, standardised data should be used for each chemical to solve this issue and make the HI a more robust tool.

For mixtures where the components have a similar mechanism of action and the compounds have a linear dose response relationship, assessment of risk using either concentration addition or independent action should give the same estimations. Independent action is rarely used as an assumption for ecotoxicity assessment methods as previous evidence has suggested concentration addition is applicable to more mixtures. Recently however, it has been suggested that a combined, tiered approach may be more appropriate for environmental risk assessment. The initial stages are used to determine whether there is a need for further, higher tier testing. It has been proposed that the first tier involves the use of concentration addition assumptions and the higher tier uses independent action if needed (Backhaus and Faust 2012; Beyer et al. 2014).

Challenges of environmental risk assessment

Interactions

As described above, the majority of regulatory risk assessments for environmental mixtures are based on the assumption of similarly acting chemicals also called concentration addition. Although for many mixtures (Table 2) this may provide a conservative measure of risk, it does not account for interactions. For chemical mixtures where the constituent chemicals influence each other's toxicity, the risk that a mixture poses to organisms in the environment can be under or overestimated using this assumption. For environmental regulation, synergistic interactions are of more concern than antagonistic ones as the mixture is more toxic to wildlife than predicted and may cause harm. Mixture effects caused by heavy metals, in particular, may be difficult to assess using traditional methods as interactions are relatively common (Vijver et al. 2011). As well as chemical interactions, there are also a number of abiotic factors relating to habitat that can combine with the effects of chemical mixtures and lead to synergy or antagonism of the mixture toxicity (Laetz et al. 2014).

Interactions in mixtures occur only in specific situations and are particular for that specific mixture, dose, organism etc. so it is generally difficult to capture them in risk assessments. In order to address this, various approaches have been developed that aim to account for interactions in mixtures. The adjusted HI or weight of evidence (WOE) HI modification incorporates pairwise assessment of possible interactions between all chemicals in a mixture. It is used when the reference levels taken for the derivation of the HI are based on a different toxic endpoint than the other chemicals in the mixture (Mumtaz and Durkin 1991; Mumtaz et al. 1993). There are also other factors that are incorporated into the WOE score such as quality of the data and type of interaction. The outcome is a numerical score that indicates whether toxicity is likely to be under or overestimated using the traditional HI method (Feron et al. 2004). This adjusted HI is limited to mixtures where all the components are known as this information is needed to enable binary interaction assessment. It is also fairly time

intensive and needs a lot of data to use. This approach has been developed for human risk assessment and although the concepts can be applied to environmentally relevant mixtures, appropriate data needs to be accessible for successful application

Methodology	Mixture Under Investigation	Reference
Whole Mixture Assessment	- Industrial wastewater containing cosmetics waste such as surfactants, preservatives and phenol derivatives	(Carbajo et al. 2015)
	- Mixture of welding fumes containing toxic metals and gases	(Sriram et al. 2015)
	- Boreal sediments containing metal emissions	(Väänänen et al. 2015)
To Toxic Equivalency Factor (TEF)	- Binary mixtures of metals containing copper, lead and cadmium	(Gao et al. 2016)
	- Binary mixtures of benzo[*a*]pyrene and dibenzo[*a*,l]pyrene as well as complex mixtures of PAHs	(Jarvis et al. 2013)
Hazard Index (HI)	- Air samples collected from classrooms	(Mishra et al. 2015)
	- A mixture of nine phthalates in food items	(Chang et al. 2014)
	- Pesticide mixtures on fruit and vegetable	(Jensen et al. 2015)
Adjusted or Weigh of Evidence HI	- Predicted environmental mixture of 15 antibiotics	(Marx et al. 2015)
Physiologically Based Pharmacokinetic (PBPK) modelling	- Mixtures of trihalomethanes from reclaimed water	(Niu et al. 2015)
	- A mixture of 109 chemicals in gasoline	(Jasper et al. 2016)
	- Mixture of pesticide residues	(de Sousa et al. 2014)

Table 2 - Examples of recent applications of common risk assessment methods and the mixtures they were used to evaluate.

Another approach that aims to predicting mixture toxicity and risk by including interactions is the physiologically based pharmacokinetic (PBPK) model. PBPK modelling aims to predict pharmacokinetic interactions in mixtures at the tissue level by assessing interactions of binary pairs of chemicals in the mix. It works on the assumption that interactions at a binary level can be used to predict interactions in the whole, more complex mixtures (Hertzberg and MacDonell 2002). The model views an organism as a set of connected tissue compartments and factors such as metabolism, uptake and interactions are included to provide a more realistic biological basis for mixture assessment (Krishnan et al. 2002). It has been shown that PBPK modelling is also able to predict changes of chemical concentration in tissues due to mixture interactions (Haddad et al. 2000; Haddad et al. 1999). This type of model is amenable to various types of data and in theory can be adapted to incorporate many types of toxicological endpoints, interactions etc. (Haddad et al. 2001).

Unfortunately, interaction based risk prediction methods require trained specialists to carry out the assessments and a wealth of data on which to model mixture effects. Even for human risk assessment, a great deal more development is needed and even more so to make them applicable in an environmental context. Therefore, these methods are not currently able to be used for standard protocol for risk assessments meaning that mixture interactions may still go unidentified (Table 3).

Multiple species assessment

The main difference between mixture risk assessment for humans and for the environment is that the latter requires consideration of multiple species. The aim of environmental risk assessment is to protect the ecosystem as a whole, not just individuals. This can present significant problems when trying to predict the risk of chemical mixtures. Assessment is hampered by a lack of knowledge of chemical mechanism of action as well as the potency in all species in a community as toxicity of mixture components will differ depending on

physiological and pharmacokinetic differences between organisms (Backhaus and Faust 2012).

Methodology	Pros	Cons
Whole Mixture Assessment	- Interactions between components are captured - Can be used to study poorly characterised mixtures such as sludge or sediment	- Cannot extrapolate assessment to any other mixtures - Can only be used for stable mixtures - No mechanistic information can be determined - Cannot identify component(s) responsible for effects
Toxic Equivalency Factor (TEF)	- Relatively simple to use - Easier to standardise than other methods	- Chemicals in question must exert toxicity via the AhR so only applicable for certain agents - Assumes that the equivalent toxicity between the chemical and the reference is the same at all concentrations
Hazard Index (HI)	- Flexible as exact mechanism of action does not have to be determined - Different types of data can be used as risk reference levels - Uses well characterised 'acceptable risk levels' for reference	- Use of uncertainty factors can result in inconsistencies in the hazard quotient
Adjusted or Weigh of Evidence HI	- Accounts for interactions in mixture - Can be used when chemicals have different toxicological endpoints	- Mixture must be fully characterised - Time and data intensive
Physiologically Based Pharmacokinetic (PBPK) modelling	- Accounts for interactions in mixture - Can incorporate various data types, toxicological endpoints, interactions etc.	- Needs a trained specialist to perform - Requires a lot of data for model

Table 3 - A summary of the pros and cons of the most commonly used mixture risk assessment methods.

Toxicity of substances may also vary dramatically between different life stages e.g. egg, larvae or adult of the same organism. Such variations in sensitivity can be caused by metabolism differences if detoxification or activation enzymes are involved in the mechanism of action. There may also be alterations in target sites that are needed for the mixture to exert toxicity (Escher et al. 2011). Overall, this presents a very complex situation with many factors to be accounted for in the characterisation of mixture effects.

Many environmental regulation approaches involve the use of predicted no effect concentrations (PNECs). (Jin et al. 2011) PNECs are generated from laboratory based standardised tests performed on the most sensitive organism in the ecosystem which are then adjusted to account for factors such as inter-laboratory variation. Such organisms are from different trophic orders and an assumption is made that protection at lower levels of the ecosystem will confer safety to higher trophic species. In Europe, standard REACH (Registration, Evaluation, Authorisation and restriction of Chemicals) regulation requires that three species data sets are provided, one from each of the main trophic levels; primary producer, primary consumer, secondary consumer. For example, for aquatic ecosystems it is required that data for an algal species, a crustacean and a fish species are provided (Agency 2008). The extrapolation of this data is often very restricted as it does not account for inherent dissimilarities between species of different trophic levels. As well, there may even be large variances between organisms within the same trophic categorisation. Furthermore, the assumption of protection to higher trophic species does not necessarily hold for chemicals that bioaccumulate up the food chain. The essential need to consider multiple species in a community has meant that typical regulatory mixture assessments are often unsuitable and may underestimate the risk of ecotoxic mixture effects.

There have been proposals for methods that aim to look at risk assessment at a community level rather than an individual level. The recent development of a tissue residue approach suggests a tiered method. The first tier assesses the toxicity of a mixture in individual species

and then the second and third tiers use tissue residue data to derive a level which would provide protection to a specified percentage of organisms in a community (Dyer et al. 2011). Further to this species specific distributions (SSDs) take species sensitivities and predict the fraction of species in the total community which will experience toxic effects from mixtures using a known statistical distribution (de Zwart and Posthuma 2005; Posthuma and De Zwart 2006). Another way to address the issue might be the use of Adverse Outcome Pathways. These models aim to use mechanistic data relating to single chemicals and mixtures to integrate population level responses into risk assessments (Dent et al. 2015; Kramer et al. 2011).

So far, these methods are still being developed and have come under some criticism due to inconsistencies in the derivation of sensitivity data. However with further work they could have substantial implications for regulatory risk assessment of environmental mixtures providing more exposure data is generated for use in SSDs etc.

Simple *vs.* complex mixtures

The approaches that are used to assess the risk of simple mixtures, those that have fewer than ten components, may not be appropriate to evaluate the potential toxicity of complex mixtures. Complex mixtures can have in excess of hundreds of chemical constituents, not all of which may have been identified. Mixtures with numerous components are also more likely to change over time and more likely to have potential interactions between chemicals. Many risk assessment approaches, particularly those focused on human health protection, are based on the assumption of binary pair toxicity predicting the mixture effects of an overall mixture. In an environmental context where there are so many potential combinations, often with unidentified components, assessment approaches need to consider how to handle highly complex mixtures that are composed of potentially innumerable chemicals.

It is possible that grouping compounds within complex mixtures may make them more manageable for risk assessment. Grouping can be done on the basis of toxicological or structural similarity to form assessment or risk groups. Two suggested methods that can be used to handle the prediction of complex mixture risk are the top *n* and pseudo top *n* approaches (Feron et al. 1998; Groten et al. 2001). The top *n* approach identifies a given number ('*n*') of the most risky chemicals in the mixture, for example the top ten components that pose the most toxic risk might be characterised. The pseudo top *n* identifies the top classes of chemical that present the most risk. Then by grouping chemicals based on similarities such as mechanism of action, a chemical is identified to represent each class. Once the actual top or pseudo top *n* chemicals have been identified, the risk of the mixture can be assessed using the same methods as are used for simple mixtures. It is assumed that the overall mixture risk is captured by focusing on the most risky chemicals (Feron and Groten 2002). A method comparable to this has previously been used in a human health risk assessment framework and it is possible this kind of approach could be used for environmental mixtures as well (Johnson and DeRosa 1995).

Depending on the amount and quality of data, it may also be possible to use PKBK approaches to extrapolate data from simple, binary mixtures to predict the toxicity of those with more than two constituents. Using pairwise interaction data as building blocks, PBPK models can add further 'connections' to incorporate more components providing there is qualitative data available on the mechanism of action. Theoretically such a model could be applied to a mixture with as many components as desired (Krishnan et al. 2002). This type of method requires a certain amount of data and as complete exposure data for such a large number of possible combinations will not always be available (or possible to obtain), computational approaches have been developed with the aim to bridge this gap (Kim et al. 2013). Although there is still significant work needed they represent a promising new approach to more accurate risk assessment of complex environmental mixtures. This is particularly important as although the field of simple mixture risk assessment is now

advancing well, the development of methods for dealing with complex mixtures is often lagging behind due to a lack of good quality, qualitative data.

Future focus

As this review has shown, there are a number of challenges specific to the assessment of environmental mixtures that further complicate an already difficult task and when considering whether we can accurately predict the risk of environmental mixtures. It becomes apparent that this may only be possible to do in data rich situations where components and mechanisms of action etc. have been characterised. There is an obvious necessity in all types of mixture risk assessment for data gaps, such as chemical mechanisms of action and species sensitivities, to be identified and perhaps a systematic approach implemented towards rectifying them. Further development of predictive models is another key step towards improved risk assessment of mixtures. Models that account for biological factors such as metabolism and body distributions, *e.g.*, the PBPK model have been suggested as offering the most refined method for predictive purposes and are increasingly being used (DeWoskin and Thompson 2008). Although it has been pointed out that such models need validation using commonly encountered chemical mixtures, once such data is generated, they might be used for standard regulatory assessments (DeWoskin and Thompson 2008; Teuschler 2007).

For environmental mixture assessment, focus towards better modelling of ecosystems and involvement of multiple species in environmental risk prediction has already begun with methods such as the SSD. There are a number of assumptions made by currently used methods that may work for human risk assessment but are not applicable to environmental mixtures. For example, data on toxicity endpoints is often only needed for one lifestage (often the adult stage) whereas some mixtures may be more toxic to developing organisms (Breitholtz et al. 2006). Also, due to the large number of organism in ecosystems, toxicity data is often only used from a select few species. However, these species may not be representative of the ecosystem as a whole, for example aquatic assessment methods for

mixtures use data from *Daphnia* which have a number of unique characteristic such as asexual reproduction (Buikema et al. 1976). Although pitfalls like these need to be amended, for ecotoxic chemicals, assessment at the population level is much more relevant than looking at toxicity at the individual level. The use of data from mesocosms or model ecosystems may offer a better solution when looking at mixture effects of a community so higher tier assessment models will require more attention and work in the future (Koshikawa et al. 2007). Finally, consideration must be given to external, abiotic factors in the ecosystem such as exposure route. Part of the risk characterisation of chemicals relies of determination of a dose response relationship in a specific media but in the environment, organisms can be exposed to chemicals via food, air, water etc. The route by which organisms are exposed to chemicals in a mixture will affect the overall toxicity and incorporation of partitioning and route information will improve the accuracy of our risk assessments. The development of multimedia fate models has been suggested as a dynamic option for looking at chemical fate and mixture exposure route and will likely see further progress in the future (Gouin and Harner 2003).

There are many areas where mixture risk assessment methods need concerted effort and work in order to make them more useable for ecotoxic mixtures. By focusing on those issues which significantly impair the accuracy of risk assessments, it may be possible for new and improved models to overcome such issues and ultimately be used in a regulatory context to ensure mixture toxicity in the environment are not above acceptable levels.

Conclusions

In the environment, organisms are simultaneously exposed to a great variety of chemicals with diverse properties. The way in which chemicals in a mixture influence the overall toxicity depends on many factors including their concentration, target site and mechanism of action. The toxicity of a mixture can be predicted using toxicity data on either the individual components or the mixture as a whole. Current approaches that aim to characterise the risk of

chemical mixtures use component based methods based on concentration addition such as TEFs and HIs. Chemicals in mixtures such as pharmaceuticals, heavy metals and pesticides can cause detrimental health effects to organisms and for those mixtures where there are interactions between the components, these traditional risk assessment methods may lead to an underestimation of toxicity which could endanger wildlife.

Currently, our ability to accurately predict the ecotoxic effect of chemicals in mixtures is restricted by major challenges, such as multi-species consideration and a lack of consideration of interactions, which hinder the development of better predicative models. The vast number of factors that need to be considered and then incorporated into risk assessments makes it appear an almost insurmountable task. However, the establishment of newer assessment methods such as PBKB and SSD models aim to overcome issues that make traditional risk assessments unsuitable for environmental mixtures. There is considerable focus on how such methods can be improved for risk assessment in general but future work will also need to address developing models that are more suited to the specific and daunting task of environmental mixture risk assessment such as population level assessment models and consideration of highly complex, poorly defined mixtures.

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Chapter 3. A baseline spectral study of predatory bird tissues

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Manuscript for submission

Contribution:

- Birds were sacrificed and tissues dissected and fixed by the Patuxent Wildlife Research Center.
- I prepared, processed and acquired data for all samples including conducting computational analysis.
- I prepared the first draft of the manuscript.

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A baseline spectral study of predatory bird tissues

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Abstract

Birds of prey are apex predators and often have long lifespans which makes them ideal sentinel species for researching the effects of environmental pollutants. However, little work has focused on understanding the basic biochemistry of predatory bird tissues. Vibrational spectroscopy techniques are powerful exploratory tools which can confer information on the structure and composition of biological samples at the biochemical level. In this study, ATR-FTIR and Raman spectroscopy have been used as complimentary techniques to analyse untreated American kestrel tissues, in order to establish tissue specific spectral signatures containing fundamental biochemical information. The largest spectral peaks were principally due to protein and lipid vibrations as these are abundant molecules in biological tissues. However, peaks were also detected which reflected the function and metabolism of the tissues under investigation; spectra from the brain had large spectral contributions from lipids which are abundant in this tissue, the cardiac and skeletal muscle spectra had peaks assigned to collagen, the liver tissue had multiple absorbances from glycogen and the gonad tissues had numerous peaks associated with DNA content. Furthermore, it was found that subtle sex-dependant differences in spectral signatures from brain, heart, kidney, skeletal muscle and gonad tissues could be identified. Biochemical information such as protein-to-lipid content, lipid saturation and membrane integrity can also be derived from spectral ratiometric analysis. Studies such as this not only demonstrate the potential of vibrational spectroscopy techniques to profile the biochemistry of biological samples but knowledge of the underlying biochemistry is also essential for successful use of predatory birds in experimental settings.

Introduction

Vibrational spectroscopy techniques are high-throughput, non-destructive, cost efficient and do not require the use of hazardous or environment-damaging reagents. They are being increasingly utilised across many scientific disciplines including medicine (Clemens et al. 2014), environmental science (Malins et al. 2006) and microbiology (Kohler et al. 2015) as they can confer structural and conformational information at the molecular level. Such techniques are able to detect the presence of biochemical bonds in a sample based on the vibrational modes of those bonds when excited due to absorption of radiation. Absorbance by biochemical bonds can be related back to molecular structure thus providing information on biomolecules such as proteins, carbohydrates and lipids within a sample (Kelly et al. 2011). Two commonly used vibrational spectroscopy approaches are Fourier-transform infrared (FTIR) and Raman spectroscopy. FTIR methods are used to investigate infrared (IR)-active molecular bonds which have an electric dipole moment and so exhibit movement when interrogated with IR light. These bonds absorb photons from polychromatic light in the mid-IR region and are excited to a higher energy state leading to various vibrations such as stretching, bending and scissoring (Bellisola and Sorio 2012). When the interferogram derived from this undergoes a Fourier-transform, the resulting spectrum shows absorbance of the IR light at specific wavelengths depending on the biochemical bonds present within the sample (Baker et al. 2014a). Raman spectroscopy is a complimentary technique which functions on a similar basis but measures the excitation of molecules using monochromatic light in the near-IR region. When a chemical bond is excited by the light and absorbs a photon, it is then excited to a higher energy state. If the photon is released and it does not return to its ground energy state, a shift in the energy of the system is said to have occurred due to Raman (or inelastic) scattering (Butler et al. 2016). This shift in energy is measured at the detector and results in a spectrum where bands at specific wavelengths relate to chemical bonds. Raman scattering is a rare phenomenon which occurs in less than 1% of excitations but Raman spectroscopy is a very powerful technique which is less affected by water within the samples,

making it apt for biological investigations (Baena and Lendl 2004). Vibrational spectroscopy techniques produce large, complex datasets so spectral measurements are usually coupled with computational analysis to allow interpretation of results (Trevisan et al. 2012).

IR and Raman spectroscopy methods can be used to biochemically analyse a variety of biological substrates including cells (Llabjani et al. 2010), tissues (Abdel-Gawad et al. 2012) and biofluids (Baker et al. 2016). Traditionally, clinical investigation has focused on differentiation between normal and diseased states for example identifying the differences between normal and cancerous tissue (Gajjar et al. 2013b), particularly at tumour boundaries (Matousek and Stone 2013), as well as identification of cancer grades (Ollesch et al. 2016). In a similar manner, environmental studies often attempt to determine indicators of exposure to pollutants in samples (Cakmak et al. 2006). By comparing diseased samples or those exposed to pollutants to normal controls, vibrational spectroscopy techniques allow researchers to identify spectral markers of exposure and/or disease and thus rapid differentiation is possible. These techniques are regarded as advantageous as to gain such information, at the molecular level, is often time-consuming and expensive when using other methodologies (Martin et al. 2010). Although investigations are frequently focused on experimental samples which are diseased, cancerous, exposed etc., appropriate analysis of normal, control tissue often underpins the success of such work. In fact, study of 'normal' tissue is not only important for comparison but also for biochemical and metabolic discrimination between organs and tissues. A number of studies have also utilised the power of IR and Raman spectroscopy to analyse and detect markers of tissue and organs within animal models such as mice (Huang et al. 2011b) and rats (Staniszewska et al. 2014). Tissues within the body have differing molecular composition and metabolic systems which allows spectroscopy techniques to differentiate between them (Staniszewska-Slezak et al. 2015). The brain, for example, is rich in lipids and proteins and uses glucose as its fuel (Yehuda et al. 1999). The cardiac and skeletal muscles are characterised by the presence of collagen fibrils and highly ordered proteins and skeletal muscle has stores of glycogen but this is much rarer in the heart which

primarily metabolises fatty acids (Bailey et al. 1979; Lopaschuk et al. 2010). The kidneys filter and reabsorb glucose from the blood whereas the liver is the metabolic centre of the body, storing glycogen, synthesizing fatty acids and breaking down amino acids from proteins (Berg et al. 2002). The gonads can vary largely between species but they contain the genetic material needed for reproduction. Thus, analysis of normal, control samples using vibrational spectroscopy techniques is not only essential for comparison to treated or diseased samples but also for investigating the fundamental biochemistry of tissues (Krafft et al. 2008). The native variations between tissues can be quickly and easily detected from spectral profiles provided by vibrational spectroscopy. Although tissue is the most common sample type for this type of analysis, spectroscopy techniques can also be used to profile samples such as blood (Zou et al. 2016) and feathers (Llabjani et al. 2012) for which rapid analysis may be even more important.

Predatory birds are ideal environmental sentinels as they are at the top of their food chain and are long-lived for their size (C.H. Walker 2012; Katzner et al. 2006). Although many studies have focused on the effect of exposure to contaminants (Crosse et al. 2013) or disease (Cooper 2008) in predatory bird tissues, there has not been as much investigation into the biochemical profiles of such tissues. In this study, we have obtained brain, heart, skeletal muscle, kidney, liver and gonad tissues from healthy, untreated American Kestrels (*Falco sparverius*) with the aim to determine the biochemical compositions and differences between those tissues. This will also provide the first baseline spectral study of control predatory bird tissues and will define fundamental information on the biochemistry of those tissues.

Materials and Methods

Kestrel Samples

American kestrels (*Falco sparverius*) were kept in outdoor enclosures containing a shaded roof, perches, a food tray and a water bowl and fed day old cockerels ad libitum. Tissues from 8 captive-bred, adult kestrels (4 male and 4 female, n=8, see S.I. Table S1) that were humanely euthanised using carbon dioxide were used in this experiment. The kestrels used in this study were control birds and so were not exposure to any chemical contaminants. After euthanasia the brain, heart, pectoral muscle, kidneys, liver and gonads were immediately dissected from the carcass of each kestrel, rinsed with PBS to prevent carry-over of excess blood etc. and then stored in 10% buffered formalin. Tissues were stored in a volume of buffered formalin that was at least ten times that of the sample and left for at least 48 hours to allow tissues to fully infuse with fixative. Samples were transferred into 70% ethanol and stored at 4˚C before use. Slices of ~0.5 mm were taken from each tissue using a Stadie-Riggs manual tissue slicer (Thomas Scientific, Swedesboro, NJ, USA) and a carbon steel cutting blade. Tissue slices were transferred onto low-E glass slides (Kevley Technologies, Chesterland, OH, USA) and desiccated at room temperature before spectroscopy.

Spectroscopy

For ATR-FTIR, 10 spectra per sample were taken from distinct areas of the slide using a Tensor 27 FTIR spectrometer with a Helios ATR attachment (Bruker Optics Ltd, Coventry, UK) containing a diamond crystal with a sampling area of 250 µM x 250 µM. Spectra were measured at a resolution of 8 cm^{-1} with 32 co-additions and a mirror velocity of 2.2 kHz which resulted in a 3.84 cm^{-1} data spacing with zero-filling. A new background spectrum was taken prior to each sample and the crystal was cleaned with distilled water between samples. For Raman, 10 spectra were acquired from the same samples using a Renishaw InVia spectrometer (Renishaw Plc, Gloucestershire, UK) with a 785 nm excitation laser and a 1200 mm^{-1} grating. Spectra were obtained using 100% laser power, 35 seconds exposure time and 2

accumulations. Raman spectra were not acquired from liver and kidney tissues due to intrinsic fluorophores which could not be overcome using photobleaching. Before spectral measurements, the system was calibrated using a silicon source.

Pre-Processing and Computational Analysis

Pre-processing and computational analysis was performed using the IRootLab toolbox (http://trevisanj.github.io/irootlab) in Matlab. ATR-FTIR and Raman spectra were cut to regions of interest, either the biological cell fingerprint region (1800-900 cm^{-1}) or the fatty acid and lipid region (3800-2500 cm^{-1}). Spectra were pre-processed by baseline correction using second order differentiation with a Savitzky-Golay smoothing filter and vector normalising. The use of second derivatives allows overlapping peaks to be resolved meaning that previously 'hidden' peaks can be used for analyses. Thus, second derivatives of ATR-FTIR and Raman spectra were used to identify biochemical signatures for each tissue and ratiometric markers. Spectra were mean centred and Raman spectra were wavelet denoised before exploratory computational analysis. Principal Component Analysis (PCA) was used as a data reduction technique, to transform each spectral dataset into a linear point or PC which captures variance. This was paired with Linear Discriminant Analysis (LDA) which optimises inter-class differences whilst reducing intra-class variation. The optimum number of PCs to retain was determined using the PCA pareto tool and LDA was cross validated to prevent overfitting of data. PCA-LDA produces scores and loadings which were used to look at overall tissue differences and differences between male and female tissues. Statistical analyses were carried out in GraphPad Prism 4 software (GraphPad Software Inc, CA, USA). Differences between scores of organs and ratios were calculated using two-way, repeated measures ANOVA tests with the sex of the bird set as a factor in the analysis and Tukey post-hoc multiple comparison corrections. The differences between male and female scores were calculated using two-tailed *t*-tests. All analyses were done using sample means rather than individual spectra to avoid pseudoreplication.

Results and Discussion

Fingerprint Spectral Profiles

Predatory birds are an important environmental sentinel species but historically, there has been little work focused on the biochemistry of avian tissues. In this work, we have studied control American kestrel tissues using ATR-FTIR and Raman spectroscopic techniques to construct spectral baselines and thus obtain fundamental information concerning biochemical structure and metabolism.

In order to allow visualisation of biochemical information from ATR-FTIR and Raman spectra, mean second derivative analysis of the spectra was performed. Using second derivatives removes contributions from the baseline and takes into account the linear and constant components of the spectrum thus allowing overlapping peaks to be resolved. In the resulting spectrum, the newly revealed peaks cross the origin and become negative (Mark and Workman Jr 2010). In this study, two main spectral regions have been analysed; the fingerprint region of 900-1800 cm^{-1} where the majority of biomolecules vibrate and also the 2500-3800 cm^{-1} region which is associated with fatty acids, triglycerides and other lipids. In S.I. Fig. S1, second derivatives of ATR-FTIR and Raman fingerprint spectra have been displayed together on the same graph, although this is not ideal for visualisation of each tissue, it does allow areas of large differences in absorbance to be identified in the spectra. For example, from the ATR-FTIR second derivatives it is noticeable that around 1750 cm^{-1} brain, heart, skeletal muscle and gonad tissue have higher absorbances. Skeletal muscle has several higher absorbance peaks in protein associated areas such as ~1650 cm^{-1} and ~1550 cm^{-1}. It can also be seen that brain has a specific peak at ~1450 cm^{-1} that is not seen in other tissues. Furthermore, in the higher areas of the spectra, the tissues generally have the same absorbance pattern, different largely in the amount of absorbance. However, in areas between 900 cm^{-1} and 1100 cm^{-1} each tissue appears to have a distinct pattern of absorbance. This has also been observed in spectra from rat tissue homogenates (Staniszewska et al. 2014) and may suggest

that this area is important in distinguishing tissue specific spectral signatures. Raman second derivatives (S.I. Fig. S1B) appear noisier due to the increased wavenumbers measured using this technique but large absorbances are still clearly visible. Large absorbance peaks from brain tissue are seen across the spectrum at ~1450 cm^{-1}, ~1300 cm^{-1}, ~1120 cm^{-1} and ~1050 cm^{-1}. Another large peak can also be seen at ~1025 cm^{-1} from the heart samples. ATR-FTIR second derivatives from the lipid region (S.I. Fig. S2C) show that tissues have the same pattern of absorbance but the extent of the absorbance at the two main peaks in the 3000-2800 cm^{-1} varies by tissue type. Heart and gonad spectra have the highest absorbances in this region as they are lipid rich organs. For full identification of biochemical spectral contributions, mean second derivative spectra were separated by tissue type (see Fig. 1 and 2). Details of all second derivative peak wavenumbers and their biochemical assignments for each tissue can be seen in S.I. Table S2 and S.I. Table S3 respectively for ATR-FTIR and Raman spectra in the fingerprint region and S.I. Table S4 for ATR-FTIR spectra in the lipid and fatty acid associated region.

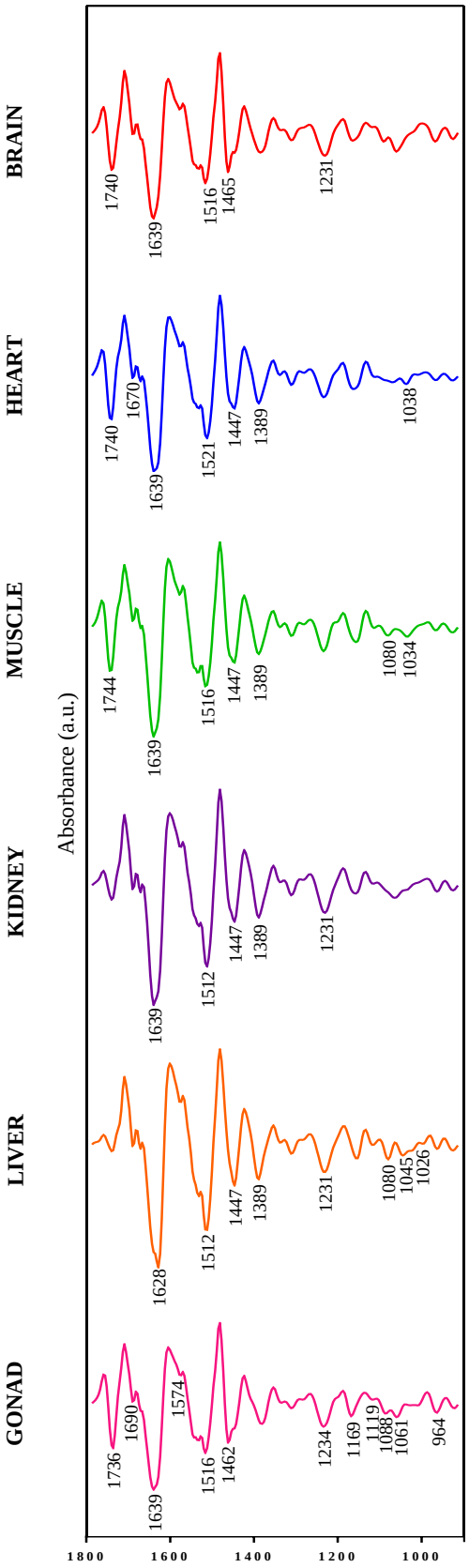


Figure 1 – ATR-FTIR second derivative spectra from the fingerprint region (900-1800 cm^{-1}) of American kestrel brain, heart, skeletal muscle, kidney, liver and gonad tissues with the five largest peaks as well as other peaks of interest highlighted by the peak wavenumber.

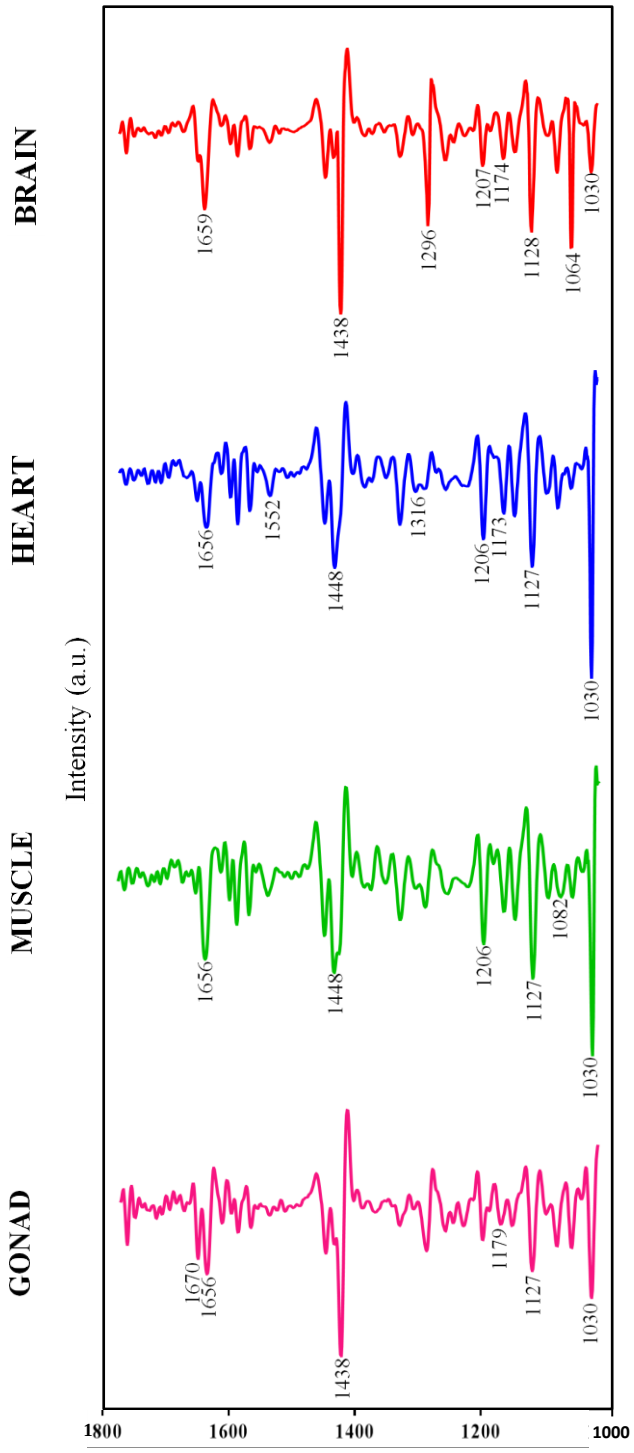


Figure 2 - Raman second derivative spectra from the fingerprint region (900-1800 cm^{-1}) of American kestrel brain, heart, skeletal muscle, kidney, liver and gonad tissues with the five largest peaks as well as other peaks of interest highlighted by the peak wavenumber.

Brain

From the mean ATR-FTIR second derivative spectra from American kestrel brain tissue (Fig. 1), it can be seen that the five largest absorbance peaks correspond with wavenumbers of 1740

cm^{-1}, 1639 cm^{-1}, 1516 cm^{-1}, 1465 cm^{-1} and 1231 cm^{-1}. These wavenumbers are associated with C=O stretching of lipids, Amide I and Amide II vibrations, CH_2 scissoring of lipids and asymmetric phosphate vibrations respectively. This indicates that the brain is largely characterised by the protein and lipid vibrations. This is consistent with what we understand about the composition of brain tissue which is rich in both of these biomolecules, white matter in particular which makes up 60% of brain mass is 49-66% lipid due to high myelin content (O'Brien and Sampson 1965). The protein content of the brain can vary depending on the species but has been found to be between 9-13% in rat and human brains (Banay-Schwartz et al. 1992). Analysis of Raman second derivative spectra from the fingerprint region (Fig. 2) also reveals a number of large intensities related to protein and lipid vibrational modes. The top five peaks and their wavenumber assignments from these second derivatives were 1438 cm^{-1} (CH_2 deformation of lipids), 1296 cm^{-1} (CH_2 deformation), 1128 cm^{-1} (C-N stretch of proteins), 1064 cm^{-1} (C-C stretch of acyl lipid chains) and 1659 cm^{-1} (C=O, Amide I). Raman spectroscopy is a complementary technique to ATR-FTIR and identifies similar intensities from a sample but due to its superior resolution, finer biomolecular details may be detectable. In the Raman spectra, there are three additional peaks of interest at 1207 cm^{-1}, 1174 cm^{-1} and 1030 cm^{-1} which are related to amino acids within the brain samples. These wavenumbers are associated with tryptophan and phenylalanine, tyrosine and phenylalanine and C-H and C-N bending of phenylalanine respectively. Thus, Raman is able to detect amino acid contributions from the proteins in brain samples, particularly intensities from phenylalanine which is a strong Raman scatterer. Peaks from phenylalanine and tryptophan can come not only from protein but also as they are biogenic amines and exist freely in brain tissues where they can function to influence brain chemistry. Tryptophan was only detected in second derivatives from brain and gonad tissues. Tryptophan is critical for the production of serotonin and has a higher affinity for the brain than for its blood transporter protein which results in it crossing the blood-brain barrier (Richard et al. 2009). Phenylalanine is also important as it forms part of dopamine synthesis (Montgomery et al. 2003). Many of the wavelengths identified here by Raman spectroscopy have also been reported from human brain (Daković et al. 2013) which

agrees with current thinking that differences in the composition and structure of human and avian brains are not as large as once thought (Clayton and Emery 2015).

Heart and Skeletal Muscle

Both cardiac and skeletal (pectoral) muscle were analysed in order to ascertain whether their spectral signatures differ using ATR-FTIR and Raman spectroscopy. The main absorbance peaks from both muscle tissues are also associated with protein and lipid but with a heavier bias towards protein vibrations as muscles are protein enriched tissues. The largest ATR-FTIR second derivative spectral peaks from heart samples (Fig. 1) were 1740 cm^{-1} (C=O stretching of lipids), 1639 cm^{-1} (Amide I), 1521 cm^{-1} (Amide II), 1447 cm^{-1} (CH_2 bending from protein and lipid) and 1389 cm^{-1} (CH_3 bending of protein methyl group). Interestingly, the heart has one of the largest absorbances at 1740 cm^{-1} designated as the C=O stretch of lipids, possibly as the heart primarily metabolises fatty acids as its fuel source rather than glucose (Lopaschuk et al. 2010) and fatty acids have a C=O moiety that forms part of the carboxyl group. There is a much smaller peak at 1038 cm^{-1} from glycogen which the heart does have metabolic reserves of but in very low amounts (Pederson et al. 2004). The mean second derivative spectrum from the heart also has another small peak at 1670 cm^{-1} which is associated with β-sheet structures of Amide I. Such absorbances are likely due to the highly ordered secondary structure of proteins that form muscle fibres in the heart (Trinick 1994). Raman second derivatives (Fig. 2) show peaks at 1030 cm^{-1} (Phenylalanine), 1127 cm^{-1} (C-N stretch of protein), 1206 cm^{-1} (hydroxyproline and tyrosine residues of collagen), 1448 cm^{-1} (CH_2 and CH_2CH_3 deformation) and 1656 cm^{-1} (C=C of lipids and Amide I). This is reflective of those peaks identified by ATR-FTIR which were also largely protein related with some contribution from lipid molecules. As seen in spectra from the brain samples, Raman spectroscopy allows some of the amino acid in the proteins to be identified. In the spectra from heart samples, intensity peaks at 1173 cm^{-1}, 1206 cm^{-1} and 1552 cm^{-1} can be seen, indicating respectively that tyrosine and phenylalanine, hydroxyproline and tyrosine and tryptophan residues are found in the heart. This is telling of the heavy protein content of muscle tissues. There are also two peaks seen at

1206 cm^{-1} and 1316 cm^{-1} which identify collagen within the samples, an essential part of connective tissue in muscles (Caulfield and Borg 1979).

Second derivatives of ATR-FTIR spectra from skeletal muscle (Fig. 1) show a similar pattern of absorbance to that derived from heart tissue. Skeletal muscle is characterized by five peaks at 1744 cm^{-1} (C=O stretching of esters), 1639 cm^{-1} (Amide I), 1516 cm^{-1} (Amide II), 1447 cm^{-1} (CH_2 bending) and 1389 cm^{-1} (CH_3 bending of protein methyl group). It also has a peak at 1080 cm^{-1} which is a major vibrational mode of glycogen. Glycogen is typically found in skeletal muscle as part of its metabolism is fuelled by glucose as well as some fatty acids. Collagen is even detectable in the ATR-FTIR spectra in this tissue as shown by a peak at 1034 cm^{-1}. Collagen also forms one of the largest intensity peaks, at 1206 cm^{-1}, in the Raman second derivative spectra (Fig. 2) and another peak at 1082 cm^{-1} is also due to the collagen content of the muscle tissue. The other main Raman peaks are seen at 1656 cm^{-1} (C-C stretching from lipids and Amide I), 1448 cm^{-1} (CH_2 and CH_2CH_3 deformation), 1127 cm^{-1} (C-N stretching of protein) and 1030 cm^{-1} (phenylalanine).

Kidney and Liver

ATR-FTIR spectra of American kestrel kidney and liver tissues were also obtained. Unfortunately, Raman spectra of these organs could not be acquired due to autofluorescence from intrinsic fluorophores which masked the underlying spectral signatures. Autofluorescence is a known limitation of Raman spectroscopy in the near infrared region (a 785 nm laser was used here) as there are some organic molecules within tissues such as urea, lactate and elastin which exhibit high amounts of fluorescence (Gaggini et al. 2015; Huang et al. 2011b). Selection of different sampling areas and photobleaching of the sample before measurements were taken did not allow recovery of spectral peaks from tissues. However, ATR-FTIR spectroscopy is not affected by these issues and clear spectra were able to be measured for both kidney and liver. The main peaks in the fingerprint ATR-FTIR second derivative spectra (Fig. 1) of the kidney were at 1639 cm^{-1}, 1512 cm^{-1}, 1447 cm^{-1}, 1389 cm^{-1}

and 1231 cm^{-1} which have assignments of Amide I, C-H bending of Amide II, CH_2 bending in proteins and lipids, CH_3 bending of protein methyl groups and asymmetric phosphate vibrations respectively. Thus, the kidney is largely characterised by vibrations associated with the structure of proteins. The second derivative ATR-FTIR spectra (Fig. 1) from liver show that the five largest peaks are the same as those seen in the kidney spectra except that the Amide I peak which is at 1639 cm^{-1} is shifted to 1628 cm^{-1} in liver tissues. This indicates that the liver is also typified mostly by protein related vibrational modes and overall, the largest spectral absorbances are similar to those obtained from kidney tissues. However, the liver spectrum has noticeably more peaks in the lower (900-1200 cm^{-1}) region than other tissues, especially the kidney. This is mainly due to absorbances from carbohydrates as the liver is the main glycogen storage organ in the body. There are peaks at 1080 cm^{-1}, 1045 cm^{-1} and 1026 cm^{-1} in the liver second derivative spectrum which are absorbances assigned to glycogen (Matthäus et al. 2008). This combination of peaks is only seen in liver spectra and signifies the heavy glycogen contributions from the organ. There is also a noticeably smaller peak in kidney and liver spectra in the ~1740 cm^{-1} region than observed in other organs which signifies a smaller amount of fatty acids in those tissues. This has also been observed in the kidneys of rats in similar experiments (Staniszewska et al. 2014).

Gonads

The gonads of the kestrels were also studied using both ATR-FTIR and Raman spectroscopy. For the purposes of obtaining an average spectral fingerprint both male and female gonads are analysed here and sex-related differences are explored later. The main absorbances seen in the ATR-FTIR second derivative spectrum (Fig. 1) are associated with protein and lipid vibrations, as shown by peaks at 1736 cm^{-1} (C=O stretching of lipids), 1639 cm^{-1} (Amide I), 1561 cm^{-1} (Amide II), 1462 cm^{-1} (CH_2 bending of lipid) and 1234 cm^{-1} (Amide II). Similarly, intensity peaks from Raman spectroscopy (Fig. 2) also suggest the presence of protein and lipid molecules with peaks at 1656 cm^{-1} (C=C stretch of lipids and proteins), 1670 cm^{-1} (C=C stretch of proteins), 1438 cm^{-1} (CH_2 deformation of lipid), 1127 cm^{-1} (C-N stretching of

protein) and 1030 cm^{-1} (C-H and C-N bending of phenylalanine). The lipid enriched nature of gonads has been documented in many species (Henderson and Almatar 1989; Parisi et al. 2011; Suloma and Ogata 2012) but in birds it may be particularly important as in preparation for the breeding season, lipid needs to be deposited into female follicles to form future egg yolks. Breeding can also effect the amount of lipid in the testes as during sexually inactive periods, large amounts of lipid accumulate in the interstitial cells (Bowles 2006). As cells within the gonads contain genetic material needed for reproduction, there are a number of peaks in the spectra which are associated with DNA. In the ATR-FTIR spectrum, there are six peaks with assignments related to DNA which can be seen at 1690 cm^{-1} (nucleic acids), 1574 cm^{-1} (adenine), 1119 cm^{-1} (phosphate stretching from DNA), 1088 cm^{-1} (vibration of phosphate I in DNA), 1061 cm^{-1} (C-O stretching of deoxyribose) and 964 cm^{-1} (C-O and C-C stretching of deoxyribose). In the Raman second derivative spectrum, there is a peak at 1179 cm^{-1} which is due to cytosine and guanine vibrations. There is also an absorbance band in the ATR-FTIR second derivative at 1169 cm^{-1} which is associated with C-O vibrations of glycomaterials and proteins and is only seen in the gonad spectrum. This may be a vibrational mode related to glycoprotein hormones such as gonadotropins and their receptors which act on the gonads.

Spectral Profiles in the 3800-2500 c m^{-1} Region

The spectra were also analysed outside of the biological cell fingerprint region as the area of 3800-2500 cm^{-1} may be of interest when studying biological material. This region contains vibrational modes largely related to lipid macromolecules such as triglycerides and fatty acids as well as some contribution from proteins. Cross-validated PCA-LDA scores (S.I. Fig. S2A and 2B) indicate which tissues are different from each other in this region as indicated by separation along the linear discriminant (LD) 1 axis. Two way, repeated measures ANOVA tests (with the sex of the bird set as a factor) of the scores showed that the brain was significantly different (P<0.01) from all other tissues except gonad. The brain is known to be a lipid rich organ and second derivative spectra from the fingerprint region (Fig. 1) also

showed that the brain and gonad had the highest absorbances at wavenumbers associated with fatty acids. The heart, muscle and kidney tissues overlap considerably along LD1 and so are not significantly different. As seen in the fingerprint region, these tissues give rise to spectra which have many protein associated absorbances and a similar amount of contribution from lipids. As before, second derivative spectra were used to resolve hidden peaks. From the combined second derivative (S.I. Fig. S2C), it is clear that there is less variation in this part of the spectrum than is seen in the fingerprint region and the tissues largely follow the same absorbance pattern. The variation between tissues is mostly seen in the difference in the amount of absorbance at each peak with brain and gonad having the highest absorbances as they are known to contain more lipid. Liver and kidney appear to have the smallest absorbances in the 2500-3800 cm^{-1} region. In the second derivative spectra separated by tissue type (Fig. 3), there are three large absorbance peaks identifiable as 2851 cm^{-1}, 2924 cm^{-1} and 2963 cm^{-1} which are assigned to symmetric CH_2 stretching, C-H stretching and symmetric CH_3 stretching respectively. Thus, the largest absorbances in this region are caused by presence of methyl and methylene groups in the samples. These three peaks are consistent in spectra from all tissues, however, in muscle the peak at 2851 cm^{-1} is shifted to 2855 cm^{-1} which is associated with symmetric and asymmetric CH_2 vibrations. There are also a number of smaller peaks which are also seen in spectra from all tissues at 3063 cm^{-1} (C_2 aromatic stretching) and 3279 cm^{-1} (symmetric O-H stretching). In some tissues, there is a small shoulder peak between the larger methyl and methylene peaks and the wavenumbers appear to be specific to the tissue type. They are found at 2878 cm^{-1} in brain and 2874 cm^{-1} in kidney which are both associated with symmetric CH_3 vibration of lipid acyl chain as well as 2870 cm^{-1} (CH_3 vibration) in liver and 2893 cm^{-1} (CH_3 stretch in triglycerides) in gonad. Overall, there is not as much variation in absorbances in the 2500-3800 cm^{-1} region but there are still small differences between some tissues which may contribute to a tissue specific spectral signature. The amount of absorbance particularly in the peaks at 2924 cm^{-1} and 2851/5 cm^{-1} may be more useful for discrimination.

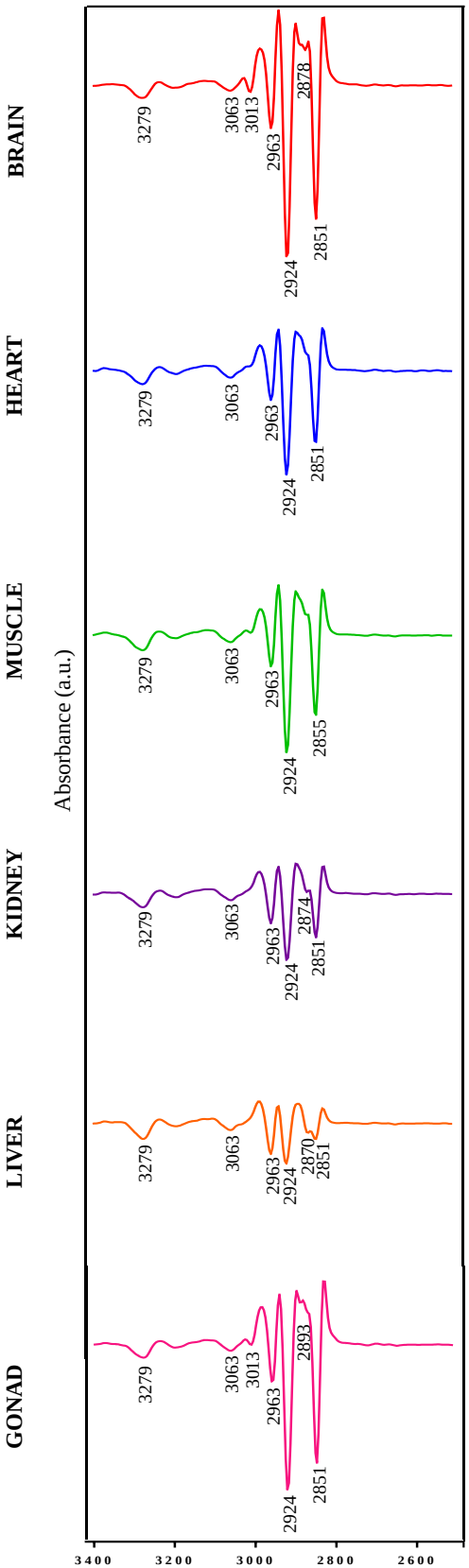


Figure 3 - ATR-FTIR second derivative spectra from the fatty acid and lipid region (3800-2500 cm^{-1}) of American kestrel brain, heart, skeletal muscle, kidney, liver and gonad tissues with the five largest peaks as well as other peaks of interest highlighted by the peak wavenumber.

Spectral Ratios

In order to gain further insight into the biochemical profile of the tissues, ratiometric analysis of some spectral features was performed. The protein-to-lipid ratio is a simple and well known ratio from which the researcher can infer the amounts of protein and lipid in a sample. Such metrics are usually determined using traditional techniques which are advantageous as they give absolute values but are often more time consuming and costly (Szalontai et al. 2000). For the ATR-FTIR protein-to-lipid ratio (Fig. 4A), the absorbance at the 1650 cm^{-1} peak was used as a marker of protein content as it is associated with C=O stretching from the Amide I moiety of proteins. Absorbance at 1740 cm^{-1}, C=O stretching of lipids and phospholipids was used as the lipid marker. In Fig. 4A, it is clear to see that liver has the largest ratio value and thus has the greatest protein content. This is mirrored in the second derivative spectra which indicated that liver tissue had many absorbance peaks associated with proteins and relatively less lipid contributions. The liver is also the site of amino acid metabolism and absorbs the bulk of dietary amino acids from the blood (Berg et al. 2002) which may contribute to the large protein content, especially as the American kestrels were fed a protein rich diet of meat. The liver protein-to-lipid ratio was significantly different to that of all the other tissues (P<0.01) but as its value was so large, the ratios of the other tissues was also analysed without the liver to reveal further information. The kidney had the second largest ratio value and accordingly, the second highest protein content. Thus, with the liver removed from the analysis, it was significantly different from the other tissues (S.I. Table. S7). The brain and then the gonad had the smallest protein-to-lipid ratios which is reflective of their lipid rich nature. For the Raman protein-to-lipid ratio, the peaks at 1689 cm^{-1} (Amide I) and 1739 cm^{-1} (C=O ester of lipids) were used. The Raman ratios (Fig. 4B) show similar values but seem to suggest that there is more protein content in the brain and gonad than suggested from ATR-FTIR ratios. However, only the brain and the muscle were significantly different (P<0.05) and from the second derivatives these tissues were suggested as lipid dense and the protein dense, respectively (liver and kidney were not measured using Raman). The heart and gonad tissues had larger standard errors using Raman and so are not significantly different.

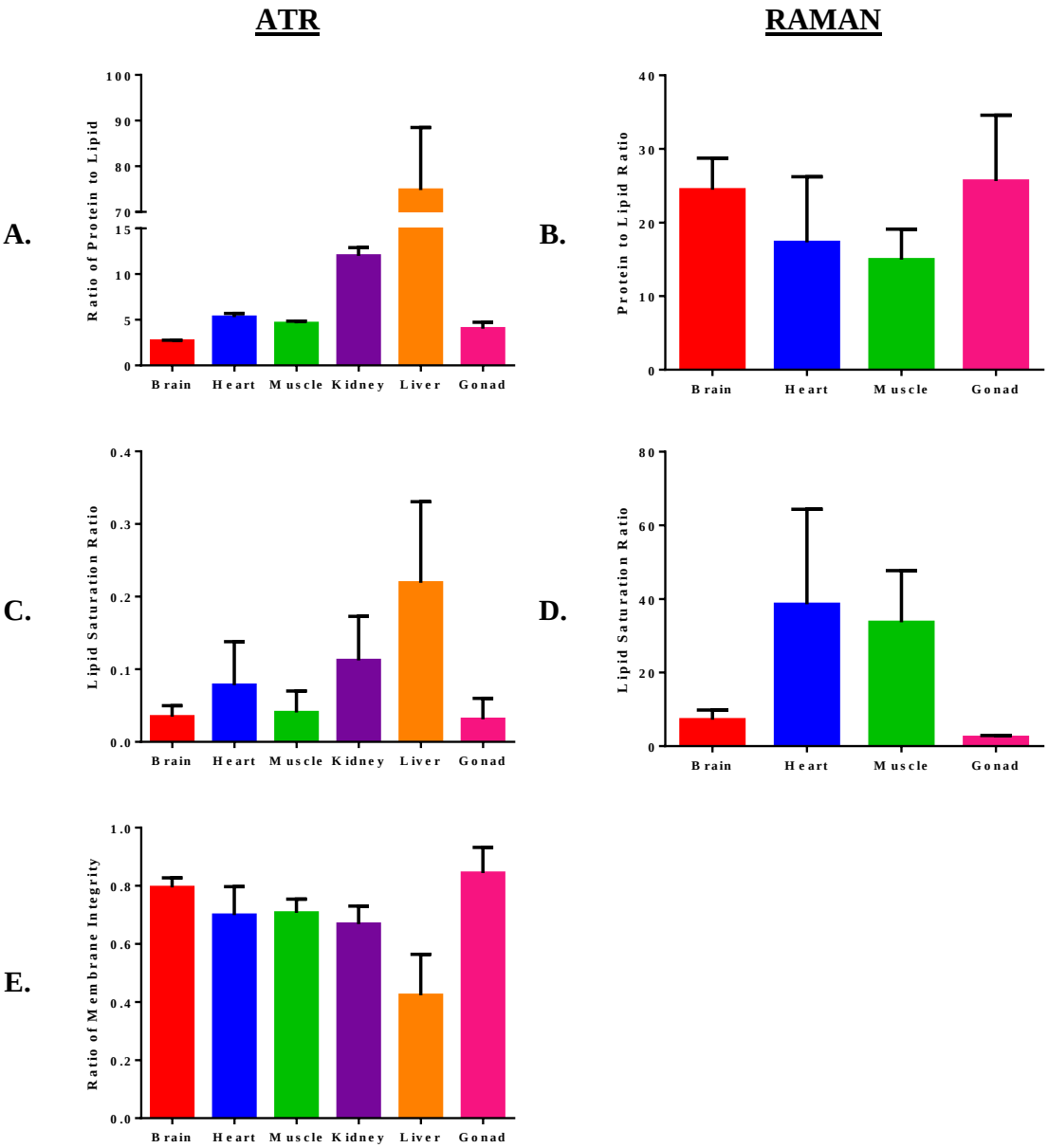
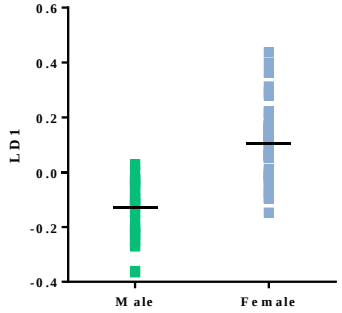


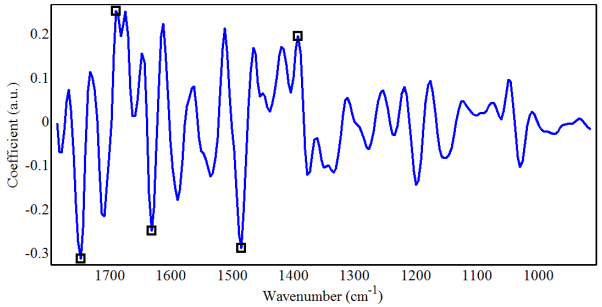
Figure 4 – Ratiometric analysis of absorbance at wavenumbers taken from pre-processed ATR-FTIR and Raman spectra of American kestrel tissues. (**A**) ATR-FTIR protein-to-lipid ratio (1650 cm^{-1}/1740 cm^{-1}); (**B**) Raman protein-to-lipid ratio (1689 cm^{-1}/1739 cm^{-1}); (**C**) ATR-FTIR lipid saturation ratio (3012 cm^{-1}/2924 cm^{-1} + 2851 cm^{-1}); (**D**) Raman lipid saturation ratio (1670 cm^{-1}/1448 cm^{-1}); (**E**) ATR-FTIR membrane integrity ratio (2922 cm^{-1}/2851 cm^{-1}). Black bars represent the standard error of the mean (n = 8).

Lipid saturation ratios were also analysed to provide additional information on the structure of lipids in tissues. For ATR-FTIR spectra, this was calculated by the ratio of the olefinic groups of lipids and fatty acids (3012 cm^{-1}) to the sum of asymmetric (2924 cm^{-1}) and symmetric (2851 cm^{-1}) CH_2 vibrations (Staniszewska et al. 2014) (Fig. 4C). This indicates that there are

more saturated lipids in the liver than in other tissues (P<0.01) and the brain and gonad have the highest amount of unsaturated lipids. It is known that unsaturated lipids such as omega-3 fatty acids play an important role in the functioning of the brain (Bourre 2004) and it is also thought that having more unsaturated lipids may play a part in the defence of tissues against damage particularly that mediated through oxidative stress (Naudí et al. 2013). As the brain has a relatively low capacity for damage repair compared to other organs, unsaturated lipid content may be an important part of its protection. The wavenumber assignments for the lipid unsaturation ratio are different for Raman spectroscopy which ratios the absorbance at 1670 cm^{-1} (v(C=C) of lipids) to 1448 cm^{-1} (CH_2 of lipids) (Wu et al. 2011a). From this ratio (Fig. 4D), it is also evident that the brain and gonad have the smallest amount of saturated lipids and there are more saturated lipids in heart and skeletal muscle tissues. The presence of more unsaturated lipids in the brain and gonad has also been documented in zebrafish using the same technique (Li et al. 2015). Finally, to assess the membrane integrity the ATR-FTIR ratio of absorbance at 2922 cm^{-1} (asymmetric stretching of CH_2 in acyl lipid chains) to 2851 cm^{-1} (symmetric CH_2 stretching) was calculated (Fig 4E) (Staniszewska et al. 2014). The main difference between the tissues is seen the decreased ratio value in liver. This indicates a higher degree of membrane disorder and thus more motional freedom in acyl chains of lipids which has also previously been found in FTIR spectra of rat liver (Melin et al. 2001).

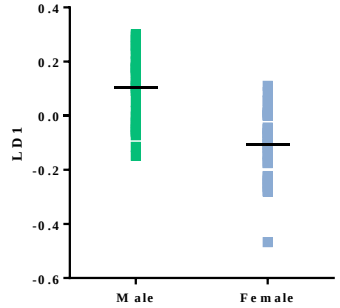
HEART

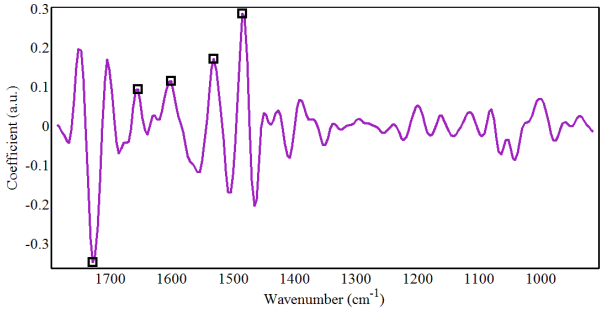




Wavenumber (cm^{-1})	Assignment
1748	*v*(C=C) of fatty acids & lipids
1690	Base carbonyl stretching and ring breathing
1632	C-C stretch of phenyl
1485	C-H deformation
1393	CH_3 bending of proteins

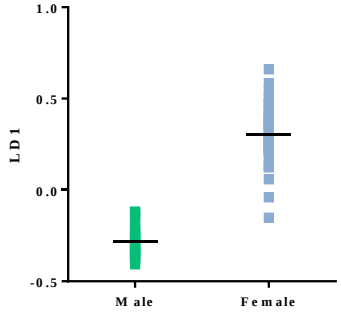
KIDNEY

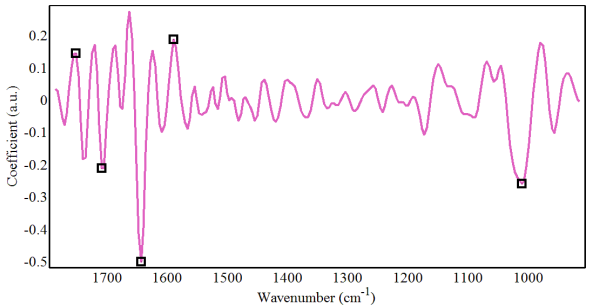




Wavenumber (cm^{-1})	Assignment
1728	C=O vibration
1655	α-helix, C=O, C-N & N-H of Amide I
1601	C=N of cytosine, N-H of adenine
1531	C=N & C=C stretching
1485	C-H deformation

GONAD

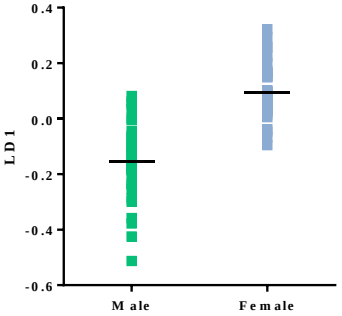


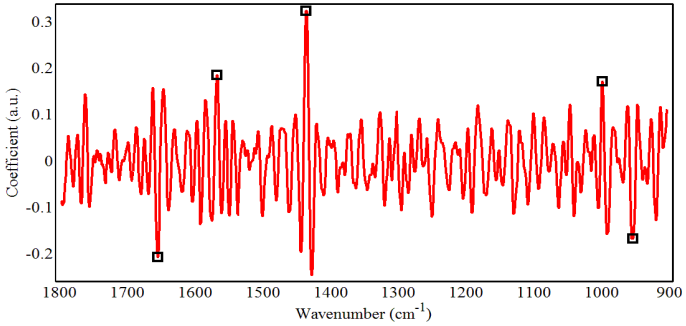


Wavenumber (cm^{-1})	Assignment
1751	*v*(C=C) of fatty acids & lipids
1709	C=O of thymine
1643	C=O stretch of Amide I
1589	C-C stretch of phenyl
1011	C-O stretch of deoxyribose

Figure 5 – Sex-related differences in tissues identified by ATR-FTIR spectroscopy. (**A**) Significantly separated (as assessed by two-tailed *t*-tests, $P<0.05$) male and female scores along LD1; (**B**) Loadings along LD1 showing absorbance peaks responsible for differences between male and female scores with the largest five peaks highlighted; (**C**) Wavenumbers from the five largest peaks in LD1 loadings with tentative assignments.

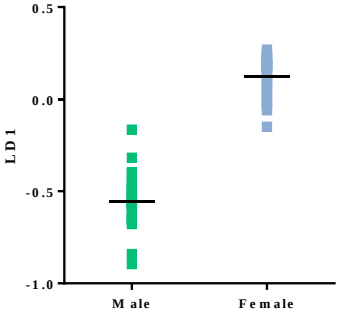
BRAIN

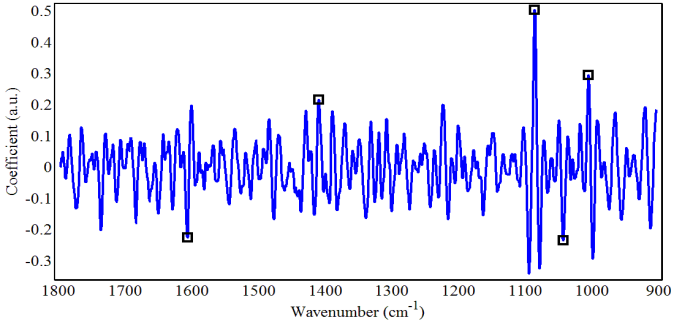




Wavenumber (cm^{-1})	Assignment
1655	α-helix (Amide I), C=C of lipid
1568	Tryptophan, COO^-
1436	CH_2 & CH_3 bending
999	C-O ribose, C-C
954	$\nu_s(CH_3)$ of α-helix proteins

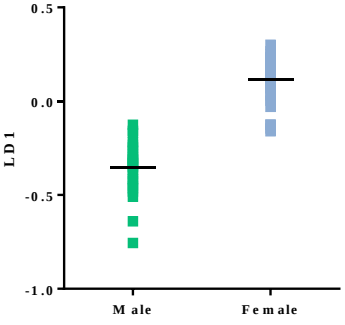
HEART

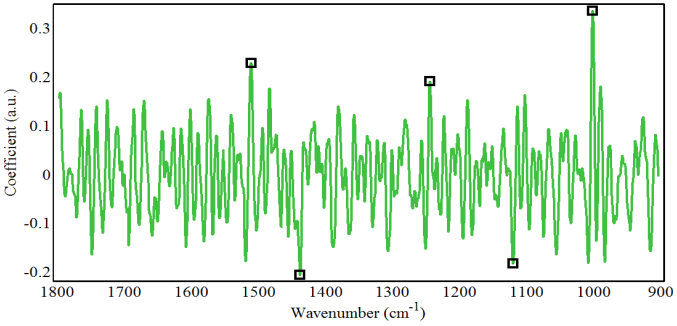




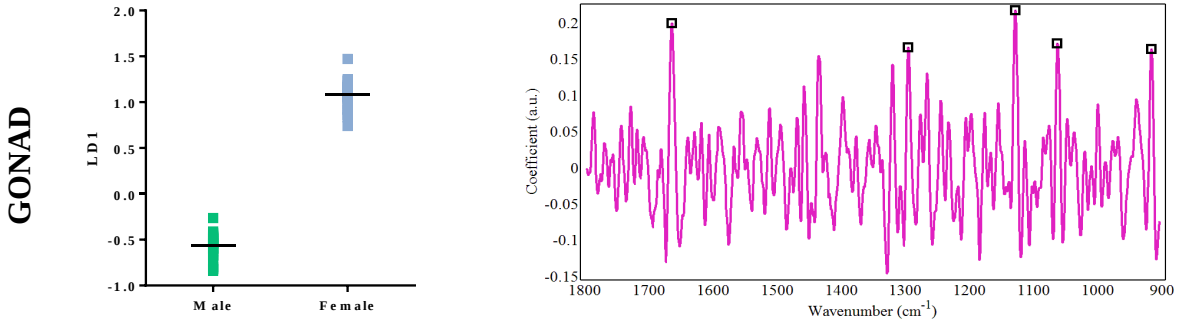
Wavenumber (cm^{-1})	Assignment
1607	Tyrosine & phenylalanine
1410	Methyl groups from collagen
1087	Carbonate, phosphate, ν(C-C) of acyl backbone in lipid
1043	Proline from collagen
1006	Phenylalanine

MUSCLE





Wavenumber (cm^{-1})	Assignment
1511	Cytosine
1438	CH_2, acyl backbone in lipids
1244	Amide III, asymmetric phosphate, collagen
1121	C-O band of ribose, ν(C-C)
1002	C-C, phenylalanine



Wavenumber (cm^{-1})	Assignment
1664	Amide I
1294	Methyl twisting, CH_2 deformation
1128	C-N stretching of protein & C-O stretching of carbohydrates
1063	C-C stretch of lipid
916	Deoxyibose vibration

Figure 6 - Sex-related differences in tissues identified by Raman spectroscopy. (**A**) Significantly separated (as assessed by two-tailed *t*-tests, $P<0.05$) male and female scores along LD1; (**B**) Loadings along LD1 showing intensity peaks responsible for differences between male and female scores with the largest five peaks highlighted; (**C**) Wavenumbers from the five largest peaks in LD1 loadings with tentative assignments.

Sex-Dependant Differences

As equal numbers of female and male birds were selected for this experiment, sex-related differences in tissues were investigated. There were few easily discernible differences in PCA-LDA scores along LD1, LD2 and LD3 with all samples imputed but splitting the samples by sex was found to increase the separation of scores (S.I. Fig. S3 and S4, S.I. Table S6 and S7). The ATR-FTIR 3D scores plot from male birds shows separation along LD1 and LD3 of brain (P<0.01) and gonad (P<0.01) scores away from other tissues as well as separation of heart scores along LD2. In the female scores plot, there is less overall separation but gonad, heart and brain scores show less overlap with other tissue scores. Raman PCA-LDA scores separated by sex (S.I. Fig. S4) show even better cluster separation especially in male plots where heart and muscle scores significantly co-cluster away from other tissue scores along LD1 (P<0.01). In the female scores plot, there is obvious separation of gonad scores along LD2 (P<0.05) with little overlap and co-clustering of heart and brain scores away from other tissue scores along LD1 (P<0.01) and LD2 (P<0.05). Overall, the ATR-FTIR and Raman PCA-LDA scores suggest that there may be sex-dependant variations particularly in brain, heart and gonad tissues.

Differences between male and female PCA-LDA scores along LD1 were analysed using unpaired *t*-tests and where the differences were found to be significant, the loadings were investigated to understand which biochemical differences were responsible for separation of the scores. Using ATR-FTIR, sex-related differences in heart, kidney and gonad were identified (Fig. 5) and Raman spectroscopy identified differences in brain, heart, muscle and gonad tissues (Fig. 6). The ATR-FTIR loadings showed that the top five absorbance peaks driving difference between male and female birds were related to protein molecules in the samples and one peak was from fatty acid and lipid absorbance. The top peaks from Raman identified the alterations responsible as amino acids (phenylalanine and tyrosine) in proteins, the acyl backbone from lipids and two absorbance peaks associated with collagen. There are a number of reported biochemical differences between male and female hearts that could be

responsible for this variation. Sex-related differences in heart tissue have been reported in other species and are thought to have implication for heart disease risks. There are a number of proteins in the heart including receptor molecules which are known to be increased in females (Gabel et al. 2005) and protein loss in the heart may also occur differentially between sexes. In humans, increased myocardial cell loss over time has been reported as up to 1g per year more for males than females (Olivetti et al. 1995). Differences in the expression of collagen as well as other structural proteins have also been reported with collagen mRNA levels increased as much as 300% in the hearts of female rats (Rosenkranz-Weiss et al. 1994). Differences between male and female gonads were also identified using both ATR-FTIR and Raman spectroscopy. The major wavenumbers responsible were associated with Amide I, CH_2 and C-N vibrations of proteins, C=O of thymidine and deoxyribose in DNA as well as C-C and C=C bonds in fatty acids and lipids. Differences in gonads were expected as there are well known alterations between testes and ovaries. For example, there are a number of specific cell types that are only found in the testes such as Leydig and Sertoli cells which may have their own spectral contributions (Deviche et al. 2011; Guibert et al. 2011). Also as mentioned previously, depending on the point in the reproductive cycle, both ovaries and testes can have drastically altered lipid composition (Aire 1997; Bowles 2006).

ATR-FTIR kidney scores were also significantly different between male and female birds. The alterations responsible for this difference were largely protein related with the top five peaks being associated with protein structure including Amide I and α-helix content, cytosine and adenine vibrations from DNA molecules and C=O vibration from lipids. Multiple factors have been determined as implicated in sex-related kidney differences including receptor density (Sabolić et al. 2007), varying renal isoenzyme subunits (Butera et al. 1989) as well as altered size and structure of the kidneys due to size sexual dimorphism (Baylis 2005; Jean-Faucher et al. 1987). Raman spectroscopy was also able to detect sex-dependant differences in brain and skeletal muscle tissues. The main peaks in the brain loadings which drive this separation are largely associated with α-helix, Amide I, tryptophan, CH_2 and CH_3 vibrations of

proteins as well as C=C from lipids and C-O from ribose. Differences in male and female brains have been a hotly debated topic and these alterations may be due to differing neurochemistry or brain structure (Ngun et al. 2011). Increased grey matter has been identified in some parts of the female brain in humans (Luders et al. 2009) and this in turn effects the protein and lipid composition of the brain. Finally, peaks from Raman skeletal muscle loadings which explain the variance between males and females are associated with a range of biomolecules including the lipid acyl backbones, phenylalanine and Amide I from proteins, collagen, ribose and cytosine. It is possible that this is simply due to differences in size and mass of muscles between male and female birds. American kestrels are sexually dimorphic in size with females being larger which may account for these alterations which cover a broad range of biomolecules.

Conclusions

In conclusion, ATR-FTIR and Raman spectroscopy are complimentary techniques that can be utilised to obtain tissue specific profiles of a broad range of American kestrel tissues in the fingerprint region (900-1800 cm^{-1}). Although the major peaks from second derivative tissue spectra were often related to protein and lipid biomolecules in the samples, there are also important absorbance differences and peaks of interest that confer information regarding the biochemical structure and metabolism of tissues. For example, the brain spectra showed it contained abundant lipids, the heart and skeletal muscles had peaks that are derived from collagen, the liver spectra had multiple glycogen absorbances and spectra of the gonad tissue had an increase in the number of DNA associated peaks. The higher frequency region of the spectrum (3800-2500 cm^{-1}) associated with fatty acids and triglycerides may also useful in distinguishing between tissues. There is much less variation in wavenumber peaks in this region of the spectrum but the differing absorption at the peaks assigned to methyl and methylene groups may be discriminatory. Being lipid-rich tissues, the brain and gonad had the largest absorbances in this region with the liver and kidneys having the smallest. Further biochemical information, such as protein/lipid content, lipid saturation and membrane

integrity, can be gained through the use of spectral ratios. Through calculating ratios of absorbance between two or more peaks, we were able to confirm that liver contains the most proteins and the brain and gonad have the highest lipid content. We could also determine that saturated lipids accumulate in the liver whereas are the brain and gonad contain mostly unsaturated lipids which may be protective for these tissues. Finally, spectral signatures of some tissues were found to be dependent on the sex of the bird. The brain, heart, kidney, muscle and gonad tissues were found to be significantly different between males and females using ATR-FTIR and/or Raman spectroscopy. The findings of studies such as this are essential to extend our understanding of how tissue type and underlying biochemistry can affect spectral results. Further work should focus on using vibrational spectroscopy techniques to analyse the spectral biochemistry of other tissues such as lung, skin and intestine. Investigation into the use of additional spectroscopy methods such as surface enhance Raman or employment of an excitation laser with a longer wavelength will also be beneficial to reduce autofluorescence from liver and kidney tissues.

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Supplementary Information:

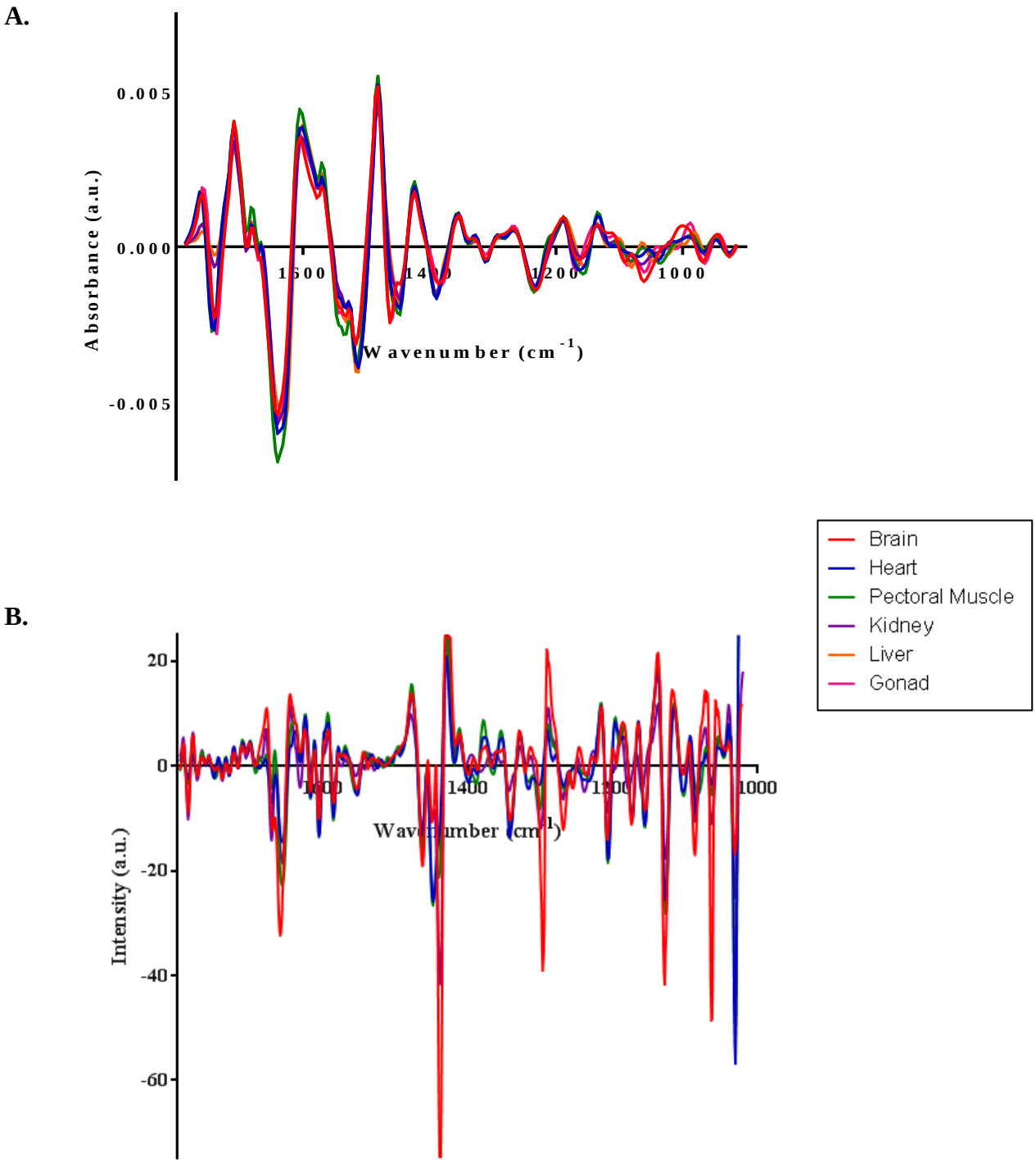
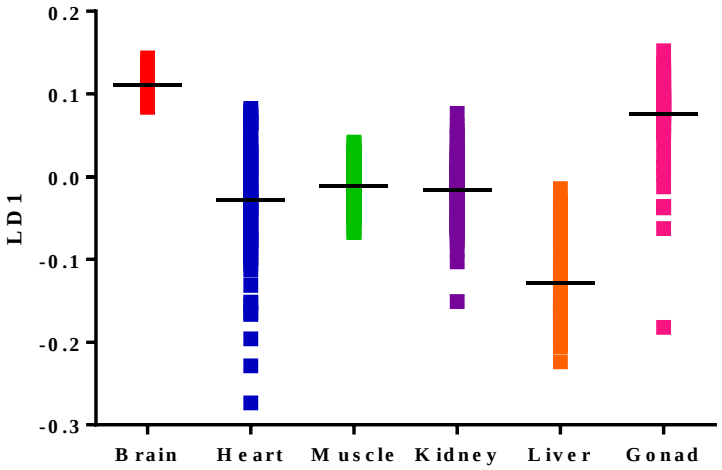


Figure S1 – Pre-processed second derivative spectra in the fingerprint region (900-1800 cm^{-1}) from American Kestrel tissues interrogated with ATR-FTIR (**A**) and Raman (**B**) spectroscopy

A.



B.

Organ comparison	Significance
Brain *vs.* Heart	*P*<0.01
Brain *vs.* Muscle	*P*<0.01
Brain *vs.* Kidney	*P*<0.01
Brain *vs.* Liver	*P*<0.01
Heart *vs.* Liver	*P*<0.01
Muscle *vs.* Liver	*P*<0.01
Muscle *vs.* Gonad	*P*<0.05
Kidney *vs.* Liver	*P*<0.01
Kidney *vs.* Gonad	*P*<0.01
Liver *vs.* Gonad	*P*<0.01

C.

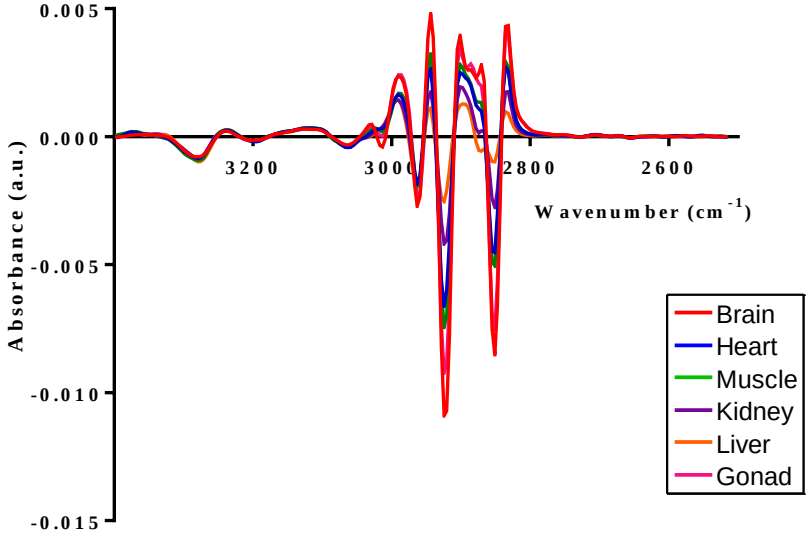


Figure S2 – (**A**) LD1 cross-validated PCA-LDA scores, (**B**) significance of PCA-LDA scores as assessed by two-way, repeated measures ANOVA with the sex of bird as a factor and Tukey's post-hoc multiple comparisons correction and (**C**) second derivative spectra from American kestrel tissues interrogated in the fatty acid and lipid region (3800-2500 cm^{-1}) using ATR-FTIR spectroscopy.

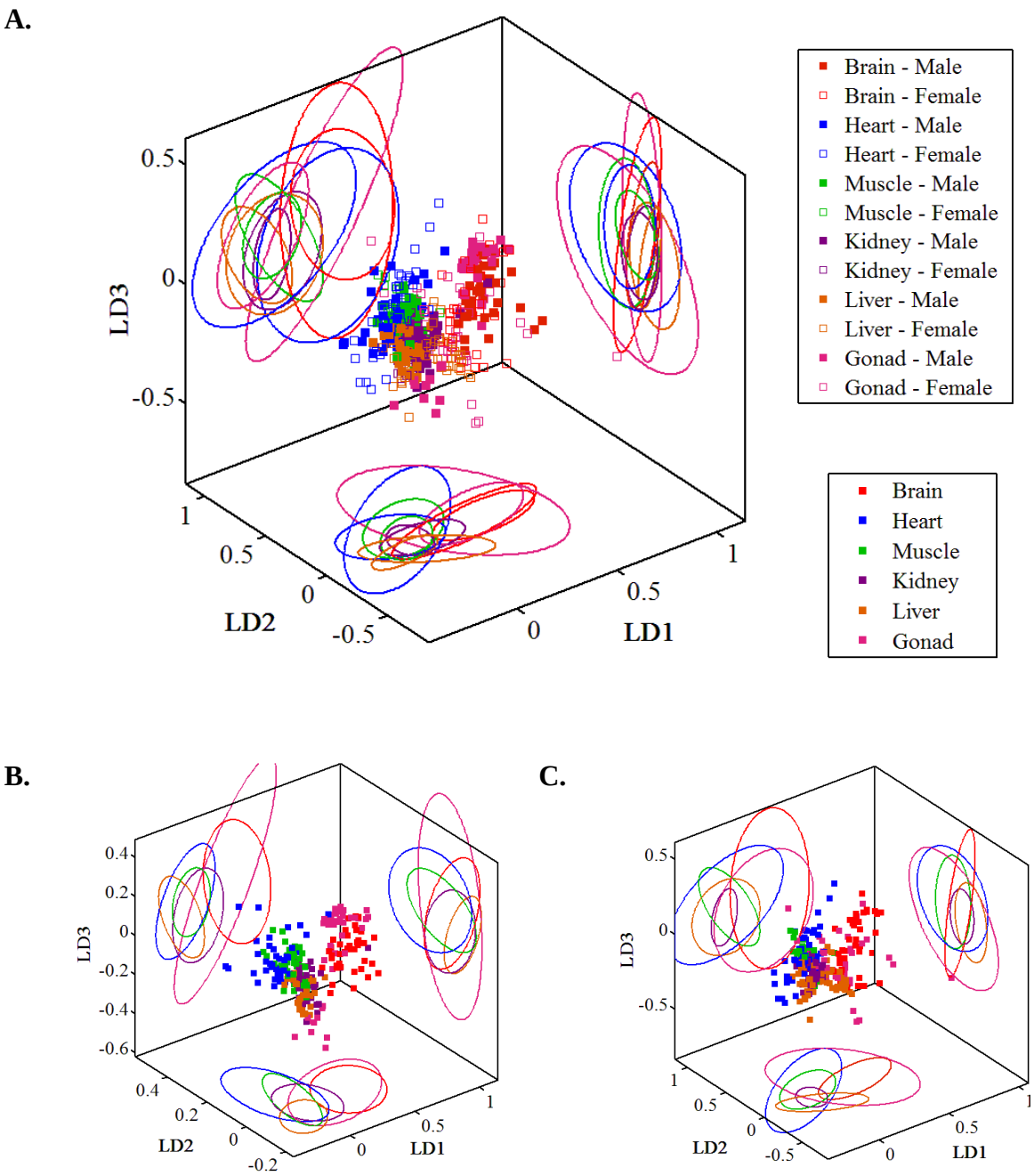


Figure S3 – 3D cross-validated PCA-LDA scores from ATR-FTIR spectroscopy of American kestrel tissues. Rings indicate 95% confidence intervals. (**A**) All birds; (**B**) Male birds only; (**C**) Female birds only.

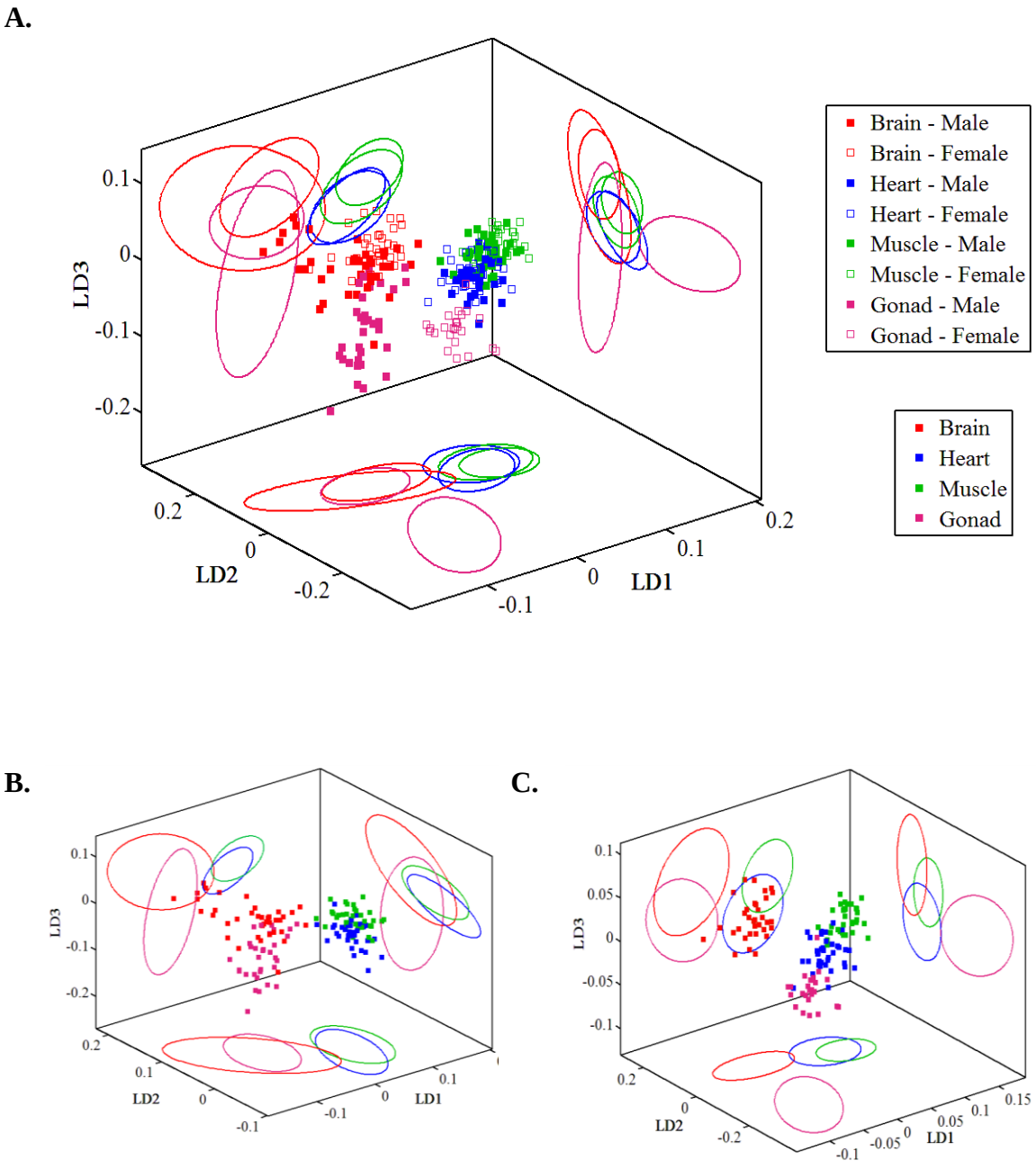


Figure S4 – 3D cross-validated PCA-LDA scores from Raman spectroscopy of American kestrel tissues. Rings indicate 95% confidence intervals. (**A**) All birds; (**B**) Male birds only; (**C**) Female birds only

Species Name	Common Name	Band number	Sex	Source	Age Class
Falco sparverius	American Kestrel	2502	Female	Captive Bred	Adult
Falco sparverius	American Kestrel	2673	Female	Captive Bred	Adult
Falco sparverius	American Kestrel	2677	Female	Captive Bred	Adult
Falco	American	2712	Male	Captive Bred	Adult

sparverius	Kestrel				
Falco sparverius	American Kestrel	2720	Male	Captive Bred	Adult
Falco sparverius	American Kestrel	2729	Male	Captive Bred	Adult
Falco sparverius	American Kestrel	2731	Female	Captive Bred	Adult
Falco sparverius	American Kestrel	2743	Male	Captive Bred	Adult

Table S1 – Control birds used for ATR-FTIR and Raman spectroscopic investigation

Wavenumber (cm^{-1})	Assignment	Brain	Heart	Muscle	Kidney	Liver	Gonad
1744	Ester C=O stretching			✓			
1740	C=O stretching of lipids	✓	✓		✓	✓	
1736	C=O stretching (lipids)						✓
1690	Nucleic acids carbonyl stretching	✓		✓	✓		✓
1670	Amide I (anti-parallel β-sheets) and *v*(C=C) of lipids and fatty acids		✓		✓	✓	
1639	Amide I	✓	✓	✓	✓		✓
1628	Amide I					✓	
1578	Ring C-C stretch of phenyl	✓		✓	✓	✓	
1535	C=N and C=C stretching			✓			
1574	C=N of adenine						✓
1531	Amide II		✓		✓	✓	✓
1530	C=N and C=C stretching	✓					
1521	Amide II		✓				
1516	Amide II	✓		✓			
1512	Amide II, C-H bending				✓	✓	
1465	CH_2 scissoring of lipid acetyl chain	✓					
1462	CH_2 lipid bending						✓
1447	CH_2 bending (protein and lipid)		✓	✓	✓	✓	
1389	CH_3 bending of protein methyl group		✓	✓	✓	✓	
1385	δCH_3 and C-O, C-H and N-H vibrations	✓					
1381	CH_3 bending of lipid						✓
1312	Amide III	✓	✓	✓	✓	✓	✓
1234	Amide III		✓	✓			✓
1231	Asymmetric phosphate	✓			✓	✓	
1169	C-O of glycomaterials and proteins						✓
1165	CC, COH and CO vibrations	✓	✓				
1157	C-O stretching of protein and carbohydrates			✓	✓	✓	
1119	Symmetric P-O-C stretching					✓	✓
1115	Symmetric P-O-C stretching			✓			
1092	Phosphate II	✓					
1088	Phosphate I (DNA)						✓
1080	C-O stretch of Glycogen			✓		✓	
1069	C-O stretching of ribose		✓				
1065	C-O stretching of phosphodiester and ribose				✓		
1061	C-O stretch in deoxyribose	✓					✓
1045	O-H bending of glycogen					✓	
1038	CH_2OH of glycogen		✓				
1034	Collagen			✓			
1026	Glycogen					✓	
968	C-O and C-C in deoxyribose	✓	✓	✓			
964	C-O and C-C in deoxyribose				✓	✓	✓

Table S2 – Tentative wavenumber assignments for peaks in the fingerprint region (1800-900 cm^{-1}) of pre-processed second derivative ATR-FTIR spectra from American kestrel tissues. Assignments were selected from Movasaghi et al (2008) and Strong et al (2016).

Wavenumber (cm^{-1})	Assignment	Brain	Heart	Muscle	Gonad
1789	C=O vibrations	✓			
1786	C=O stretching				✓
1670	Amide I, C=C stretching				✓
1659	C=O, Amide I	✓			
1656	C=C of lipids and Amide I		✓	✓	✓
1631	Amide I				✓
1605/6	Ring C-C stretch of phenyl	✓	✓	✓	✓
1584/5	C=C olefinic stretch	✓	✓	✓	✓
1555	Amide II			✓	
1552	*ν*(C=C) of tryptophan		✓		
1463/4	δCH_2	✓	✓	✓	✓
1448	CH_2CH_3 deformation, CH_2 deformation		✓	✓	
1438	CH_2 deformation of lipid	✓			✓
1399	C=O symmetric stretch, CH_2 deformation		✓	✓	
1342	CH_2 deformation (protein and carbohydrates)	✓	✓	✓	✓
1316	Twisting mode of collagen		✓		
1301	Triglycerides, CH_2 and C-H of lipids			✓	
1296	CH_2 deformation	✓			
1299	CH_2 deformation (lipids)				✓
1267/8	Amide III, C-H lipid	✓	✓		
1264	Triglycerides (fatty acids)			✓	✓
1239	Amide III				✓
1207	Tryptophan and phenylalanine in protein	✓			✓
1206	Hydroxyproline, tyrosine (collagen)		✓	✓	
1173/4	Tyrosine, phenylalanine, C-H bend in proteins	✓	✓	✓	
1179	Cytosine and guanine				✓
1160	C-C and C-N stretching in protein				✓
1155	C-C and C-N in protein	✓	✓	✓	
1127/8	C-N stretching of protein	✓	✓	✓	✓
1102	Phenylalanine in protein			✓	
1086/7	C-C acyl backbone of lipid	✓	✓		✓
1082	Carbohydrate residue of collagen			✓	
1064	C-C stretch and acyl lipid chains	✓	✓		
1063	C-C skeletal stretch random conformation			✓	✓
1030	C-H and C-N bending of phenylalanine	✓	✓	✓	✓

Table S3 – Tentative wavenumber assignments for peaks in the fingerprint region (1800-900 cm^{-1}) of pre-processed second derivative Raman spectra from American kestrel tissues. Assignments were selected from Movasaghi et al (2007).

Wavenumber (cm^{-1})	Assignment	Brain	Heart	Muscle	Kidney	Liver	Gonad
3279	Symmetric O-H stretching	✓	✓	✓	✓	✓	✓
3063	C_2 aromatic stretching	✓	✓	✓	✓	✓	✓
3013	*v*=CH of lipids	✓					✓
2963	CH_3 vibration modes	✓	✓	✓	✓	✓	✓
2924	C-H stretch	✓	✓	✓	✓	✓	✓
2893	CH_3 symmetric stretch						✓
2878	Symmetric CH_3 vibration of acyl lipid chains	✓					
2874	Symmetric CH_3 vibration of acyl lipid chains				✓		
2870	Symmetric CH_3 vibration				✓	✓	
2855	Symmetric and asymmetric CH_2 vibrations			✓			
2851	Symmetric CH_2 stretch	✓	✓		✓	✓	✓

Table S4 – Tentative wavenumber assignments for peaks in the fatty acid and lipid region (3800-2500 cm^{-1}) of pre-processed second derivative ATR-FTIR spectra from American kestrel tissues. Assignments were selected from Movasaghi et al (2008).

Spectroscopy	Ratio	Organ Comparison	Level of Significance
ATR-FTIR	Protein-to-Lipid	Brain *vs.* Liver	*P*<0.01
		Heart *vs.* Liver	*P*<0.01
		Muscle *vs.* Liver	*P*<0.01
		Kidney *vs.* Liver	*P*<0.01
		Gonad *vs.* Liver	*P*<0.01
ATR-FTIR	Protein-to-Lipid (Liver removed)	Brain *vs.* Kidney	*P*<0.01
		Heart *vs.* Kidney	*P*<0.05
		Muscle *vs.* Kidney	*P*<0.01
		Kidney *vs.* Gonad	*P*<0.01
Raman	Protein-to-Lipid	Brain *vs.* Muscle	*P*<0.05
ATR-FTIR	Lipid Saturation	Brain *vs.* Heart	*P*<0.01
		Brain *vs.* Kidney	*P*<0.01
		Brain *vs.* Liver	*P*<0.01
		Heart *vs.* Liver	*P*<0.01
		Muscle *vs.* Kidney	*P*<0.05
		Muscle *vs.* Liver	*P*<0.01
		Kidney *vs.* Liver	*P*<0.01
		Kidney *vs.* Gonad	*P*<0.01
		Liver *vs.* Gonad	*P*<0.01

Table S5 – Significant differences between spectral ratios derived from ATR-FTIR and Raman spectroscopy of American kestrel tissues. Significance was determined by a two-way, repeated measures ANOVA with the sex of bird as a factor and Tukey's post-hoc multiple comparisons correction

All Samples		
Linear Discriminant (LD)	**Organ Comparison**	**Level of Significance**
LD1	Brain *vs.* Heart	$P<0.01$
	Brain *vs.* Muscle	$P<0.01$
	Brain *vs.* Kidney	$P<0.01$
	Brain *vs.* Liver	$P<0.01$
	Heart *vs.* Gonad	$P<0.01$
	Muscle *vs.* Gonad	$P<0.01$
	Kidney *vs.* Gonad	$P<0.01$
	Liver *vs.* Gonad	$P<0.01$
LD2	Heart *vs.* Liver	$P<0.05$
	Heart *vs.* Gonad	$P<0.05$
Male Samples		
LD1	Brain *vs.* Heart	$P<0.05$
	Brain *vs.* Muscle	$P<0.05$
	Brain *vs.* Kidney	$P<0.01$
	Brain *vs.* Liver	$P<0.05$
LD2	Heart *vs.* Liver	$P<0.05$
LD3	Muscle *vs.* Kidney	$P<0.05$
Female Samples		
LD1	Brain *vs.* Muscle	$P<0.05$
	Brain *vs.* Kidney	$P<0.01$
	Brain *vs.* Liver	$P<0.01$
	Muscle *vs.* Gonad	$P<0.05$
	Kidney *vs.* Gonad	$P<0.05$

Table S6 – Significant differences between cross-validated PCA-LDA scores from American kestrel tissues along LD1, LD2 and LD3 from ATR-FTIR spectroscopy. Significance was determined by a two-way, repeated measures ANOVA with the sex of bird as a factor and Tukey's post-hoc multiple comparisons correction.

All Samples		
Linear Discriminant (LD)	**Organ Comparison**	**Level of Significance**
LD1	Brain *vs.* Heart	$P<0.01$
	Brain *vs.* Muscle	$P<0.01$
	Brain *vs.* Kidney	$P<0.01$
	Heart *vs.* Gonad	$P<0.01$
	Muscle *vs.* Gonad	$P<0.01$
LD2	Brain *vs.* Heart	$P<0.01$
	Brain *vs.* Muscle	$P<0.01$
	Brain *vs.* Gonad	$P<0.01$
	Heart *vs.* Gonad	$P<0.05$
	Muscle *vs.* Gonad	$P<0.01$
LD3	Brain *vs.* Heart	$P<0.01$
	Brain *vs.* Muscle	$P<0.01$
	Heart *vs.* Muscle	$P<0.01$
	Heart *vs.* Gonad	$P<0.01$
Male Samples		
LD1	Brain *vs.* Heart	$P<0.01$
	Brain *vs.* Muscle	$P<0.01$
	Heart *vs.* Gonad	$P<0.01$
	Muscle *vs.* Gonad	$P<0.01$
LD2	Brain *vs.* Heart	$P<0.05$
LD3	Muscle *vs.* Kidney	$P<0.05$
	Heart *vs.* Muscle	$P<0.01$
Female Samples		
LD1	Brain *vs.* Heart	$P<0.01$
	Brain *vs.* Muscle	$P<0.01$
	Heart *vs.* Muscle	$P<0.05$
LD2	Brain *vs.* Heart	$P<0.05$
	Brain *vs.* Muscle	$P<0.05$
	Brain *vs.*Gonad	$P<0.05$
LD3	Brain *vs.* Heart	$P<0.05$
	Brain *vs.* Muscle	$P<0.01$
	Heart *vs.*Muscle	$P<0.01$

Table S7 – Significant differences between cross-validated PCA-LDA scores from American kestrel tissues along LD1, LD2 and LD3 from Raman spectroscopy. Significance was determined by a two-way, repeated measures ANOVA with the sex of bird as a factor and Tukey's post-hoc multiple comparisons correction.

Chapter 4. Levels of organochlorine pesticides are associated with amyloid aggregation in apex avian brains

Kelly A. Heys, Richard F. Shore, M. Glória Pereira and Francis L. Martin.

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Contribution:

- Wild, dead birds were collected and dissected by the Predatory Bird Monitoring Scheme (PBMS).
- Chemical analysis of brain tissue was previously performed at Centre for Ecology and Hydrology, Lancaster.
- I conducted all experiments for the study.
- I prepared, processed and acquired data for all samples including conducting computational analysis.
- I prepared the first draft of the manuscript.

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Kelly A. Heys Prof. Francis L. Martin

Levels of organochlorine pesticides are associated with amyloid aggregation in apex avian brains

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Abstract

Organochlorine (OC) pesticides pose a significant environmental risk to wildlife and humans and have been associated with Alzheimer's disease (AD). This study aims to spectroscopically analyse brains from free-flying birds and link the results to OC exposure and consequent amyloid aggregation. As long-lived apex predators, predatory birds represent a sentinel species similar to humans. Therefore, the results have implications for both species and may also add to our understanding of the role OC pesticides play in the development of AD. Brains of wild sparrowhawks were analysed using ATR-FTIR and Raman spectroscopy and Congo red staining; results were correlated with OC pesticide concentrations in livers. Effects of OC exposure were sex and age dependant and associated alterations were seen in lipids and protein secondary structure. A shift from α-helix to β-sheet conformation of proteins indicated that concentrations of OC pesticides > 7.18 µg/g may lead to cerebral amyloid aggregation.

Introduction

Organochlorine (OC) insecticides are a large and diverse class of compounds, many of which are highly lipophilic and persistent in the environment. They have attracted a lot of attention in the past due to wide-spread usage between the 1940s and 1970s and the ensuing recognition that some OCs have significant detrimental effects on the environment (Blus et al. 1974; C.H. Walker 2012). OC insecticides were banned in many countries during the 1990s due to environmental and human health concerns but are still used in many developing countries, particularly to control malaria (Ali et al. 2014). Since the global OC usage decreased due to substance control, there have been many questions regarding the long-term effects of environmental OC exposure. Even in the past three years, OC concentrations have been detected and measured in human blood samples from around the world (Lam et al. 2015; Wang et al. 2013) as well as in many species of fish (Ameur et al. 2013; Yohannes et al. 2014) which is thought to represent a significant source of dietary OC intake. Recently, OCs have also been determined in environmental compartments including air (Meire et al. 2016), water (Temoka et al. 2016), and soil (Arienzo et al. 2015) and have been detected in various environmental species such as dolphins (Arienzo et al. 2015), bears (Romanić et al. 2015) and mussels (Galvao et al. 2014). Such studies demonstrate that these persistent chemicals are still a current environmental concern, both for wildlife and for the human population.

OCs are known neurotoxins which is the mechanism by which they are able to control pest populations. They alter sodium and potassium channels, in particular causing persistent opening of sodium channels which allows constant firing of action potentials (O'Reilly et al. 2006). The brain is particularly vulnerable to their effects as it is rich in lipids and has a low capacity for detoxification. Thus, OCs have been implicated in the development of neurodegenerative diseases including Alzheimer's disease (AD) and Parkinson's disease (PD). AD patients have been reported to have higher serum levels of dichlorodiphenyldichloroethylene (DDE, a metabolite of DDT), whilst elevated levels of β-

hexachlorocyclohexane have been found in patients with PD (Richardson et al. 2014). AD is characterised by the presence of aggregated amyloid-β protein plaques as well as neurofibrillary tangles composed of phosphorylated tau protein in the brain (Perl 2010). The associated cognitive decline is caused by neuronal death and loss of synapses due to the presence of these protein aggregates. Many factors have been attributed to the causation of AD including genetic factors, ageing and exposure to chemicals such as OCs (Manivannan et al. 2015). Environmental exposure to a number of chemicals, including OC pesticides, is thought to cause the aggregation of amyloid-β proteins by inducing cellular oxidative stress. OCs may directly alter NADH/NAD^+ levels or interact with the respiratory chain in mitochondria leading to an increase in cellular reactive oxygen species (ROS) levels (Thany et al. 2013). This results in oxidation and aggregation of cellular proteins including amyloid-β.

AD is a major health issue in ageing Western populations so research to further our understanding of the disease is essential. Although many studies aim to link chemical exposure with the aetiology of AD, investigations are often limited by availability of post-mortem brain tissue and environmental exposure data for those samples (Zaganas et al. 2013). In this study, we have obtained sparrowhawk (*Accipiter nisus*) brain samples that have been analysed for concentrations of various OC pesticides, [see Supplementary Information (SI) Table S1] allowing for further analysis of potential OC-mediated amyloid aggregation. Birds of prey, such as sparrowhawks, are apex predators and occupy a niche at the top of their food chain in the same way that humans do. Also, free-flying predatory birds encounter cumulative, real world OC exposures making them ideal sentinels in which to study environmental pollutants. Many predatory bird species have been previously used as sentinel species to study environmental contaminant such as OCs, PBDEs, PCBs and heavy metals in tissue samples as well as using feathers, blood and eggs (Gómez-Ramírez et al. 2014; Newton et al. 1993). Using vibrational spectroscopy techniques which are able to identify alterations at the biomolecular level, we aim to determine cellular alterations caused by high and low OC exposures. In line with current thinking, we will also investigate whether exposure to OC

pesticides can be linked with amyloid aggregation in sparrowhawk brain samples using spectroscopy, staining and immunoassay methodologies. To our knowledge, this is a unique study that attempts to couple chemical exposure data and spectral data with cerebral amyloid aggregation in birds.

Methods and materials

Brain samples

Sparrowhawk (*Accipiter nisus*) brain samples were obtained from the Predatory Bird Monitoring Scheme (http://pbms.ceh.ac.uk) which receives dead birds for analysis from members of the public in the UK. On receipt, the Sparrowhawk brains were removed from the carcasses and stored in a tissue archive at -18˚C. The brain samples for this study were chosen from the archived tissues from Sparrowhawks that died between 1979 and 1990 in the UK. The Sparrowhawk livers had been previously analysed for wet weight OC pesticide concentrations using previously reported analysis methods and so the brain samples used were ranked according to the total OC pesticide concentrations found in the livers of the same birds. Total OC pesticide concentrations ranged from 0.56 to 82.31 µg/g in liver (see SI Table S1). 58 brain samples (n=58) were ranked and selected so that there were two main sample groups; 30 with 'high' total OC concentrations (15 male, 15 female) and 28 with 'low' total OC concentrations (15 male, 13 female). Details of samples used are included in SI Table S1.

Spectral signal of Amyloid fibril

In order to investigate the spectral signal from amyloid, Aβ1:42 fibres were interrogated using Raman spectroscopy. 50 µM of Aβ1:42 was aggregated for one week in 10 mM phosphate buffer and then spun down in an airfuge system (Beckman Coulter, (UK) High Wycombe, UK) for 1 h at 125,000 x g to pellet fibres. The pellet was resuspended in 100 µl of distilled water and 10 µl was deposited onto gold coated glass slides (Platypus Technologies, WI, USA).

Spectroscopy analysis of Amyloid fibrils

For each sample, 1g of brain material was spectroscopically analysed on an infrared-reflective, low-E slide (Kevley Technologies, Chesterland, OH). For ATR-FTIR spectroscopy, five spectra were obtained per slide using a Bruker TENSOR 27 FTIR spectrometer with Helios ATR attachment containing a diamond IRE of 250 µm x 250 µm (Bruker Optics, Coventry, UK). The ATR-FTIR was set to attain spectra with 8 cm^{-1} spectral resolution and 32 co-additions, allowing 3.84 cm^{-1} spectral data spacing. Mirror velocity was set to 2.2 kHz. After each sample was analysed, the diamond was cleaned with distilled water and a new background was taken to account for environmental deviations. For Raman spectroscopy, samples were interrogated using an InVia Renishaw Raman spectrometer, containing a 785nm excitation laser, coupled with a charge- coupled device (CCD) and Leica microscope systems (Leica Microsystems, Milton Keynes, UK). Before each session of taking spectra, the spectrometer was calibrated using a silicon source. Seven spectra were taken per sample using 100% laser power, 30 seconds exposure time and 2 accumulations with a 1200 1 mm^{-1} grating. Spectra of amyloid fibres were taken at 100% laser power, 35 seconds exposure time and 3 accumulations. Spectra were pre-processed and analysed using the IRootLab toolbox (http://trevisanj.github.io/irootlab/) with Matlab 2013a (The Maths Works, MA, USA). Firstly, all spectra were cut to the fingerprint region of 900-1800 cm^{-1}. ATR-FTIR spectra were pre-processed by baseline correction using 2nd order differentiation, vector normalisation and then mean centred. Spectral data from Raman were pre-processed in the same manner but spectra were wavelet denoised before mean centering. In order to extract features from the large spectral dataset, principal component analysis (PCA) and linear discriminant analysis (LDA) were used. PCA was used as a data reduction technique and optimal number of PCs was input into LDA to minimise intra-class variation. Leave-one-out cross-calculation was employed to avoid overfitting data. Tentative wavenumber assignments were given to loadings using FTIR and Raman assignments reported (Movasaghi et al. 2007; Movasaghi et al. 2008). In order to investigate the secondary structure of proteins in brain samples from high and low OC concentration groups, deconvolution of the mean amide I peak

was performed using PeakFit v4.12 software (Systat Software Inc, San Jose, California). Using the software, spectra underwent baseline correction and were cut to the amide I region of 1600-1700 cm^{-1}. Second derivatives of spectra were used to identify subpeaks that were 'hidden' within the amide I peak. Final deconvolution of subpeaks was performed using Gaussian peak-fit functions with 20% smoothing. The r^2value of each fitted curve was maintained above 0.999.

Staining Amyloid fibrils

All reagents were purchased from Sigma (Sigma-Aldrich, Dorset, UK) unless stated otherwise. A Congo red stock solution was made up in 100 ml of 80% ethanol using 0.3 g of Congo red and 0.3 g NaCl. This was diluted to a working solution with the addition of 1 ml of 1% NaOH. For each sample, 1 g of brain material was transferred onto a glass microscope slide and stained with Congo red working solution for 15 min. Slides were then rinsed in dH_2O before differentiation in alkaline alcohol and counterstaining with haematoxylin. Following this, they were briefly exposed to blueing reagent and rinsed with tap water. Slides were viewed using a 15x Reflachromat objective on a Thermo Nicolet Continuμm microscope with cross-polarising filters, fitted with an Olympus U-TV0.5XC-3 video camera. Microscopy image contrast was adjusted and then sharpened with a 0.55 weight Unsharp mask using ImageJ software (http://imagej.nih.gov/ij/).

Quantifying Amyloid Aβ1:42

To obtain tissue lysates suitable for ELISA, 100 mg of brain tissue was homogenized, on ice, in 500 µl of TBS with 1% triton X-100 and 2 mM EDTA. Tissue homogenates were then centrifuged for 20 minutes at 13000 RPM. The supernatant was transferred to a fresh tube and stored at -80˚C. The ELISA was performed using a colorimetric BetaMark x-42 ELISA kit from Biolegend (London, UK). The reagents, standards and test samples were diluted and prepared as outlined in the manufacturer's instructions. 50 µl of sample was loaded into each well along with 50 µl of horseradish peroxidase detection antibody and incubated overnight at

4˚C. The next day, wells were washed 5 times and incubated, in the dark, with the tetramethylbenzidine substrate for 50 minutes at room temperature. The plate was read at 620 nm using a Tecan Infinite 200 Pro microplate reader (Tecan, Männedorf, Switzerland). Results were analysed by construction of a 4PL standard curve and interpolation using Graphpad Prism 4.

Statistical analyses

GraphPad Prism 4 (GraphPad Software Inc., CA, USA) was used to carry out statistical tests unless otherwise stated. Two-tailed, unpaired *t*-tests in order to compare PCA-LDA scores from two sample groups (High *vs.* Low) or to compare absorbance at specific peak locations in the case of CySS:Protein ratio and amyloid peak analysis. Two-way ANOVAs with Sidak multiple comparison tests were performed to compare PCA-LDA scores and account for interactions with other independent variables (Male *vs.* Female or Adult *vs.* Juvenile). *t*-tests and ANOVAs were done using sample spectral means rather than on individual spectra to avoid pseudoreplication. Normality of data was checked using D'Agostino-Pearson omnibus normality tests. Canonical correspondence analysis (CCA) was performed using XLSTAT (Addinsoft, New York, USA). CCA is a direct gradient analysis technique and can be used to detect species variation patterns which are caused by a provided set of environmental variables and thus explain variation in samples. Data was arranged into a species data table composed of spectral data and a table of environmental variables composed of OC concentration data. The CCA was run using 1000 random permutations.

Results

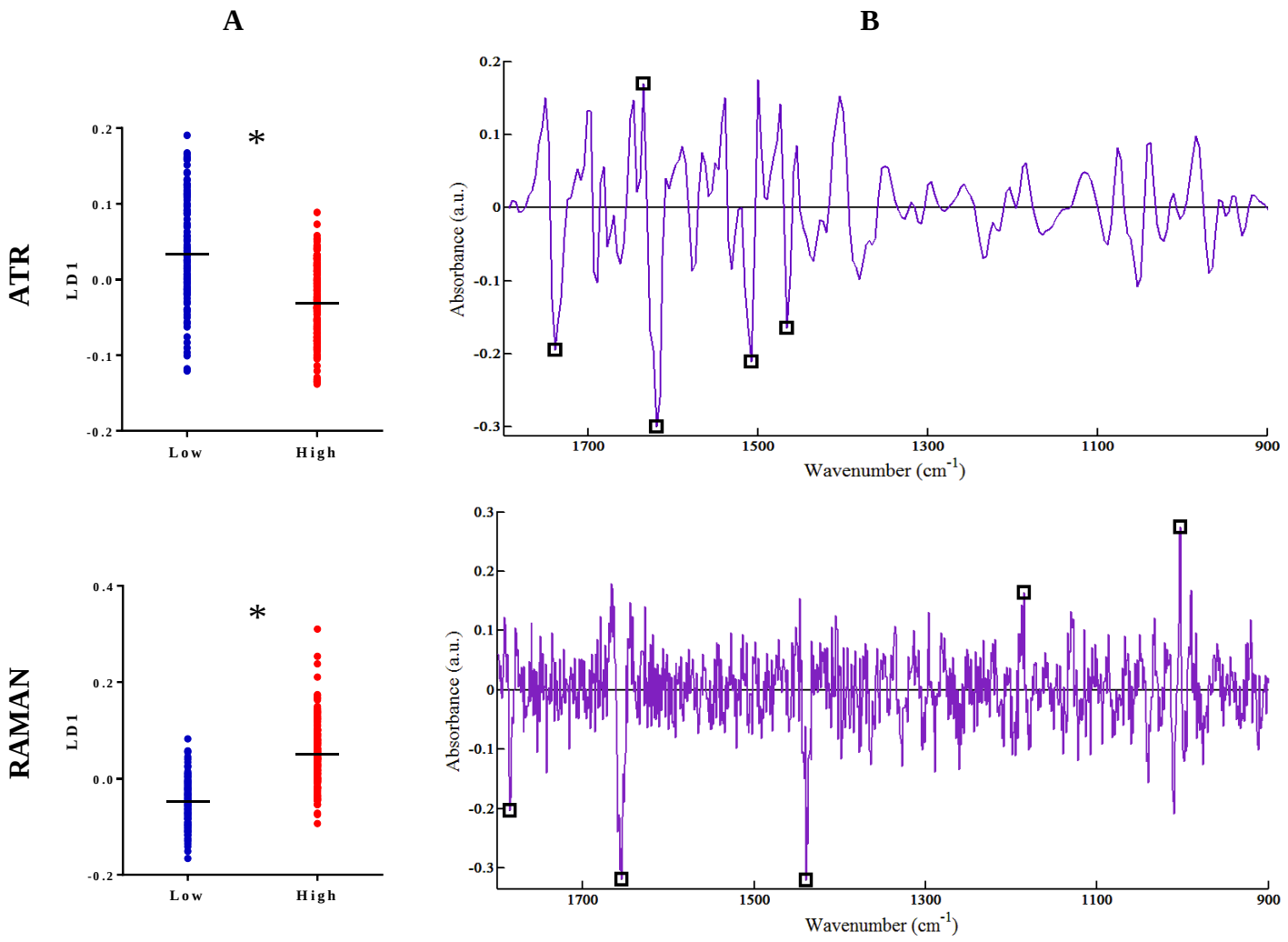
Spectral analysis of OC exposed brain samples

To assess the effects of high and low OC pesticide exposure, sparrowhawk brain samples were interrogated using ATR-FTIR and Raman spectroscopy and analysed using cross-calculated PCA-LDA. High and low OC exposure groups were found to have significantly

different spectral features. Fig. 1A shows one-dimensional (1D) scores plots from PCA-LDA of ATR-FTIR and Raman spectra which illustrate the difference between the two exposure groups along linear discriminant (LD) 1. In scores plots distance denotes dissimilarity, thus we can see that diverse spectral alterations were induced by low (below 2.03 μg/g) and high (above 7.18 μg/g) OC concentrations as demonstrated by the differences between group means and in distribution patterns. Unpaired, two-way t-tests using sample means (rather than spectral replicates) verified that the high and low exposure groups were significantly different at the $P<0.01$ level. Analysis of corresponding ATR and Raman LD1 loadings (Fig. 1B) was performed to detect biospectral alterations that were responsible for the separation observed between groups in the scores plot. The top five peaks which contributed with the most magnitude to the observed variation were identified and tentative wavenumber alterations were assigned (Fig. 1C). ATR-FTIR identified the top five wavenumber alterations in areas associated with C=O stretching and CH_2 vibrations of lipids (1740 cm^{-1}; 1466 cm^{-1}) and in regions associated with alterations in protein secondary structure. These comprised of changes in Amide I and Amide II spectral areas (1620 cm^{-1}; 1508 cm^{-1}) as well as alterations in β-sheet structures within the Amide I region (1636 cm^{-1}). Wavenumber alterations detected by Raman spectroscopy confirmed that variation between the two exposure classes was due to alterations in C=O and CH_2 lipid regions (1785 cm^{-1}; 1440 cm^{-1}) and spectral regions corresponding with protein secondary structure as changes in the α-helix structures of Amide I (1654 cm^{-1}) were detected. Raman spectral analysis also determined that alterations in asymmetric phosphate stretching vibrations from DNA (1185 cm^{-1}) and the amino acid phenylalanine (1003 cm^{-1}) contributed importantly to the difference between low and high OC pesticide exposed brain samples.

Information was available on the age and sex of the birds from which brain samples were obtained so spectra were re-classified accordingly so that possible age and sex dependant effects of OC exposure could be investigated. The significance of differences between PCA-LDA scores along LD1 was assessed using two-way ANOVAs with Sidak multiple

comparison tests in order to account for the other independent variables (sex, age, OC concentration). *P* value results from these analyses can be seen in S.I.Table 2. ATR-FTIR and Raman PCA-LDA scores plots that were classed by sex (S.I. Fig. 2A) show that there were significant ($P<0.01$) differences between male and female scores. Group means are significantly separated and distribution of spectra is altered along LD1. Sex-dependant effects were also analysed individually in high and low OC exposure groups. Raman scores from samples exposed to high OC concentrations were significantly separated by sex along LD1 at the $P<0.01$ level (S.I. Fig. 2B). However, there was no significant difference found between male and female ATR-FTIR scores in the high OC group as there was a large amount of overlap between the two classes along the LD1 axis. Significant ($P<0.01$) separation between male and female scores was revealed in the low OC exposure group using both spectral methods (S.I. Fig. 2C). The effects of OC exposure were also found to be influenced by the age of the bird. Significant differences between adult and juvenile PCA-LDA scores were detected along LD1 using ATR-FTIR ($P<0.01$) and Raman ($P<0.01$) (Fig. 2A). Significant age-related differences at the $P<0.01$ level were found in both high and low OC exposure classes when analysed with Raman and at the $P<0.05$ significance level for ATR-FTIR scores from the low OC exposure class (Fig. 2B and 2C). Therefore, the spectral results of exposure to OC pesticides are influenced by sex and age of the sparrowhawk.



Wavenumber (cm-1)	Assignment
1740	C=O stretching of lipids
1636	Amide I of β sheet structures
1620	Amide I
1508	Amide II
1466	CH2 vibrations in lipids

Wavenumber (cm-1)	Assignment
1785	C=O of lipids
1654	Amide I of α-helix structures
1440	CH_2 of lipids
1185	Asymmetric phosphate
1003	Phenylalanine

Figure 1 – ATR-FTIR and Raman spectral comparison of brains samples from high and low OC exposure groups. (**A**) One-dimensional scores plots from cross-calculated PCA-LDA. Group means are represented by a black line and significance at the $P<0.01$ level as determined by unpaired, two-tailed t-tests is indicated by an asterisk. (**B**) PCA-LDA loadings along LD1 with top 5 peaks highlighted and (**C**) Tentative biochemical assignments for the top five wavenumber peaks.

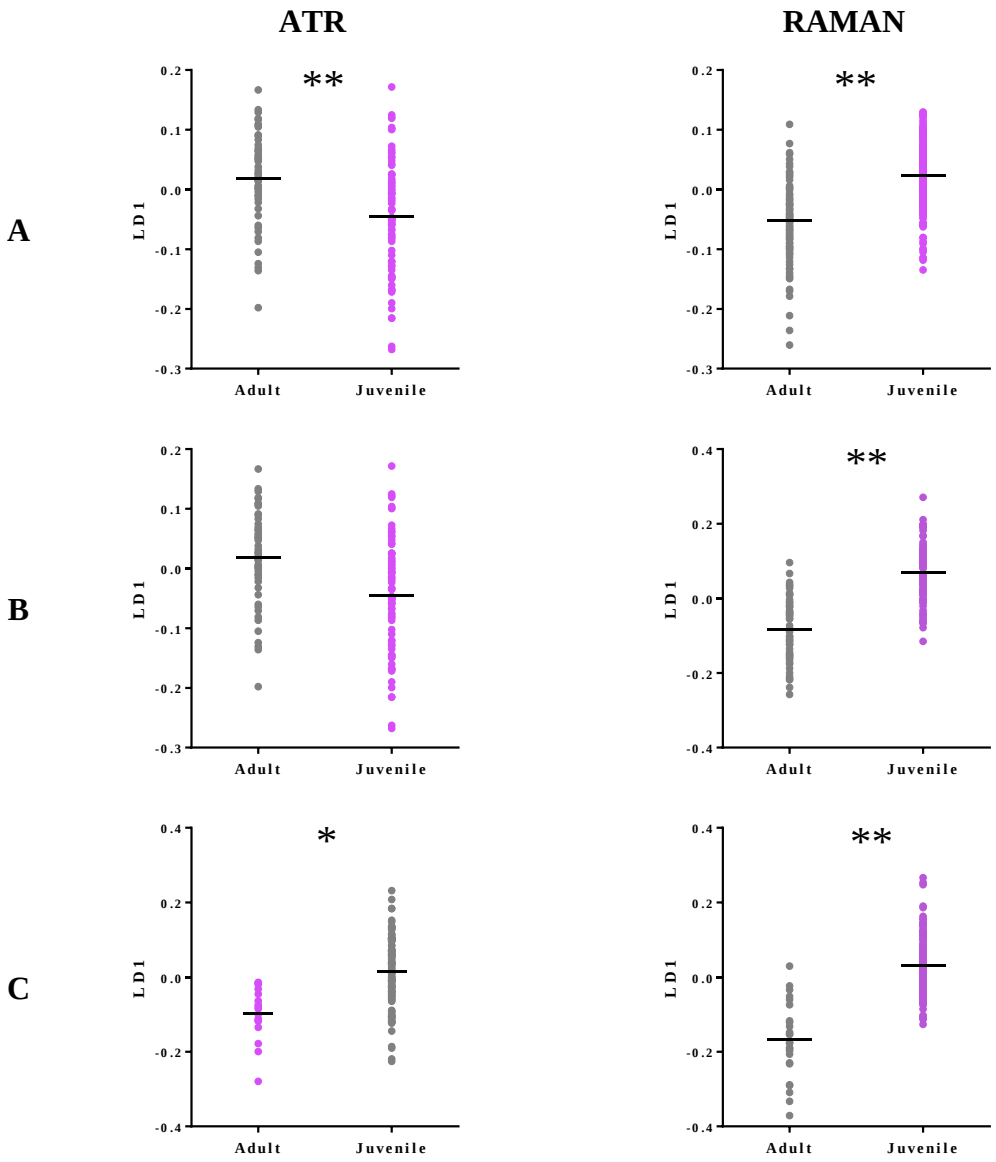


Figure 2 – One-dimensional PCA-LDA scores plots from ATR-FTIR and Raman spectroscopy of brain samples showing age dependant effects of OC exposure. (**A**) All samples (**B**) High OC exposure group samples and (**C**) Low OC exposure group samples. Group means are represented by a black line. Significance at the $P<0.05$ level as determined by two-way ANOVA with Sidak multiple comparison test is indicated by one asterisk and significance at the $P<0.01$ level is indicated by two asterisk.

Contribution of amyloid to separation of high and low OC exposed samples

To investigate the involvement of amyloid in the difference observed between high and low OC exposed brain samples, amyloid Aβ1:42 fibrils were interrogated using Raman spectroscopy (Fig. 3). The five major vibrational peaks were identified as 1671, 1447, 1342, 1216 and 1003 cm^{-1} which are

wavenumbers associated with C=C stretching, CH_2 bending, CH deformation, C-N stretching and phenylalanine respectively. Some of these peaks are similar to those responsible for the separation of high and low Raman scores, in particular those related to regions of Amide I (C=C stretching), CH_2 vibrations and phenylalanine. The Raman spectral results were analysed to see if the two OC exposure groups were separated at wavenumbers associated with amyloid spectral peaks. It was found that scores from high and low OC exposure brains were significantly dissimilar ($P<0.05$) along LD1 at all the major peaks found in the amyloid spectrum. To further investigate, the Amide I peak was deconvoluted to reveal 'hidden' subpeaks (Fig. 4A). Deconvolution of the Amide I peak from both high and low OC exposure classes resulted in five peaks related to secondary protein structure. Analysis of subpeaks from high OC exposure spectra showed that there was a lesser proportion of α-helix than observed in the low OC exposure subpeaks. Conversely, more β-sheet was contributing to the Amide I peak in the high group than seen in the low group (see SI Table 3). As it is difficult to assess whether percentages are significantly different without additional values (numerator/denominator), we cannot state a definite significance but this should be explored in future work.

Further to this, brain samples were stained with Congo red. All samples were stained and examined and four samples displayed the characteristic apple green colour (Fig. 4B). Three samples which were thin and fibrillar in appearance were from the high OC exposure group and one was from the low group. The three samples from the high group were all from male sparrowhawks (two adults and one juvenile) and the sample from the low group was from a female. Finally, to confirm if amyloid was present in any of the samples, an ELISA was performed using lysates of the brain tissues (see SI Fig. 1). Amyloid Aβ1:42 was detected in two of the brain samples but the points did not fall within the linear portion of the standard curve and so quantification would not be accurate. The two samples which contained amyloid were both from the low OC exposure group and both from juvenile female birds.

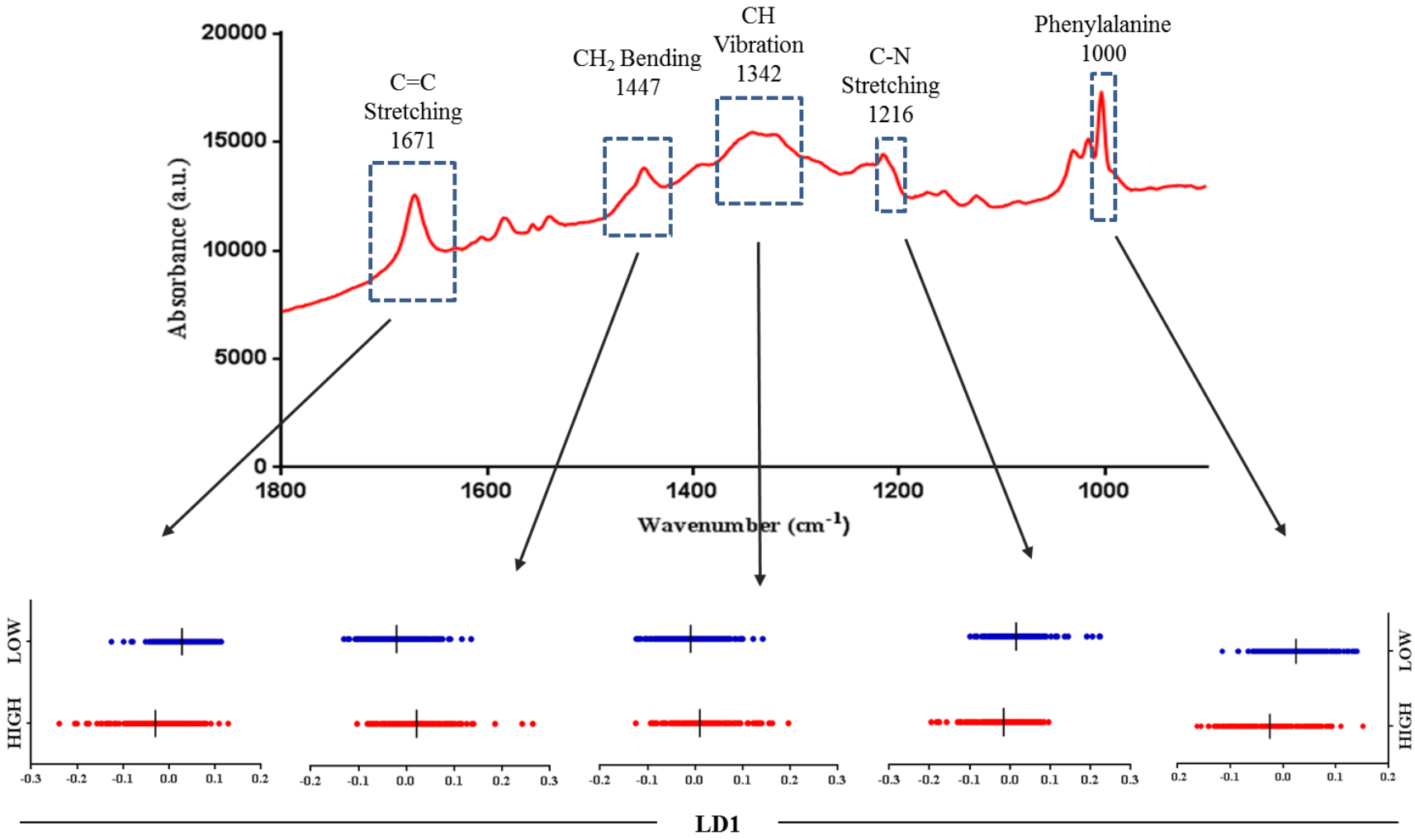


Figure 3 – Raman spectra of amyloid Aβ1:42 fibrils with main vibrational peaks highlighted in blue. ID PCA-LDA scores plots underneath show separation of high and low OC exposed brain samples at these vibrational nodes. Group scores are significantly different at the $P<0.05$ level as assessed by unpaired, two way t-tests.

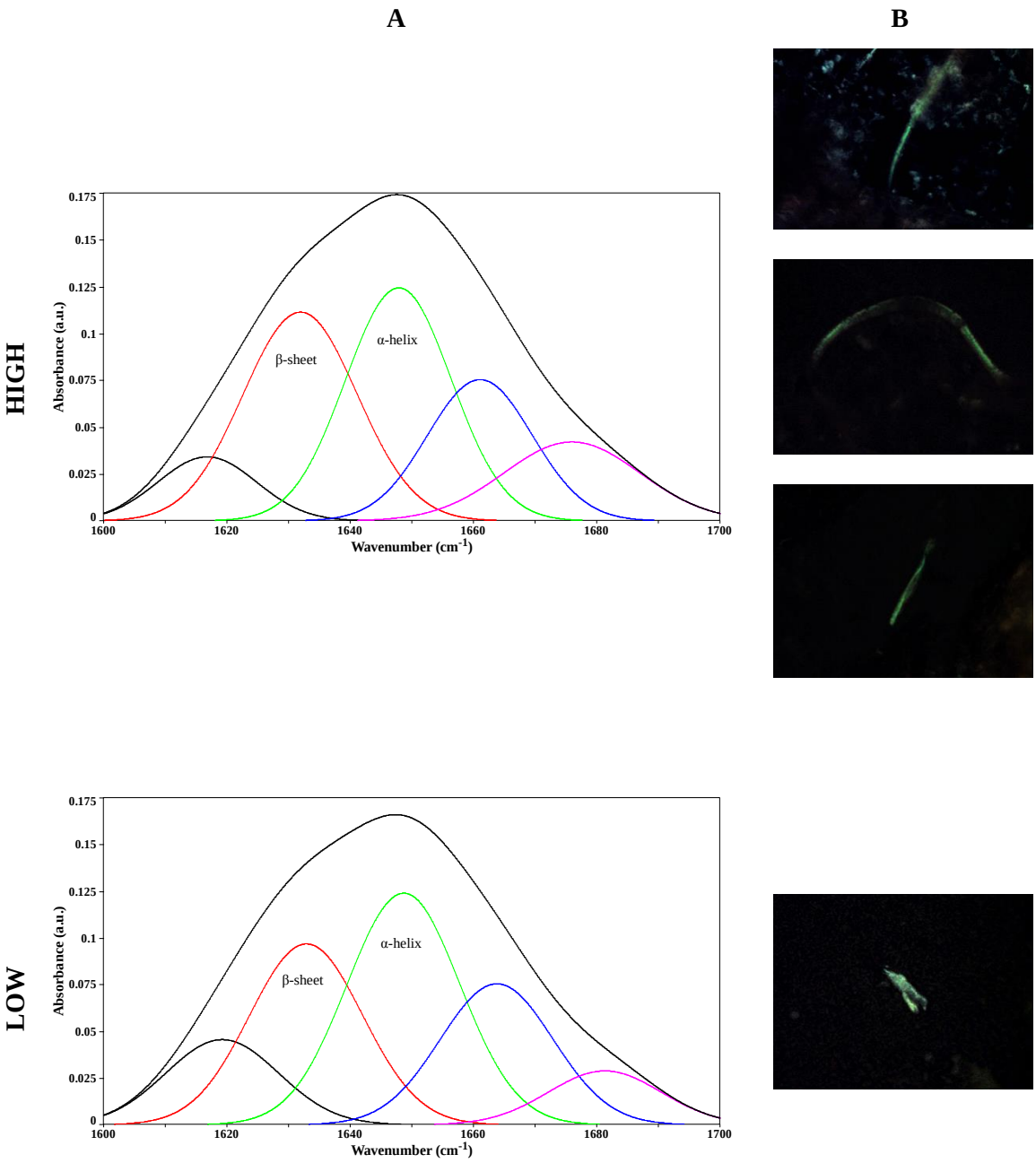
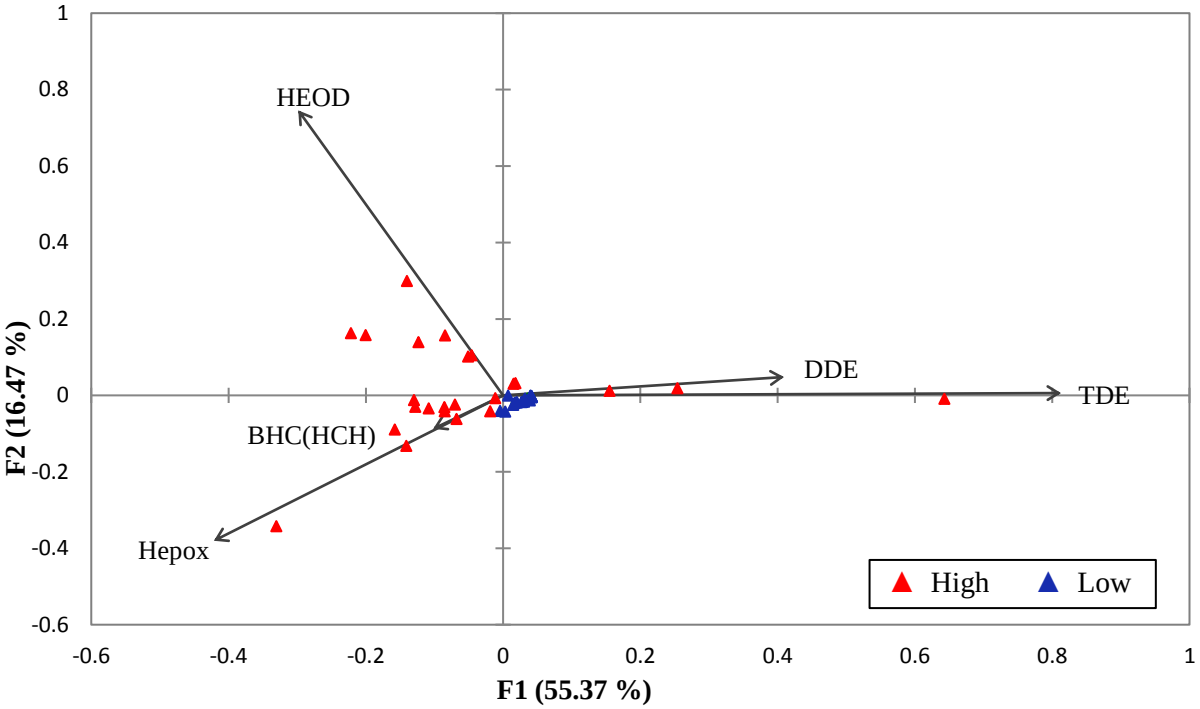


Figure 4 – (**A**) Deconvolution of the amide I peak derived from ATR-FTIR spectroscopy of high and low OC exposure groups, using second derivatives and Gaussian peak-fitting. (**B**) Microscopy images of Congo red stained brain samples, from high and low OC exposure groups, which exhibited apple green birefringence when viewed under crossed polarisers.

Correlation of spectral results and OC chemical exposure

A CCA was performed to determine if total OC pesticide exposure was having an effect on the variation seen in the spectral results between the high and low OC exposed brain samples. The analysis showed that the constrained variables explained 12.185% of the inertia observed (Fig. 5B). This means that the imputed variables, *i.e*. the chemical OC concentration data explain 12% of the variation seen in the spectral data. The remaining variance is due to other uncontrolled factors. The ordination plot (Fig. 5A) showed that points from the low OC exposure group are more strongly co-clustered than those from the high group which exhibit more scattering and variability. There is a small amount of overlap but largely, the two groups are well separated and clustered along the F1 axis. Assessment of the length and position of chemical directional arrows shows that TDE, heptachlor epoxide and HEOD are the OC pesticides that are most important and influential for the ordination. It also shows that DDE and TDE exposures are correlated as are BHC(HCH) and heptachlor epoxide exposures.

A



B

Inertia	Value	Percentage (%)
Total	0.301	100.000
Constrained	0.037	12.185
Unconstrained	0.264	87.815

Figure 5 – Correlation of chemical concentrations and ATR-FTIR spectral results from brain samples. (**A**) CCA ordination plot and (**B**) Inertia (variation) table showing correlation of spectral and chemical data from brain samples. Abbreviations as follows: BHC(HCH) - (β)Hexachlorocyclohexane; TDE – tetrachlorodiphenylethane; Hepox - Heptachlor epoxide; DDE - dichlorodiphenyldichloroethylene; HEOD – Dieldrin.

Discussion

The category of vibrational spectroscopy is composed of many techniques including FTIR and Raman methodologies which allow fast yet detailed analysis of biological samples. Such techniques are powerful tools which can be used to reveal alterations in biochemical composition and structure at the molecular level (Baker et al. 2014a). Infrared spectroscopy

has previously been used in the monitoring and interrogation of environmental samples including assessment of the effect of water quality on tadpoles (Strong et al. 2016a), identifying biomarkers of water contamination in English sole (Malins et al. 2006) and detection of chemical exposure profiles in Egret feathers to use as a monitoring tool (Llabjani et al. 2012). Here, we have used ATR-FTIR and Raman spectroscopy to assess the effects of OC pesticide exposure and the implication of such exposures to amyloid aggregation in sparrowhawk brains. Computational analysis of spectra was carried out to allow examination of spectral scores and loadings. We have identified that the major wavenumber associated alterations that occur in response to exposure to OC pesticides arise in lipids and the secondary structure of proteins. This is consistent with what we understand about the toxic mechanism of OC pesticides which can induce alterations in a range of biomolecules including proteins and lipids by modifying endocrine and apoptotic pathways (Mrema et al. 2013). The toxicity of strongly lipophilic chemicals such as OCs is often enhanced in the brain which is lipid rich and has poor detoxification mechanisms. As modifications in lipids and the secondary structure of proteins were identified by both ATR and Raman, these alterations may represent spectral markers of OC pesticide exposure in avian brain tissue.

The spectral results of OC pesticide exposure were found to be influenced by the sex and age of the bird. Sex related differences in the body burdens of OC chemicals as well as other contaminants have previously been reported (Robinson et al. 2012). Research suggests that female body concentrations may be lower than their male counterparts due to loss of body mass during breeding and through the maternal transfer of chemicals into eggs (Zheng et al. 2015). The reported figures regarding the amount that is passed into eggs vary greatly but it is thought that as much as a third of a female's OC burden could be transferred in this way and incorporated into the lipid-containing yolk (Newton et al. 1981). Maternal transfer is also a relevant consideration for humans as secretion of OC pesticides into the breast milk of mammals including humans has been reported (Cadieux et al. 2016; Chávez-Almazán et al. 2016). The impact of age on the consequences of OC pesticide exposure has been well studied

due to reports of neurodegeneration in aged individuals who are known to have been occupationally exposed to OCs during their lifetime (Hayden et al. 2010; Kamel et al. 2007).

Exposure to OC pesticides is regarded as an important environmental risk factor in the development of AD (Ballard et al.). This study investigates the presence of amyloid in wild avian brains and attempts to link this to 'real world' OC pesticide exposures. Using ATR-FTIR and Raman spectroscopy, we have identified that alterations in proteins, primarily changes in α-helix and β-sheet content, are induced by exposure to OCs. This suggests that a significant modification in secondary protein structure occurs in response to elevated OC pesticide concentrations (those above 7.18 μg/g). This is typically observed in amyloid-β protein aggregation which is characterised by a shift from α-helix to β-sheet formation as the peptide changes to an insoluble form (Kotler et al. 2014). Congo red staining also indicated that amyloid was present in four brain samples, three of which were exposed to high concentrations of OCs. This again suggests that exposure to higher concentrations of such pesticides may lead to amyloid aggregation in brain tissue. An exploratory Aβ1:42 ELISA detected the peptide in two brain samples. Interestingly, the two birds from which these samples came from were both juvenile females and were both from the low OC pesticide exposure group. This suggests that other factors are also important in the process leading to amyloid aggregation and that possibly there are genetic factors involved. In humans, an increase in cerebral Aβ1:42 has been associated with early onset AD (Lista et al. 2014). As proposed by some 'two-hit' hypotheses, genetic influences may act as a predisposition to amyloid aggregation that is mediated by other factors such as pesticide exposure. There are, however, many other factors involved in disease progression such as smoking, brain trauma and diet (Reitz and Mayeux 2014). As there are numerous implicated factors, a CCA was performed to understand the relationship between the OC pesticide exposure and spectral results from the brain samples. This established that 12% of spectral variation was due to the influence of OCs which is a reasonable amount as environmental and spectral datasets are complex with multiple factors to consider. In conjunction with spectral analysis, this lends

evidence to the argument that exposure to elevated levels of OC pesticides are implicated in the aggregation of amyloid and possibly in the development of AD. Multiple studies have also found elevated OC pesticide levels in humans with AD (Richardson et al. 2014; Singh et al. 2013).

Despite large scientific and financial input, there is currently no cure for AD and we still do not fully understand many aspects of the disease (Association 2016). It is therefore imperative that research efforts continue to further our understanding of AD. However, many studies focusing on the role of chemical contaminants are often limited by the availably of chemical data and brain tissue from the same test subjects. Studies such as this one, aim to increase our knowledge base of the etiology of AD as predatory birds occupy a very similar position in the food chain as humans do. Being apex predators and relatively long lived makes birds of prey ideal surrogates in lieu of human experimental subjects. The use of tissue and data from wild free flying birds, via initiatives such as the PBMS, allows us to study the effects of 'real world' exposure profiles. Recent reports have also suggested that the brains of birds are more similar to those of humans than previously thought and so the avian brain has been recognised as a potential model for human neuroscience (Chen et al. 2013; Clayton and Emery 2015). The results of studies like this may also be significant for avian research as well. Although wild birds do not usually live as long as their captive counterparts, many predatory bird species such as eagles and owls are remarkably long lived and experience similar chemical exposures. If there are genetic predisposing factors as there are in humans, then younger birds and those with shorter lifespans may also be vulnerable to amyloid aggregation. Previously, an amyloid plaque has been identified in the brain of an aged captive woodpecker so there is a precedent of amyloid aggregation in the avian brain (Nakayama et al. 1999). This also demonstrates that if birds live long enough, aggregation of the amyloid protein into cerebral plaques is possible. In homing pigeons, neurodegeneration due to age related synaptic loss has been associated with reduced spatial cognitive function (Coppola et al. 2016). If predatory birds develop amyloid aggregations and suffer synaptic loss, as has been demonstrated in

humans, this could affect navigation and hunting behaviours which require spatial cognition. Predatory birds may represent a new opportunity to study amyloid aggregation and AD risk factors which has significant research implications for both humans and wildlife. This study also demonstrates that vibrational spectroscopy could be a useful tool in the investigation of OC pesticide exposure and potentially to identify markers of amyloid aggregation in brain tissue.

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Supplementary Information:

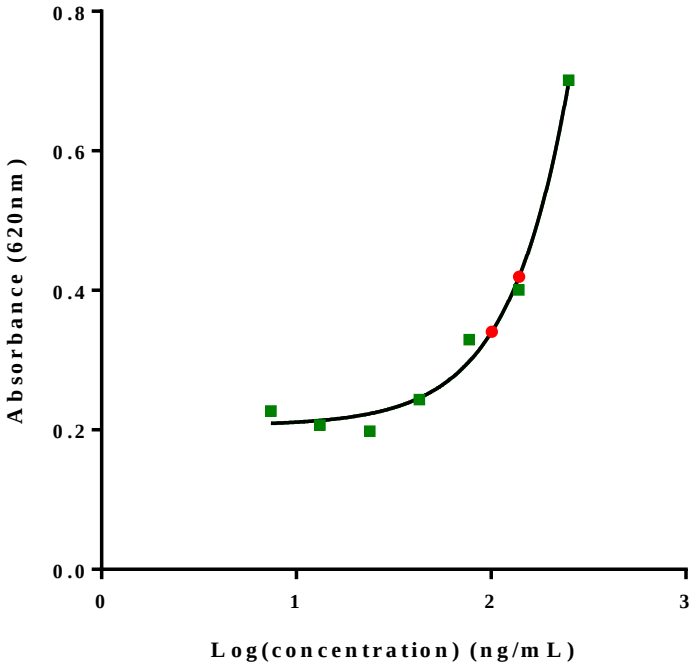

Figure 1 – Human anti-amyloid Aβ1:42 calorimetric ELISA performed on homogenised brain lysates. Absorbance was read at 620nm. Standards are indicated by green point and fitted to a 4PL standard curve with interpolated results shown in red. Samples were diluted 1:5 for the assay.

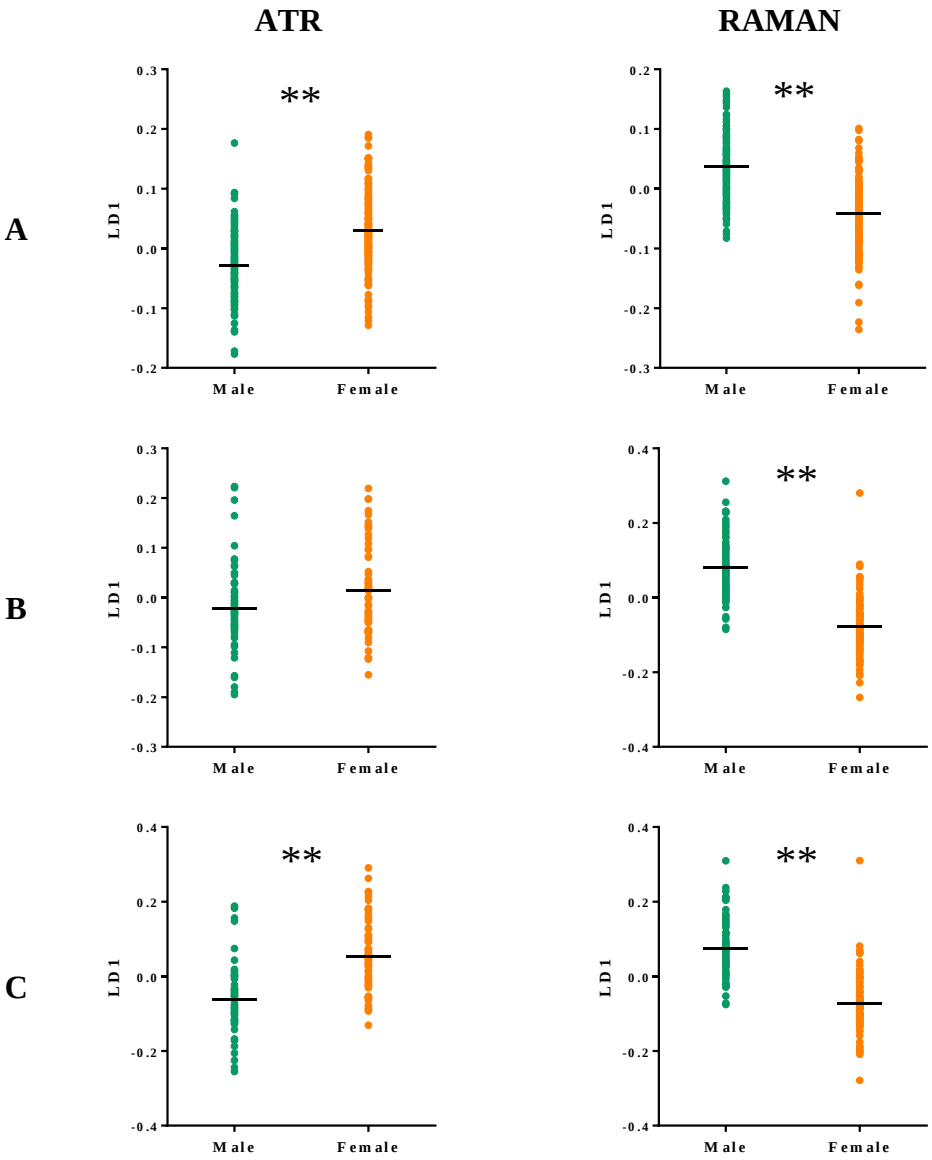

Figure 2 – One-dimensional PCA-LDA scores plots from ATR-FTIR and Raman spectroscopy of brain samples showing sex dependant effects of OC exposure. (**A**) All samples (**B**) High OC exposure group samples and (**C**) Low OC exposure group samples. Group means are represented by a black line and significance at the $P<0.01$ level as determined by two-way ANOVA with Sidak multiple comparison test is indicated by two asterisks.

Sample	Group	Age	Sex	BHC(HCH)	TDE	Hepox	DDE	HEOD	Total OC concentration
1	Low	0	0	0.01	0.01	0.19	0.27	0.08	0.56
2	Low	0	1	0.08	0.01	0.01	0.38	0.10	0.58
3	Low	0	0	0.08	0.01	0.01	0.38	0.1	0.58
4	Low	0	1	0.36	0.01	0.01	0.15	0.06	0.59
5	Low	0	1	0.03	0.01	0.01	0.53	0.01	0.59
6	Low	0	0	0.01	0.01	0.01	0.47	0.10	0.60
7	Low	0	1	0.01	0.01	0.01	0.37	0.22	0.62
8	Low	0	0	0.01	0.01	0.01	0.76	0.05	0.84
9	Low	0	0	0.01	0.01	0.31	0.09	0.50	0.92
10	Low	0	0	0.01	0.01	0.01	0.76	0.13	0.92
11	Low	0	1	0.30	0.01	0.20	0.24	0.18	0.93
12	Low	0	1	0.09	0.04	0.35	0.19	0.30	0.97
13	Low	0	1	0.31	0.01	0.23	0.38	0.1	1.03
14	Low	0	1	0.08	0.01	0.20	0.67	0.07	1.03
15	Low	0	0	0.10	0.11	0.05	0.48	0.31	1.05
16	Low	1	1	0.01	0.01	0.01	1.04	0.16	1.23
17	Low	1	0	0.17	0.01	0.10	0.95	0.01	1.24
18	Low	0	1	0.28	0.01	0.25	0.51	0.28	1.33
19	Low	0	1	0.27	0.01	0.29	0.62	0.16	1.35
20	Low	0	0	0.01	0.01	0.01	1.31	0.17	1.51
21	Low	1	1	0.01	0.01	0.08	1.33	0.15	1.58
22	Low	0	0	0.01	0.01	0.01	1.43	0.15	1.61
23	Low	0	0	0.16	0.18	0.13	1.05	0.12	1.64
24	Low	0	1	0.41	0.01	0.51	0.5	0.22	1.65
25	Low	0	0	0.01	0.01	0.01	1.42	0.30	1.75
26	Low	0	0	0.01	0.01	0.3	1.1	0.4	1.82
27	Low	0	0	0.01	0.01	0.01	1.88	0.01	1.92
28	Low	1	0	0.08	0.01	0.55	1.38	0.01	2.03
29	High	1	1	0.33	0.01	0.41	4.99	1.44	7.18
30	High	0	0	0.15	0.01	0.21	8.28	0.52	9.17
31	High	1	1	0.01	0.01	0.01	8.03	1.62	9.68
32	High	0	1	1.73	0.01	2.7	5.41	1.49	11.34
33	High	1	0	0.20	0.30	0.80	10.00	0.60	11.90
34	High	0	0	0.01	0.01	0.01	10.71	1.29	12.03
35	High	0	0	0.37	0.01	2.25	8.45	1.49	12.57
36	High	0	1	0.28	0.74	0.45	10.89	0.70	13.06
37	High	0	0	0.36	0.01	0.99	12.34	2.33	16.03
38	High	0	0	0.24	0.14	1.22	14.98	0.67	17.25
39	High	1	0	0.64	0.47	1.31	13.87	1.09	17.38
40	High	0	1	3.99	0.14	0.13	16.05	0.12	20.43
41	High	1	0	0.07	0.01	0.35	13.12	7.11	20.66
42	High	0	1	0.48	0.01	1.09	19.07	1.09	21.74
43	High	1	1	0.11	0.43	5.39	17.24	1.41	24.58

44	High	0	1	0.22	0.05	0.57	2.33	21.59	24.76
45	High	1	0	0.04	0.01	1.13	25.12	1.14	27.44
46	High	1	0	0.04	0.01	0.13	25.55	5.16	30.89
47	High	1	0	1.10	0.01	2.79	25.67	1.82	31.39
48	High	1	0	0.54	0.01	1.92	27.92	1.03	31.42
49	High	0	0	0.33	0.01	1.59	13.61	16.24	31.78
50	High	1	0	0.21	0.59	2.27	9.27	20.14	32.48
51	High	0	0	0.53	0.01	1.29	28.67	3.02	33.52
52	High	1	1	0.27	0.19	0.33	26.68	9.48	36.95
53	High	1	1	1.73	0.15	0.92	34.00	0.65	37.45
54	High	1	1	0.31	0.01	0.66	28.39	9.63	39.00
55	High	0	1	0.4	6	0.68	35.96	2.68	45.72
56	High	0	1	2.48	5.63	0.9	42.07	5.4	56.48
57	High	0	1	0.29	6.50	0.86	67.30	2.21	77.16
58	High	0	1	0.33	12.45	0.7	66.31	2.52	82.31

Table 1 – Sample summary table with chemicals measured as wet weight concentration of contaminant in liver (ug/g). Abbreviations and notation is as follows: Sex 0 – male; Sex 1 – female; Age 0 – juvenile; Age 1 – adult; BHC(HCH) - (β)Hexachlorocyclohexane; TDE – tetrachlorodiphenylethane; Hepox - Heptachlor epoxide; DDE – dichlorodiphenyldichloroethylene; HEOD – Dieldrin; Total OC concentration - BHC+TDE+Hepox+DDE+HEOD.

		OC Concentration		
	Variable	**High**	**Low**	**All**
ATR	Sex	ns	<0.01	<0.01
	Age	ns	<0.05	<0.01
Raman	Sex	<0.01	<0.01	<0.01
	Age	<0.01	<0.01	<0.01

Table 2 – *P* values from two-way ANOVA, with Sidak multiple comparison test, used to compare PCA-LDA LD1 scores with respect to age and sex.

OC Exposure Group	Peak Centre (cm^{-1})	Assignment	Percentage area (%)
HIGH	1617	Unknown	7.4
	1632	β-sheet	29.9
	1648	α-helix	31
	1661	Disordered	18.6
	1675	Turn	13.2
LOW	1619	Unknown	10.4
	1632	β-sheet	25.9
	1648	α-helix	34
	1663	Disordered	21.8
	1681	Turn	8

Table 3 – Peak centres, assignments and percentage area of protein secondary structure sub-peaks derived from amide I deconvolution of ATR-FTIR spectra of OC exposed brains.

Chapter 5. Vibrational biospectroscopy characterises biochemical differences between cell types used for toxicological investigations and identifies alterations induced by environmental contaminants

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Contribution:

- I conducted all experiments for the study.
- I prepared, processed and acquired data for all samples including conducting computational analysis.
- I prepared the first draft of the manuscript.

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Kelly A. Heys Prof. Francis L. Martin

Vibrational biospectroscopy characterises biochemical differences between cell types used for toxicological investigations and identifies alterations induced by environmental contaminants

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Abstract

The use of cell-based assays is essential in reducing the number of vertebrates used in the investigation of chemical toxicities and in regulatory toxicology assessment. An important factor in obtaining meaningful results which can be accurately extrapolated is the use of biologically appropriate cell lines. In this preliminary study, ATR-FTIR spectroscopy with multivariate analysis was used to assess the fundamental biomolecular differences between a commonly used cell line, MCF-7 cells, and an environmentally relevant cell line derived from mallard (*Anas platyrhynchos*) dermal fibroblasts. To better understand differences in basic cell biochemistry, the cells were analysed in the untreated state or post-exposure to PCB and PBDE congeners. The main spectral peaks in spectra from both cell types were associated with cellular macromolecules, particularly proteins and lipids but the spectra also revealed some cell-specific differences. Spectra from untreated mallard fibroblasts spectra contained a large peak associated with lipids. The cell-related differences in lipid and DNA were also identified as regions of spectral alteration induced by PBDE and PCB exposure. Although lipid alterations were observed in post-treatment spectra from both cell types, these may be of more significance to mallard fibroblasts, which may be due to increased intracellular lipid as determined by Nile red staining. Untreated MCF-7 cell spectra contained unique peaks related to DNA and nucleic acids. DNA associated spectral regions were also identified as areas of considerable alteration in MCF-7 cells exposed to some congeners including PBDE 47 and PCB 153. The findings indicate that in their native state, MCF-7 and mallard cells have unique biochemical differences which can be identified using ATR-FTIR spectroscopy. Such differences in biochemical composition differences, which may influence cell susceptibility to environmental contaminants and therefore influence the choice of cell type used in toxicology experiments. This is the first study to analyse the biochemistry of a mallard dermal fibroblast cell line and to use ATR-FTIR spectroscopy for this purpose. ATR-FTIR spectroscopy is demonstrated as a useful tool for exploration of biomolecular variation at the cellular level and with further development, it could be used as part of a panel of cell-based assays to indicate

when different results might be seen in environmental species compared to currently used cell lines.

Introduction

In order to protect the health of humans and wildlife, it is important that we are able to understand and estimate the toxicity of contaminants that enter the environment. To date, this has been made possible using biological and computational models that allow us to study the effects of such contaminants at various levels within an organism (de Zwart and Posthuma 2005). For regulatory purposes, it is crucial that the toxicity of pollutants is fully characterised as regulatory guidelines determine the usage of chemicals which may enter the environment (Walker et al. 2012)(Liu et al. 2015). Therefore, it is essential that we have accurate methodologies in place that allow us to assess the effects of contaminant chemicals in experimental and regulatory contexts. Current methods used to assess the effects of exposure to chemicals can be time-consuming, expensive or rely on a high degree of operator competence. Therefore, considerable effort is being invested into developing new tools for these purposes.

Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy is a high throughput and economical technique that has been used for analyses in several fields including biomedicine (Kazarian and Chan 2013), geology (Chen et al. 2015), plant science (Butler et al. 2015) and ecotoxicology (Obinaju et al. 2014). ATR-FTIR is a vibrational spectroscopy technique that can characterise biomolecules in samples by measuring the absorption of infrared (IR) by IR-active chemical bonds through their inherent dipole moment (Kelly et al. 2011). The dipole moments occur at specific wavelengths depending on the chemical bonds present. This data not only confers structural or conformational information but also by looking at alterations of specific bonds between biological samples, further information such as toxic effects of chemicals can be elucidated (Martin et al. 2010). It has previously been recognised that ATR-FTIR and other vibrational spectroscopy techniques may be useful to study the effects of chemical toxicants in biological systems (Llabjani et al. 2011). It has been used to investigate the effects of environmental contaminants including

fungicides (Strong et al. 2016), nanoparticles (Riding et al. 2012) and polycyclic aromatic hydrocarbons (PAHs) (Obinaju et al. 2015) as well as being used to investigate composition of contaminants in mixtures (Jeon et al. 2008), mixture interactions (Ewing et al. 2014) and the biological toxicity of environmental binary mixtures (Llabjani et al. 2010). ATR-FTIR spectroscopy is highly useful as it can be used to investigate the toxicity of chemicals using a wide range of biological substrates, both live and fixed, including biofluids, tissues and cells (Baker et al. 2014).

In vitro cell models are an increasingly emerging focus for toxicity research, including that using vibrational spectroscopy. Toxicity testing is required to understand how chemicals in the environment can affect organisms but the methods by which we experimentally determine an agent's toxicity and at which concentrations is a topic of some debate. The use of animal models is regarded by many as most representative as it provides toxicity information within the complexity of the whole-organism level (Stephens 2010). However, as set out by The National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3R), we must reduce the number of vertebrates used in scientific procedures and *in vitro* toxicity testing has historically been a precursor for animal testing. In the wake of the NC3R guidelines, chemical testing using cell lines has been increasingly used as a suitable surrogate (Scholz et al. 2013). Analysis of toxicity at this level has the benefit of capturing important interactions between the chemical and biological targets which happens first in the cells, before an organ or tissue effect is seen (Huang et al. 2015). On the other hand, many of the cell lines used for environmental toxicant studies are often far removed from the cell types that would be exposed to chemicals in sentinel organisms. Using commonly employed cell lines does have the benefit that the cells are well characterised but problems may arise during the interpretation of results in how these are extrapolated to meaningful conclusions regarding whole organism toxicity. Additionally, in the context of environmental research, there are many species groups, *e.g.* mammals, birds, fish and amphibians, which comprise many physiological and biochemical differences that can further complicate extrapolation of

experimental results (Leist et al. 2012). It is possible that current experimental cell lines, which may come from very different test organisms or be derived from abnormal tissues such as tumours, are not the most representative system in which to measure how a chemical contaminant will affect environmental species. The development of new cell lines, from environmentally relevant sources, could represent a new focus for investigating the toxicity of environmental contaminants (Bols et al. 2005).

The major aim of this study was to compare and evaluate two cell lines which may be used for testing the toxicity of environmental contaminants; an MCF-7 cell line which has been used for such experiments (Barber et al. 2006; Li et al. 2013) and a mallard (*Anas platyrhynchos*) dermal fibroblast cell line derived from free-living birds, representing a test system which is biologically closer to wildlife species found in the environment. Mallard dermal fibroblasts were selected as a preliminary test cell line as they are non-mammalian, from an environmentally abundant species and in an anatomical site, which would frequently come into contact with contaminants. Environmentally relevant cell lines, such as this, may provide results that can be more accurately extrapolated to environmental organisms, either when used alone or in conjunction with other cell lines. Both the fundamental biochemistry of the cells and their response to chemical contaminant exposure was analysed using ATR-FTIR spectroscopy with computational analysis methods. As described, ATR-FTIR spectroscopy is a sensitive technique which is able to differentiate between cell and tissue types (Martin et al. 2010) and therefore was used to determine cellular differences at the biomolecular level. In order to determine whether there were differences in response post-exposure to common environmental contaminants which may necessitate the use of alternative cell lines, the cells were treated with PCB 153 and PBDE congeners 47, 99 and 153 which are well-known environmental pollutants. Treatments with single agents at environmentally reported concentrations were used to simulate environmental levels. The results of studies like this may be essential for the development of accurate cell-based assays, particularly for understanding how environmental chemicals are toxic to avian species.

Methods and materials

Test agents

Stock solutions of PBDE congeners 47, 99 and 153 were purchased, pre-dissolved in nonane at a concentration of 50 µg/mL, from LGC standards (Teddington, UK). PCB 153 was purchased as a powder from Greyhound Chromatography and Allied Chemicals (Birkenhead, UK) and made up in nonane (Sigma-Aldrich, Dorset, UK). Stock solutions for chemical treatments were made up to a concentration of 2 µM in DMSO and then serially diluted in DMSO to achieve the experimental concentrations required. Vehicle controls consisted of the same amount of DMSO as used in chemical treatments, spiked with equal quantities of nonane.

Cell culture

Human MCF-7 cells were taken from an established culture derived from a frozen aliquot from a line gifted by the Institute of Cancer Research. For experiments, they were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and a penicillin and streptomycin mixture (100 U/mL and 100 µg/mL respectively). Mallard (*Anas platyrhynchos*) dermal fibroblasts (derived as previously described (Harper et al. 2011)) were grown in the same medium with the addition of 2% chicken serum (Sigma-Aldrich) and 1% non-essential amino acids (Thermo Fisher Scientific, Nottinghamshire, UK). Both cell types were cultured in a humidified atmosphere with 5% CO_2 in air, at a temperature 37°C. Cells were sub-cultured twice weekly by disaggregation with trypsin (0.05%)/EDTA (0.02%) solution before spinning at 1000 × g for 5 minutes. The resultant cell pellet was then re-suspended in fresh complete DMEM and seeded into T75 flasks for routine sub-culture or T25 for experiments. Unless stated otherwise, all cell culture consumables were purchased from Lonza (Verviers, Belgium).

Cell experiments

After seeding into T25 flasks, cultures were left for 24 hours to allow cells to attach and enter into S phase. After 24 hours, cells were treated with single agents: PBDE 47, PBDE 99, PBDE 153 or PCB 153 at concentrations of 10^{-8} M, 10^{-10} M or 10^{-12} M, by adding 25 µl of the appropriate treatment to each flask; vehicle controls were treated with 25 µl of DMSO (with nonane). Cells were exposed to treatments for 24 hours before they were disaggregated with trypsin, washed three times with 70% ethanol to remove residual media and then fixed for 24 hours in 70% ethanol. After fixation, cells were pipetted onto IR-reflective low-E glass slides (Kevley Technologies, Chesterland, OH, USA) and allowed to air dry before being placed in a desiccator for 24 hours to remove any remaining water. This experimental procedure was repeated at five different points in time over a 12-month period to give five technical replicates (n=5).

ATR-FTIR spectroscopy

Five spectra per slide were acquired using a Bruker TENSOR 27 FTIR spectrometer with Helios ATR attachment which contained a diamond IRE with a sampling area of 250 µm × 250 µm (Bruker Optics, Coventry, UK). Spectra were acquired with an 8 cm^{-1} spectral resolution with 32 co-additions, giving rise to a 3.84 cm^{-1} spectral data spacing. A mirror velocity of 2.2 kHz was used. Before each sample, a background measurement was taken to account for atmospheric changes and the diamond was cleaned with distilled water between samples.

Spectral pre-processing and computational analysis

An in-house Matlab 2013a (The Maths Works, MA, USA) toolbox called IRootLab was used for pre-processing and computational analysis of the spectra (http://trevisanj.github.io/irootlab/). Raw spectra were cut to 900-1800 cm^{-1}, which is known as the fingerprint region where most biomolecules are known to absorb IR. For biochemical analysis of the derivative spectra after cutting, spectra were Savitzky-Golay 2nd order

differentiated (2nd order polynomial, 9 coefficients) to correct baseline aberrations and to resolve overlapping peaks. For cell comparison of exposure to single agents, spectra were baseline corrected using 1st order differentiation, vector normalised and mean-centred. Principal component analysis (PCA) was paired with linear discriminant analysis (LDA) to allow exploratory analysis of treatment-induced spectral alterations. PCA was used for data reduction purposes and was optimised, to incorporate the maximum amount of variance (~95%) whilst minimising noise incorporated into LDA, using the PCA pareto tool. LDA is a supervised technique that was used to optimise inter-class separation. K-fold (where k =5), leave-one-out cross-calculation was used to avoid overfitting. Forward feature selection was also used to compare and classify cell types. It was performed using a Gaussian fit classifier on five features using 100 randomised repeats with random subsampling. Significance of analysis results was determined in GraphPad Prism 4 (GraphPad Software Inc., CA, USA). Differences between scores from the two cell types were analysed by two-way *t*-test and differences from control in dose response scores were analysed by one-way ANOVA with Dunnett's post-hoc test. Significance testing was performed using replicate means rather than all spectral data to avoid pseudoreplication.

Nile red staining

Untreated MCF-7 and Mallard fibroblast cells were stained with Nile Red to visualise cellular lipids. Nile Red stain was purchased as a powder (Sigma-Aldrich) and made up to a 1 mg/mL stock solution, which was stored in the dark at -4˚C until use. For staining, a 1:1000 working solution was made up from the stock using PBS. Cells were snap frozen by submerging in liquid nitrogen for 30 seconds before incubation for 30 minutes in 5 mL of stain. Once stained, cells were washed twice in ice-cold PBS and air-dried. Samples were imaged by confocal microscopy using a Leica DMIRE2 inverted microscope with a Leica TCS SP2 scan head. Images were obtained using a Leica HC PL Fluotar 20× objective and a numerical aperture of 0.5.

Western blotting

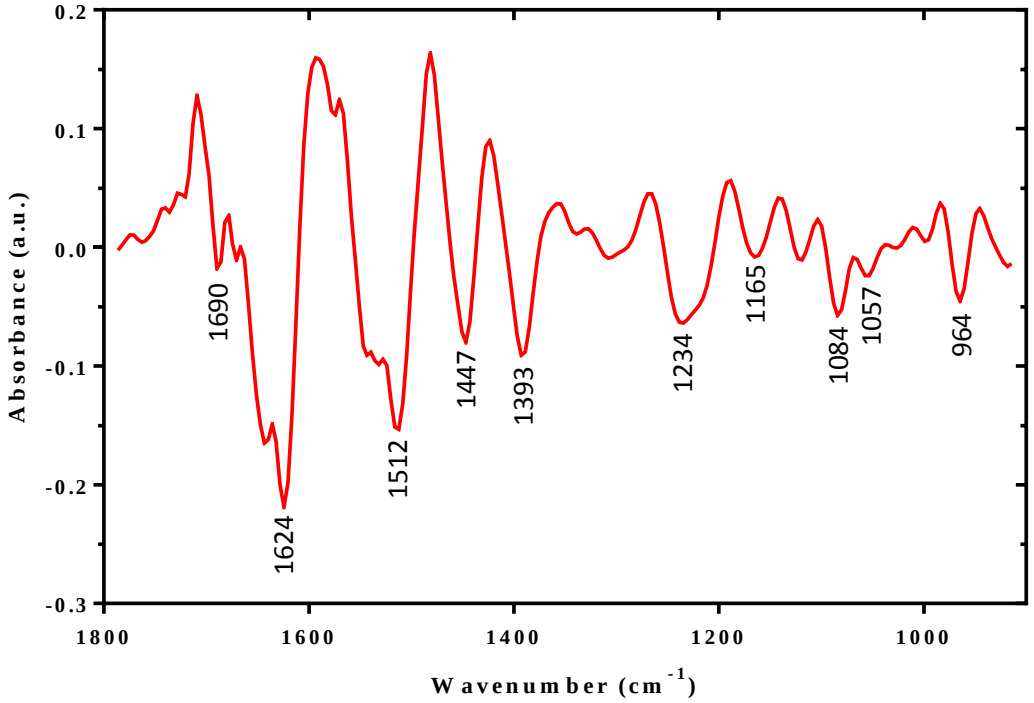
For Western blot experiments, cells were seeded in 6-well culture plates at a density of 1×10^6 cells and allowed to grow for 24 hours before being exposed to the highest concentration of chemical treatment (10^{-8} M) for 24 hours in the same manner as described for cell experiments. Cells were lysed in 300 µl of RIPA buffer (50 mM Tris, 150 mM NaCl with 0.5% sodium deoxycholate, 1% IGEPAL CA-630 and 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich). Lysates were spun at 12,000 rpm for 10 minutes and the supernatant was transferred into a clean, labelled tube. Samples were mixed 1:3 with 4× Laemmli sample buffer (containing 10% β-mercaptoethanol) and heated for 2 minutes at 95˚C. Once cooled, samples were run in a 12% acrylamide gel for 35 minutes at 180 V. Proteins were then transferred to a nitrocellulose membrane using a Bio-Rad Trans-Blot Turbo Transfer system. Membranes were blocked in a 3% solution of non-fat milk powder in Tris-buffered saline Tween-20 (TBST) for 1 hour at room temperature with constant shaking. Membranes were then incubated with rabbit anti-CYP1A1 antibody (Abcam, Cambridge, UK), at a concentration of 1:1000, overnight at 4˚C. The membranes were washed three times in TBST before incubating, at room temperature for 1 hour, with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz, CA, USA) diluted 1:10,000 in TBST. Finally, membranes were washed three times in TBTS and protein bands were quantified using Clarity Western ELC substrate on the Bio-Rad ChemiDoc XRS imaging system with β-actin as a loading control. This procedure was repeated at three different points to give three replicates (n=3). Unless stated otherwise, Western blotting related equipment and consumables were from Bio-Rad (Hercules, CA, USA).

Results and discussion

Comparison of untreated cells

In order to understand the fundamental differences between MCF-7 and mallard cells in the absence of chemical exposures, the spectra from untreated cells were compared. The second derivatives of ATR-FTIR spectra were utilised to eliminate baseline irregularities and to resolve overlapping bands, which are particularly common in the fingerprint region (900-1800 cm^{-1}) of the spectrum. Second order differentiation results in negative facing bands where the peak maxima are at the same wavenumber as the original, undifferentiated bands. This technique is extremely useful for revealing the basic biochemistry of samples, which are subject to IR spectroscopy. Fig. 1 shows the 2^{nd} derivative spectra of untreated MCF-7 and mallard cells with the ten largest peaks highlighted by peak wavenumber [tentative wavenumber assignments are found in electronic supplementary information (ESI) Table 1). There are some areas of the spectrum with key peaks, which are common to both cell types. Most of these peaks are related to structure-associated macromolecules that are conserved between cell types, such as proteins. For example, both cell spectra have a peak at 1690 cm^{-1}, which is associated with the Amide I moiety of proteins, in particular β-sheet structure vibrations. Both 2^{nd} derivative spectra have peaks at 1512 cm^{-1} due to Amide II and in-plane CH bending and at 1393 cm^{-1} due to symmetric CH_3 bending in proteins. There are also some peaks, seen in both the MCF-7 and mallard cell spectra, which are related to the same biomolecular vibrations but are shifted by a few wavenumbers. The peak associated with deformation of CH_3 groups in proteins is observed at 1447 cm^{-1} in MCF-7 cells but at 1450 cm^{-1} in mallard cells. Similarly, an Amide III protein peak is visible at 1234 cm^{-1} in the MCF-7 cell spectra and 1238 cm^{-1} in the mallard cell spectra and the peak related to C-O stretching of proteins is seen at 1165 cm^{-1} in MCF-7 cells and 1161 cm^{-1} in mallard cells. There is also a large peak between 1600-1700 cm^{-1} present in the spectra of both cell types, which represents the largest biomolecular contribution. In both cell spectra, it appears to be composed of two bands as there are two peaks visible but the absorbance of the peaks varies with cell type. In

A.



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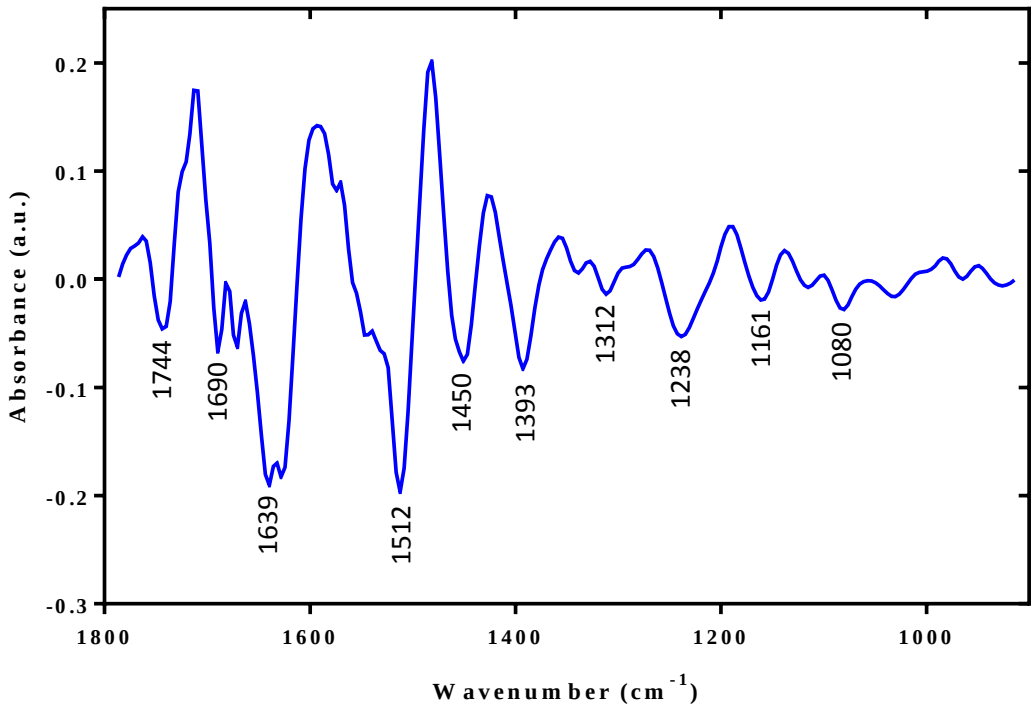


Figure 1 – Mean ATR-FTIR 2nd derivative spectra from untreated (A) MCF-7 cells and (B) Mallard cells in the biological fingerprint region of 900-1800 cm-1 with the top ten peaks labelled.

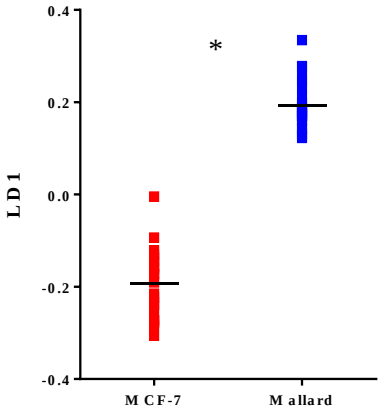
the MCF-7 cell 2nd derivative spectra, the peak is found at 1624 cm^{-1} and in the mallard cell spectrum, it is found at 1639 cm^{-1}. This may be due to slightly different vibrational modes of Amide I molecules due to varying types and proportions proteins (de Campos Vidal and Mello 2011) or due to differences in the secondary structure of proteins which can be reflected in small shifts in the Amide I band in derivative spectra (Gallagher 2009).

Possibly of more interest, there are also regions of the spectrum, which are distinctly different in MCF-7 and mallard cells. In the mallard cell spectrum, this is mainly due to the peak at 1744 cm^{-1} which is associated with C=O stretching of lipids and triglycerides. This peak is almost non-existent in the MCF-7 cell spectrum indicating that there is more intracellular lipid in mallard cells. This may be due to the nature of the mallard cells, which are fibroblasts derived from the skin. The avian epidermis has unique subkeratinocytes which produce lipids needed to keep the skin and feathers healthy (Menon and Menon 2000). This could represent an important modification seen in some avian cells and may have implication for how susceptible certain cell types are to exposure to contaminants. Dermal contact may be an important exposure route (Shore et al. 2014) and additionally, many environmental contaminants are lipophilic and may be able to sequester easier in mallard cells (Arts et al. 2009). There is also a unique peak in the mallard spectrum at 1312 cm^{-1} related to Amide III vibrations. Another peak, seen at 1080 cm^{-1} in mallard cells, is due to symmetric phosphate stretching vibrations in nucleic acids and although the 1084 cm^{-1} peak in the MCF-7 spectrum is also associated with this, the peak at 1080 cm^{-1} has also been linked to collagen presence. This may also be due to the mallard fibroblast cells originating from the skin which is known to contain collagen (Prum and Torres 2003). In the MCF-7 cell 2nd derivative spectrum, there are two unique peaks at 1057 cm^{-1} and 964 cm^{-1}, which are related to C-C or C-O stretching of deoxyribose in DNA. MCF-7 cells are epithelial cells from a mammary tumour and thus may have different DNA characteristics as a higher proportion of cells are likely to be in the S and G_2 growth phases (Hammiche et al. 2005). MCF-7 cells also have a doubling time of 29 hours whereas avian cells are generally slower growing as birds are often longer lived than

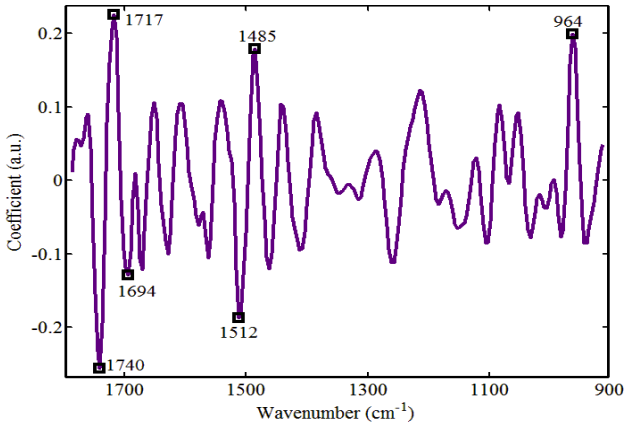
mammals of the same size (Harper et al. 2011). The mallard fibroblasts had a doubling time of ~48 hours.

Using multivariate analysis, PCA-LDA was also used to explore and visualise the differences and similarities between the two cell types. Fig. 2A and 2B shows the one-dimensional (1D) scores plots and corresponding loadings from this analysis. The scores plots indicate that untreated MCF-7 and mallard cells are significantly different ($P<0.01$) along the LD1 axis. Co-clustering and lack of overlap of scores along LD1 signifies that there is variation between the two cell types as in these types of plots, dissimilarity is indicated by increasing distance (Kelly et al. 2011). The loadings (Fig. 2B) display the absorbances of biomolecules that are responsible for the separation seen in the scores plot; the six largest peaks representing the most contributing wavenumbers have been highlighted with tentative assignments (Fig. 2D). Some of the wavenumbers in the loadings reflect the main peaks seen in the 2nd derivative spectra (Fig. 1) including the peak at 1740 cm^{-1} which is associated with C=O stretching of lipids and the deoxyribose peak at 964 cm^{-1}. Additionally there are peaks in the loadings at 1717 cm^{-1} and 1485 cm^{-1} due to nucleic acids (thymine and guanine respectively) which may be related to DNA differences seen in MCF-7 cells in the derivative spectra. There is also a lipid-related peak at 1416 cm^{-1} which as previously established, may be due to differences in cellular lipid profiles. The other peaks seen in the loadings are associated with protein variation; 1694 cm^{-1} (Amide I) and 1512 cm^{-1} (Amide II). To further explore the biochemical differences which discriminate the cell types and to validate that the ATR-FTIR spectrochemical technique is able to distinguish between them, forward feature selection (FFS) was employed as well as PCA-LDA. FFS ranks subsets of wavenumbers based on how much they contribute to the classification of the spectra into the experimental groups (Gallagher 2009). A classification rate of 100% was achieved and the feature histogram seen in Fig. 2C shows the top six wavenumbers which had the most hits when cells were classified (assignments found in Fig. 2D). This confirms that the wavenumbers responsible for discriminating between MCF-7 and Mallard cells are largely associated with lipid content.

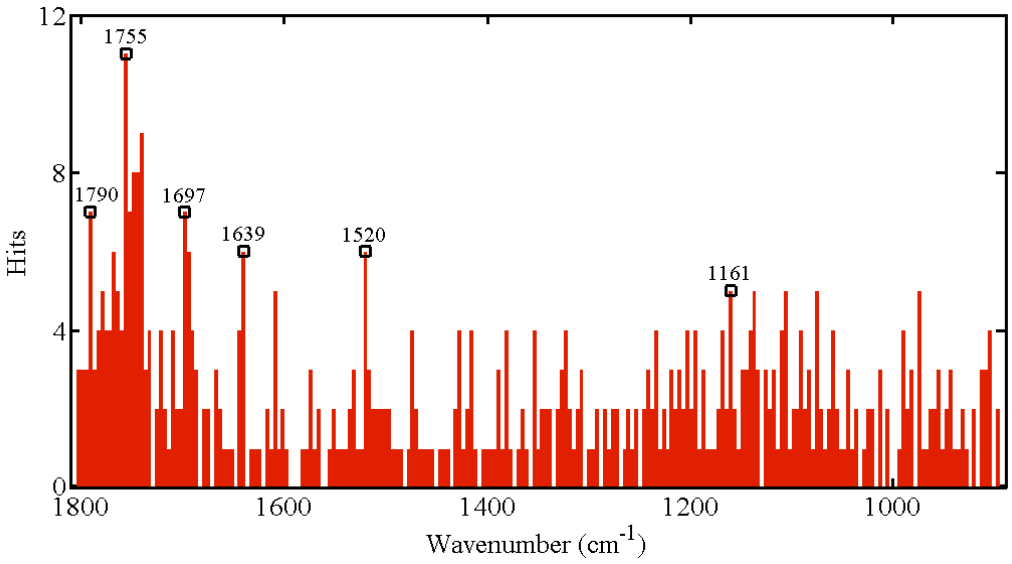
A.



B.



C.



D.

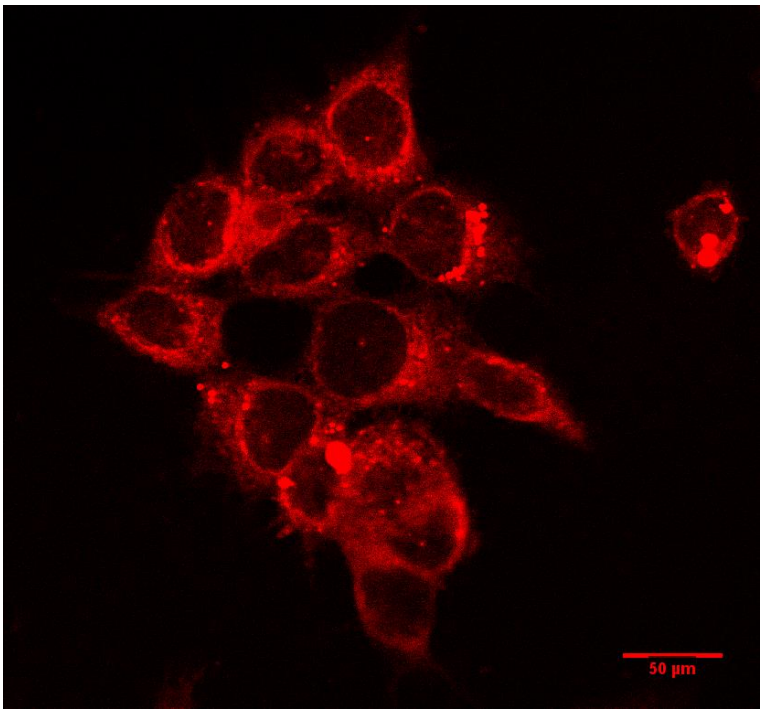
Wavenumber (cm^{-1})	Assignment
1790, 1755	ν(C=C) of lipids and fatty acids
1740	C=O stretching of lipids
1717	C=O of thymine
1697	Amide I; Guanine
1694	Amide I; β-sheet; C=O, C-N and N-H vibration
1639	Amide I
1520;1512	Amide II; CH in-plane bending
1485	Ring vibrations of guanine
1161	C-O of proteins
964	C-C or C-O of deoxyribose

Figure 2 – Comparison of untreated MCF-7 and Mallard cells. (**A**) ATR-FTIR PCA-LDA scores plot showing separation of scores along LD1, significance assessed by two-sample *t*-test ($P<0.01$ level indicated by ** symbol); (**B**) LD1 loadings describing wavenumber alterations responsible for separation in scores plots with the top six peaks highlighted; (**C**) Forward feature selection histogram resulting from 100% classification of cell type; (**D**) Tentative wavenumber assignments, derived from Movasaghi et al, 2008.

Two of the features with the most hits are observed at 1790 cm^{-1} and 1755 cm^{-1} which are both associated with vibrations of C=C bonds in lipids and fatty acids. The other features highlighted are mostly related to the protein content of the cells, which is mirrored in the loadings and 2nd derivative spectra. The other peaks in the feature histogram can be seen at 1697 cm^{-1} (Amide I), 1639 cm^{-1} (Amide I), 1520 cm^{-1} (Amide II and CH bending) and 1161 cm^{-1} (C-O vibrations from proteins).

As cellular lipid content emerged as a consistent discriminatory feature in the analysis, the cells were stained with Nile red stain and imaged using confocal microscopy (Fig. 3). Nile red is a vital stain which is highly soluble in lipids and is strongly fluorescent but only in the presence of a hydrophobic, lipid-rich environment (Greenspan et al. 1985). It is visually obvious that the staining pattern in the two cell lines differs suggesting that MCF-7 and mallard cells have dissimilar intracellular lipid distribution. In MCF-7 cells, lipids appear to be mostly accumulated around the periphery of the cells indicating that most of the lipid is found in the cell membrane. However in the mallard cells, lipids appear to be distributed throughout the cytoplasm so that the cell membrane cannot be distinguished. The nucleus is visualised as a 'hole' in the staining as less lipid is localised there. As previously suggested, the difference in lipid distribution is likely due to increased lipid content in bird skin cells, including mallards. Although this will not be a feature of all avian cell types, because such a lipid profile may provide an ideal environment for the accumulation of lipophilic chemicals it could have implications for extrapolation of results from toxicity tests using traditional cell line assays. As the cells are very different in physiology, lipidomic analysis to provide quantitative lipid values for the two cells types should be performed to confirm this. Another reoccurring feature was wavenumbers related to DNA and nucleic acid molecules in the cells, possibly as the MCF-7 cells are from a breast cancer cell line with altered gene expression patterns. MCF-7 cells have been found to express higher basal levels of cytochrome P450 enzymes compared to benign epithelium and exhibit an increased inducible response to contaminant exposure and at basal levels compared to normal epithelial cells

A.



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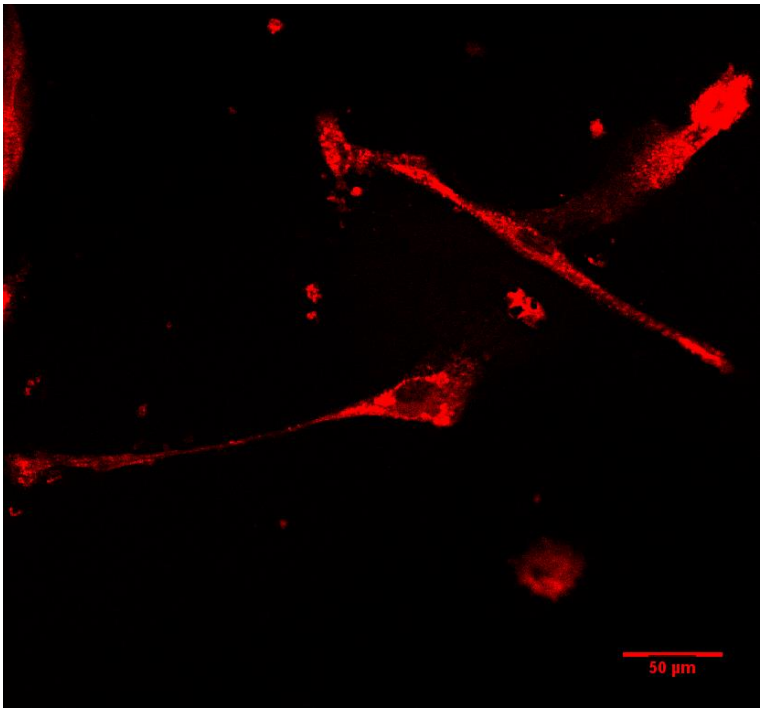

Figure 3 – Confocal microscopy images of untreated cells stained with 1:1000 Nile Red stain to show cellular lipids. Images obtained using a 20× objective and a numerical aperture of 0.5. (**A**) MCF-7 cells; (**B**) Mallard cells.

(Angus et al. 1999); hence a Western blot was performed to assess CYP1A1 levels in the absence of treatment (see ESI Fig. 1). It appeared that basal expression of CYP1A1 in mallard cells was almost half that observed in MCF-7 cells, which may indicate that metabolism of xenobiotic compounds is likely to be different. Previous work has found that MCF-7 cells preferentially express CYP1A1 (Spink et al. 1998) whereas in avian species, other isoforms such as CYP1A4 or CYP1A5 may be more important (Mahajan and Rifkind 1999). However, it is important to recognise that whilst some mallard isoforms are orthologous to mammalian CYP1A1, the degree of homology is not perfect which could interfere with the efficacy of antibody-based assays (Watanabe et al. 2013). Therefore, cytochrome P450 metabolism differences cannot be determined with certainty until the strength of avian CYP isoforms binding to CYP antibodies has been fully validated. This would also need to be fully tested in response to a number of different compounds and to investigate thetheir consequent effects on different CYP isoforms, all which may have implications for species-based susceptibility to contaminants.

Exposure to PCB and PBDE congeners

IR spectroscopy is a powerful tool that not only allows insight into the underlying biochemical structure of samples but can also be applied to reveal the effects of chemical treatments by extracting induced spectral alterations. In order to see if differences in biochemistry influence the effects on MCF-7 and mallard cells when exposed to single PCB or PBDE agents, the PCA-LDA processed spectral datasets were visualised as 1D scores plots with corresponding loadings from LD1. Scores plots of both cells treated with 10^{-8} M, 10^{-10} M or 10^{-12} M PBDE 47 (Fig. 4, see ESI Table 2) show that all cells exposed to the chemical segregate significantly (P<0.01) away from the controls. This suggests that treatment with PBDE 47 at these concentrations induces significant biochemical changes in both human and avian cells. The major wavenumber assigned alterations that contribute to this separation in mallard cells are in spectral regions associated with lipids (1767 cm^{-1}; 1713 cm^{-1}), C=O stretching of Amide I (1643 cm^{-1}), Amide II (1543 cm^{-1}; 1497 cm^{-1}) and protein CH_3

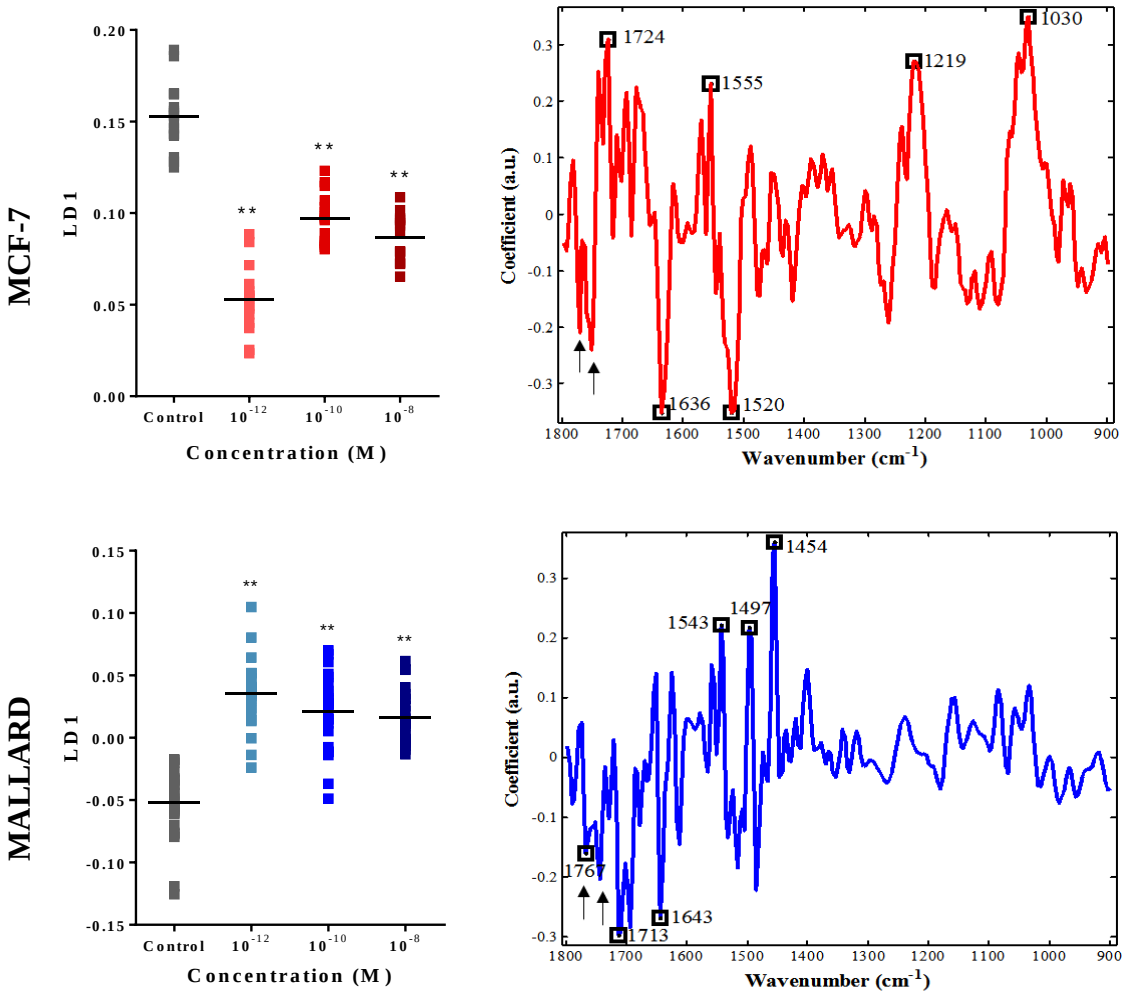

Figure 4 – Comparison of ATR-FTIR PCA-LDA scores plots and corresponding loadings plots for MCF-7 and mallard cells treated with 10^{-8}, 10^{-10} or 10^{-12} M PBDE 47. Significance from control calculated using one-way ANOVA followed by Dunnett's post-hoc test (P<0.05 level (*) or P<0.01 level (**). Arrows signify similar alterations induced in the lipid region in the range between 1751 and 1771 cm^{-1}.

deformation (1454 cm^{-1}). In MCF-7 cells, treatment with PBDE 47 is associated with fatty acids (1724 cm^{-1}), Amide I (1636 cm^{-1}), Amide II (1555 cm^{-1}; 1520 cm^{-1}), asymmetric phosphate stretching vibrations in DNA (1219 cm^{-1}) and glycogen and ribose vibrations (1030 cm^{-1}). Both cell types exhibit alterations in the lipid region of the spectrum in response to PBDE 47 exposure. In both loadings plots, two peaks with the same direction and similar magnitudes of change can be seen in the lipid region between 1751 and 1771 cm^{-1}. This suggests that PBDE47 induces similar effects on both cell types in this region of the spectrum.

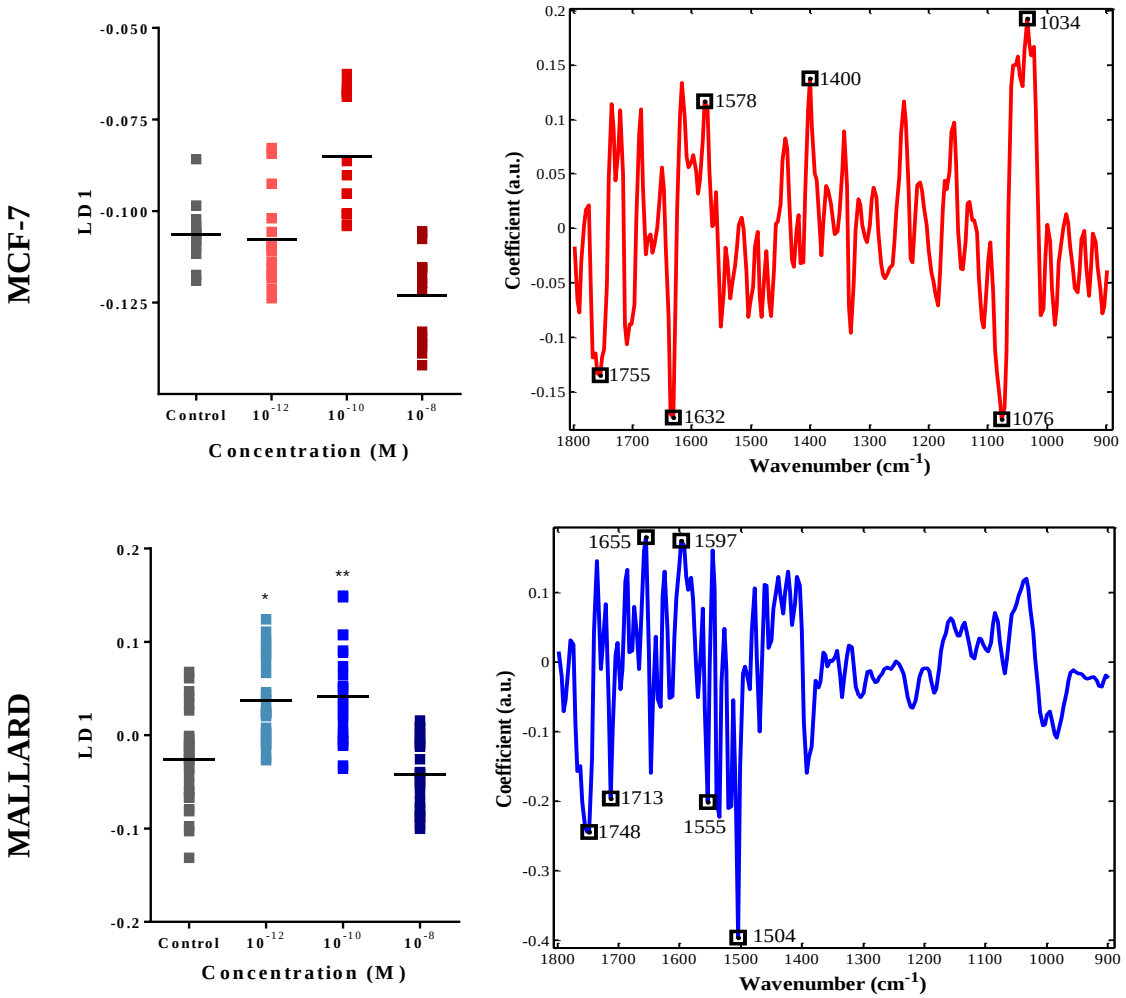

Figure 5 – Comparison of Comparison of ATR-FTIR PCA-LDA scores plots and corresponding loadings plots for MCF-7 and mallard cells treated with 10-8, 10-10 or 10-12 M PBDE 99. Significance from control calculated using one-way ANOVA followed by Dunnett's post-hoc test ($P<0.05$ level (*) or $P<0.01$ level (**)

In mallard cells, the lipid alteration associated peaks are among the largest changes induced by the chemical whereas in MCF-7 cells, these alterations are surpassed in magnitude by those associated protein and DNA. The two cell types exhibit a different range of biomarkers of exposure to PBDE 47 with alterations in mallard cells occurring in lipid and protein regions and MCF-7 cell alterations occurring in lipid, protein, DNA and carbohydrate regions. The unique DNA alterations in MCF-7 cell spectra may reflect an essential difference in cellular biochemistry between the two cell types. As seen in Fig. 5 (and ESI Table 3), similar alteration profiles appear to be seen in MCF-7 cells when exposed to PBDE 99 and those

treated with PBDE 47. MCF-7 cells treated with PBDE 99 showed alterations in peaks of lipids and fatty acids (1755 cm^{-1}), C=O vibration of Amide I (1632 cm^{-1}), Amide II (1578 cm^{-1};1400 cm^{-1}), symmetric phosphate stretching vibrations in DNA (1076 cm^{-1}) and collagen (1034 cm^{-1}). However, it is important to note that there was considerable overlap of the scores from PBDE 99-treated MCF-7 cells with the control, resulting in an insignificant result and this limits the conclusions that can be drawn from the loadings. Visually similar loadings profiles could suggest similar profiles of exposure induced by these congeners but this would

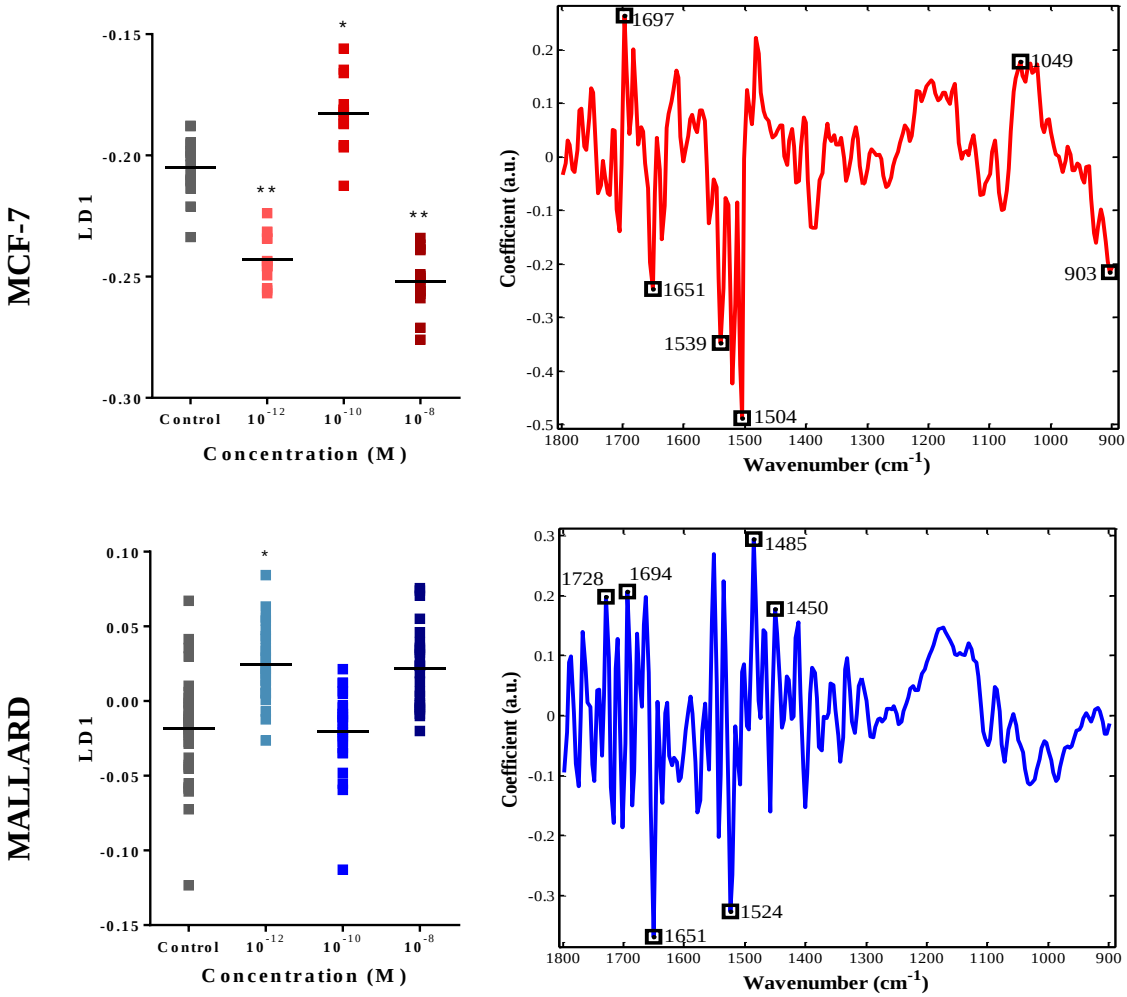

Figure 6 – Comparison of ATR-FTIR PCA-LDA scores plots and corresponding loadings plots from MCF-7 and mallard cells treated with 10^{-8}, 10^{-10} or 10^{-12} M PBDE 153. Significance from control calculated using one-way ANOVA followed by Dunnett's post-hoc test (P<0.05 level (*) or P<0.01 level (**).

need further investigation to be conclusive. In avian cells, exposure was characterised by extensive alterations in lipid and protein associated spectral regions. The major alterations were seen in C=C and C=O bonds of lipid molecules (1748 cm^{-1}; 1713 cm^{-1}), C=O, C-N or N-H bond vibrations of Amide I (1655 cm^{-1}) and Amide II (1597 cm^{-1}; 1555 cm^{-1}; 1504 cm^{-1}). The major wavenumber-associated alterations from PBDE 153-treated MCF-7 cells (Fig. 6, see ESI Table 4) are seen in Amide I (1697 cm-1; 1651 cm^{-1}) and Amide II (1539 cm^{-1}; 1504 cm^{-1}) groups from proteins as well as carbohydrates including glycogen (1049 cm^{-1}) and DNA phosphodiester stretching (903 cm^{-1}). PBDE 153 treatment induced MCF-7 cell specific alterations in carbohydrates and DNA spectral regions that are not seen in mallard cells treated with this chemical. However, alterations induced in mallard cells by PBDE 153 exposure are not explored here as the scores were not significantly separated except at a concentration of 10^{-12} M. This means that the loadings cannot be interpreted with accuracy as they will also incorporate potentially meaningless biological information from unseparated scores. Finally, alterations in MCF-7 and mallard cells treated with 10^{-8} M, 10^{-10} M or 10^{-12} M of PCB 153 can be seen in Fig. 7, see ESI Table 5. After exposure, MCF-7 cells were characterised by changes in fatty acid esters (1732 cm^{-1}), Amide I (1697 cm^{-1}; 1643 cm^{-1}), Amide II (1543 cm^{-1}; 1597 cm^{-1}) and symmetric phosphate stretching vibrations of DNA (1072 cm^{-1}) whereas mallard cells exposed to PCB 153 exhibited alterations in lipids (1709 cm^{-1}), Amide I (1694 cm^{-1}), Amide II (1535 cm^{-1}; 1497 cm^{-1}) and other structural and functional molecules including collagen and glycogen (1458 cm^{-1} and 1030 cm^{-1}, respectively) in avian cells.

Overall, spectra from both cell types treated with PBDE 47, PBDE 99, PBDE 153 or PCB 153 show alterations mostly in Amide I and II protein regions, with unique DNA alterations seen in MCF-7 spectra. Alterations in lipid-associated spectral regions were also seen in both cell types, particularly in response to PBDE 47 and PCB 153 exposures. This is consistent with what we know of these types of chemicals and the cellular changes they can stimulate. PCBs and PBDEs can induce a number of cellular effects which could cause alterations in protein in both cell types, such as induction of enzymes involved in phase I and II xenobiotic

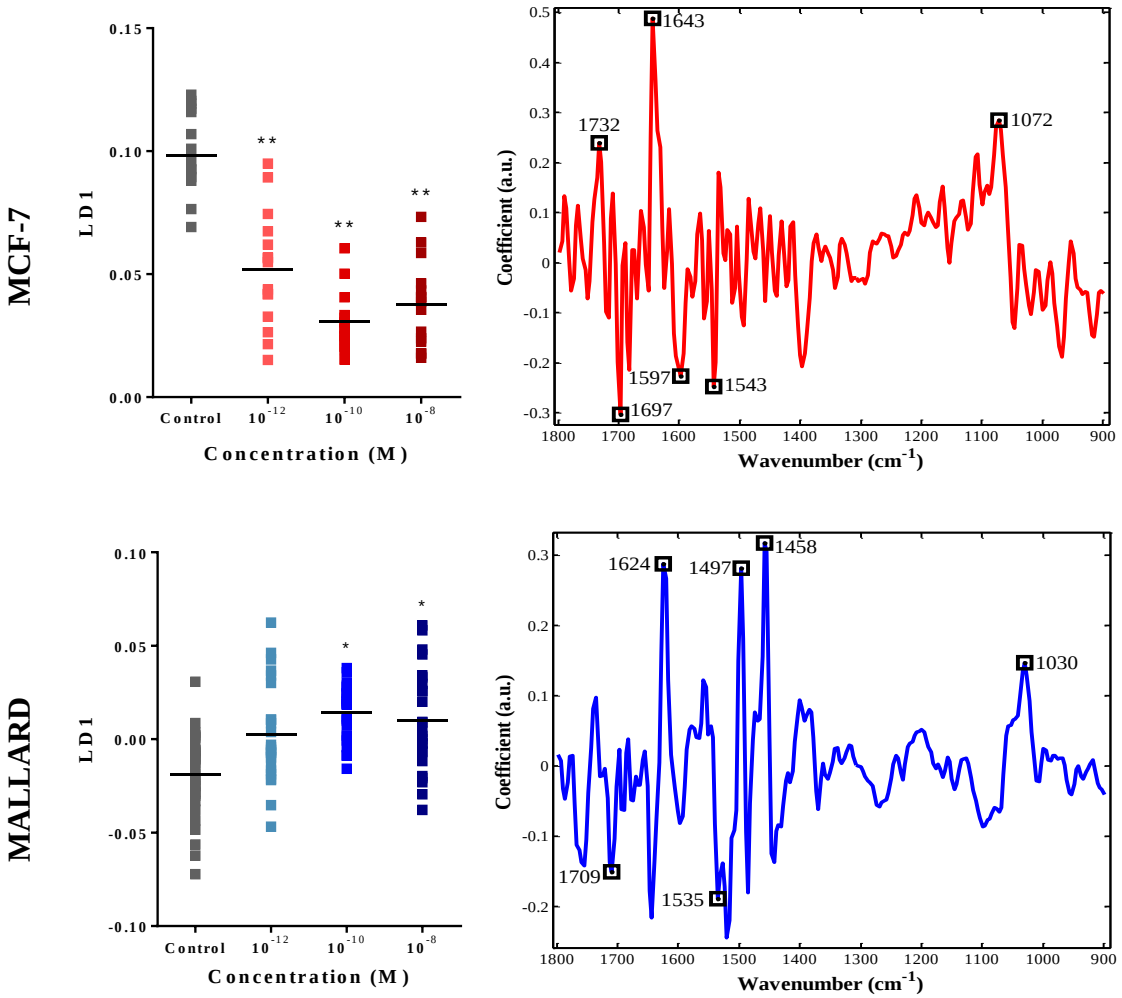

Figure 7 – Comparison of ATR-FTIR PCA-LDA scores plots and corresponding loadings plots from MCF-7 and mallard cells treated with 10-8, 10-10 or 10-12 M PCB 153. Significance from control calculated using one-way ANOVA followed by Dunnett's post-hoc test (P<0.05 level (*) or P<0.01 level (**).

metabolism and changes to regulatory or structural proteins (Alm et al. 2009). They are also lipophilic chemicals and have been shown to cause a range of toxic effects which can cause cellular lipid alterations including instigating oxidative stress and lipid peroxidation (He et al. 2008). Lipid alterations were amongst the most marked induced in mallard cells by all congeners which may indicate that they are susceptible to lipophilic compounds such as PCBs and PBDEs, that can alter lipid metabolism (Noël et al. 2014) and production of ROS leading to lipid damage (He et al. 2008). This may be due to lipid distribution throughout this cell

type, which appears to be throughout the cytoplasm as seen in Nile red-stained images. Other cell types have been found to be differentially affected by certain contaminants due to their lipid content, for example neurons with myelin sheaths are thought to be susceptible to organochlorine chemicals (Cannon and Greenamyre 2011). Untreated MCF-7 cells were found to exhibit more spectral contributions from DNA than mallard cells and correspondingly, more DNA alterations were induced by exposure to PCB and PBDE contaminants in these cells than in their avian counterparts. Both PCBs and PBDEs can damage DNA either directly or by producing free-radicals and causing oxidative stress (Ravoori et al. 2008)-(Alabi et al. 2012; Schilderman et al. 2000) and thus the native biochemical differences of MCF-7 cells may make them more susceptible to this type of toxicity. This could potentially lead to an over-estimation of toxic risk in cell-based assays using MCF-7 cells. Therefore, for cell-based toxicity testing, it may be appropriate to use a number of representative cell types to ensure accurate results. As this could potentially be time-consuming and costly, ATR-FTIR spectroscopy may have a novel application as a tool to rapidly screen multiple cell lines (Balls et al. 2012) in order to determine which cell types are differentially affected by a treatment before full toxicity testing is performed.

As with many preliminary exploratory studies, there are weaknesses in this work, which can be used to direct and improve future experiments. The concentrations used in this study were selected to simulate exposure levels of contaminants that would be encountered by organisms in the environment, particularly as one of the cell lines used is derived from a free-flying duck species. Previous work has identified spectral protein and lipid alterations in MCF-7 cells at concentrations as low as 10^{-12} M due to bimodal dose responses (Barber et al. 2006) but the majority of studies focus on concentrations above the μM range (Li et al. 2012). The mallard cell line appears to respond differently to dose response experiments than the MCF-7 cells. Therefore, some of the results are not significant particularly at lower concentrations, which limits conclusions that can be made. In order to avoid this, future experiments should focus on concentrations above the μM range before investigating the response of cells at very low

environmental levels. With the use of such small concentrations, the measurement of other sub-lethal toxicological effect markers would be also useful. This may also increase the stability of the PCA-LDA models and allow dose-response scores clusters to be consistently resolved. The results presented here were analysed using PCA-LDA as the results from PCA were not found to be significant (see ESI Figs. 2-5, Table 6).

The results from this initial spectral study indicate that MCF-7 and mallard cells have unique biochemical differences and the results of studies like this can contribute to important decisions regarding which cell types should be used in toxicology assays. This may be particularly essential when studying environmental contaminants and want to extrapolate the results to environmental species. Biochemical differences, which can be identified using ATR-FTIR spectroscopic techniques, may influence how cell types used in assays are affected by common environmental contaminants such as PCBs and PBDEs. This study demonstrates ATR-FTIR spectroscopy as an exploratory tool to investigate biochemical differences at the cellular level and may have practical application as a means to direct further work. Such techniques could be used as part of a panel of cell-based assays to indicate when results from environmentally derived cell lines differ from those obtained from traditional cell lines which might vastly differ in many aspects, e.g. metabolism, biochemistry etc. This is a preliminary study and future work is needed to understand where and how mallard fibroblast cells and other environmental cell lines can be used but with optimisation the use of IR spectroscopy to analyse environmentally relevant cell lines could improve the extrapolation of results to environmental settings in a cost and time efficient manner.

Conclusions

Differences at the molecular, cellular, tissue and/or species level can affect the toxicity of environmental chemical contaminants. Cell-based assays to investigate the toxicity of such compounds are highly useful in the effort to reduce the number of animals used in scientific procedures but appropriate cell types must be used in order to obtain meaningful results. To

investigate environmentally relevant chemicals, a cell line closer to those found in the environment may be more suitable for extrapolation of results. By using ATR-FTIR spectroscopy to analyse untreated cells, the spectra of an MCF-7 and a mallard fibroblast cell line were compared to understand cellular differences, which might affect the results of cell-based toxicity testing. The two cell types were found to share some spectral features, in the fingerprint region, largely due to cellular macromolecules such as proteins. However, each cell type spectrum also demonstrated unique differences in biochemical composition. Untreated mallard cell spectra displayed large lipid-associated peaks, possibly due to greater intracellular lipid content, which was explored in Nile red-stained images. MCF-7 cells were found to have spectral differences in the DNA and nucleic acid regions, possibly as the cell line is derived from an epithelial breast tumour. This demonstrates that ATR-FTIR spectroscopy can identify fundamental biochemical cellular differences. It is possible that these biochemical differences may also determine how environmental pollutants will affect cells and thus could influence how accurate the extrapolation of toxicity test results to environmental species will be. However, further characterisation of mallard fibroblasts would be needed. Differences in the biomolecular structure and composition of cells must be considered during such testing and if possible, multiple cell types should be analysed. ATR-FTIR with multivariate analysis can be used as a powerful tool for this purpose as it can be used to discriminate between cell types based on fundamental variances at the biomolecular level. This technique may be particularly useful as a screening tool to analyse variations in response to chemical exposure before full toxicology testing is performed. In this manner, ATR-FTIR spectroscopy would allow researchers to obtain rapid initial findings that can direct conclusive toxicological investigation. It is not possible to say from this initial study whether mallard fibroblasts are the most suitable environmental 'surrogate' for toxicology tests using mammalian cells so future work would focus on additional characterisation of mallard fibroblasts as well as investigation of cellular differences in cell lines from other environmental species such as amphibians and fish.

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Supplementary Information:

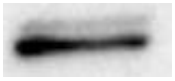
	MCF-7	MALLARD
CYP1A1		
β-actin		
Relative CYP1A1 Intensity	0.66	0.35

Figure S1 – CYP1A1 protein band intensity in untreated MCF-7 and Mallard cells as assessed by western blot. Band intensity adjusted to background intensity and to the β-actin loading control.

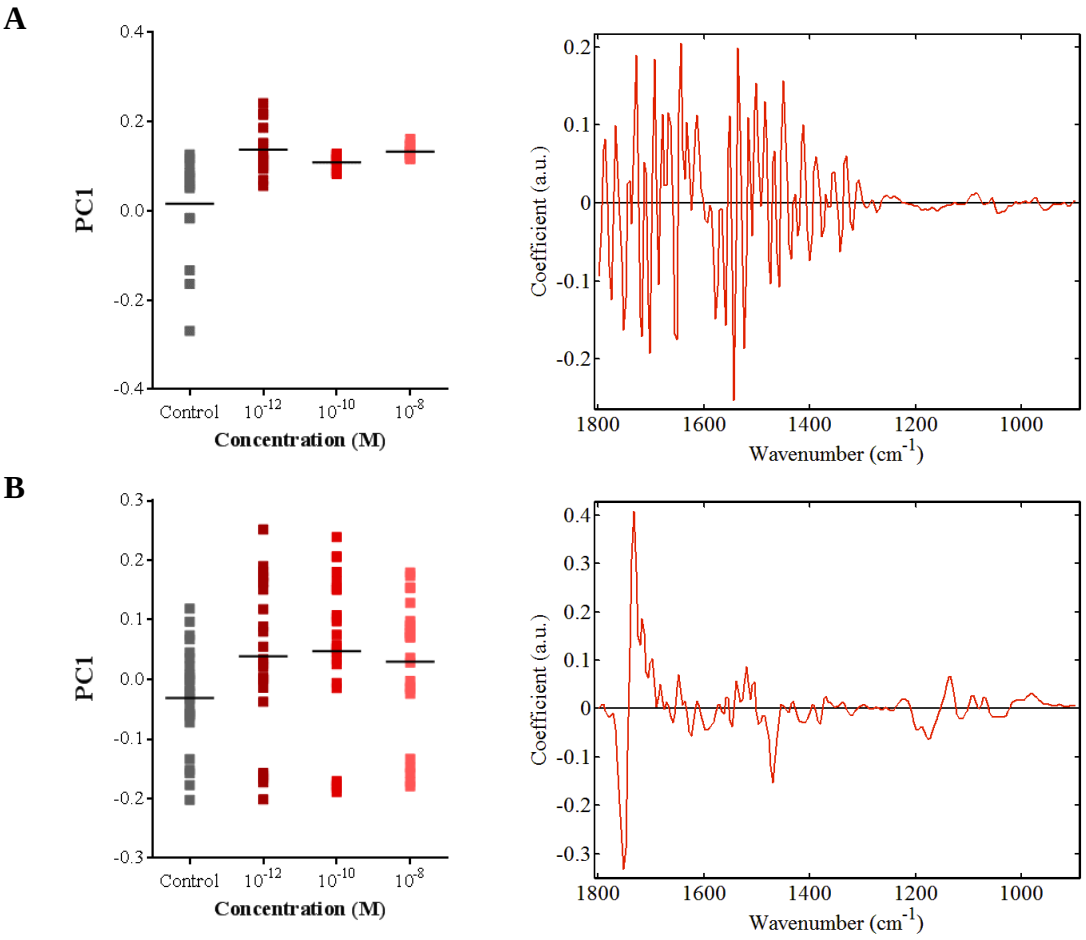


Figure S2 – ATR-FTIR PCA scores plots and loadings along PC1 from (**A**) MCF-7 cells and (**B**) mallard cells treated with PBDE 47 showing separation of scores along PC1. Separation from control was not found to be significant as assessed by one-way ANOVA.

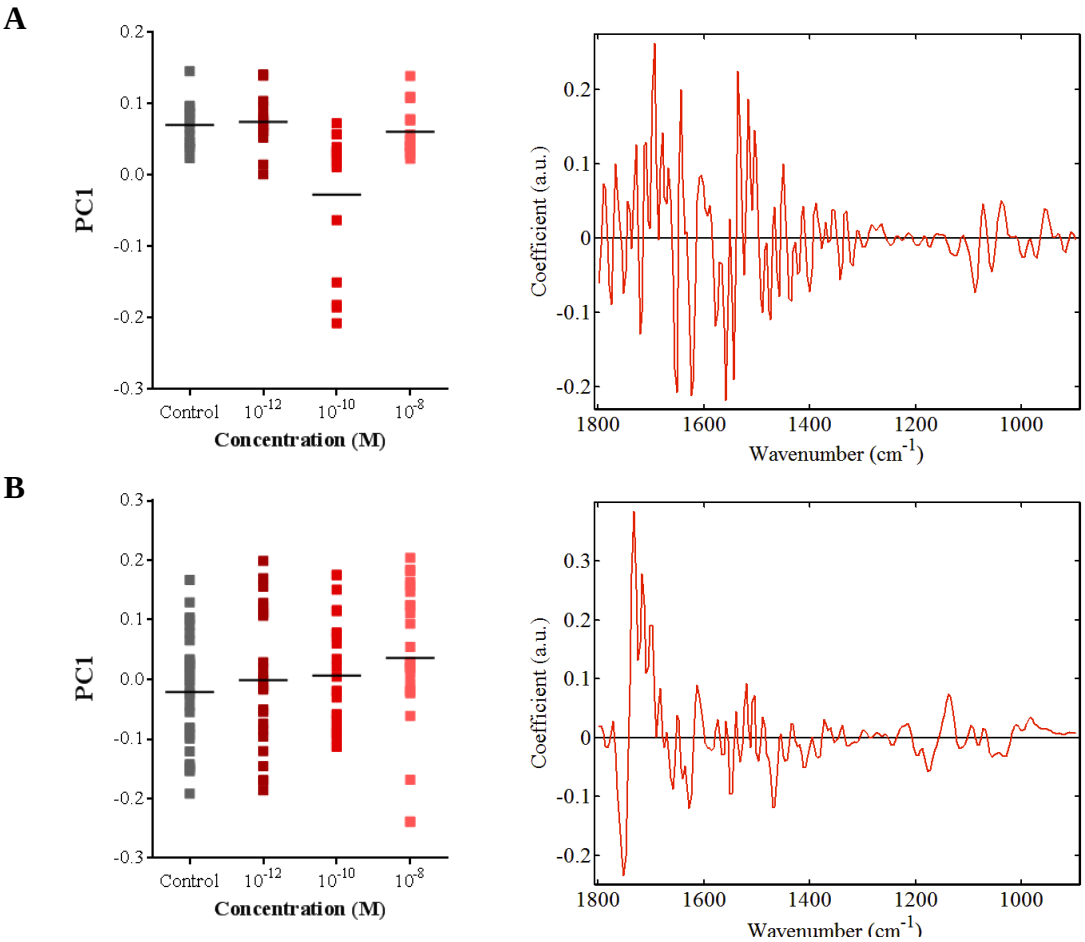


Figure S3 – ATR-FTIR PCA scores plots and loadings along PC1 from (**A**) MCF-7 cells and (**B**) mallard cells treated with PBDE 99 showing separation of scores along PC1. Separation from control was not found to be significant as assessed by one-way ANOVA.

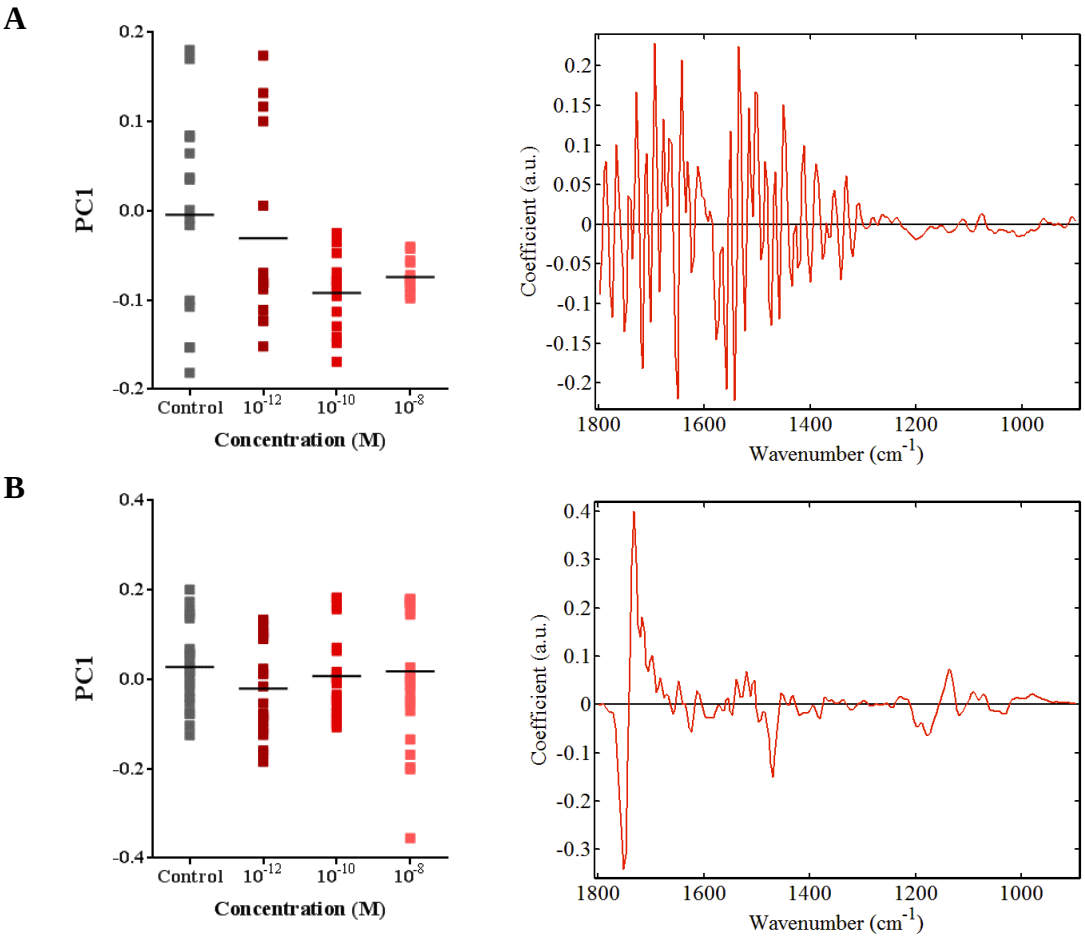


Figure S4 – ATR-FTIR PCA scores plots and loadings along PC1 from (**A**) MCF-7 cells and (**B**) mallard cells treated with PBDE 153 showing separation of scores along PC1. Separation from control was not found to be significant as assessed by one-way ANOVA.

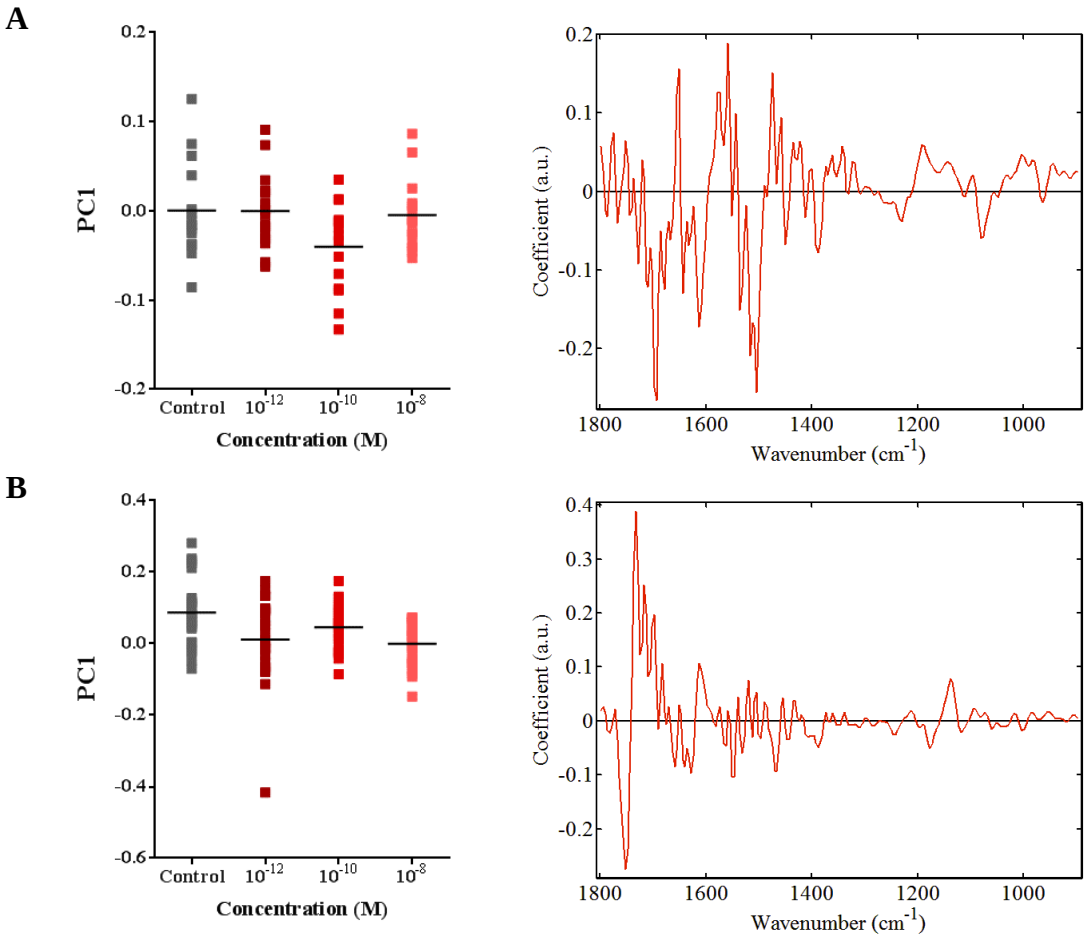


Figure S5 – ATR-FTIR PCA scores plots and loadings along PC1 from (**A**) MCF-7 cells and (**B**) mallard cells treated with PCB 153 showing separation of scores along PC1. Separation from control was not found to be significant as assessed by one-way ANOVA.

Cell Type	Wavenumber (cm^{-1})	Assignment
MCF-7	1690	Amide I; β-sheet; Carbonyl stretching
	1624	Amide I
	1512	Amide II; CH in-plane bending
	1447	Asymmetric CH_3 bending of proteins
	1393	Symmetric CH_3 bending of proteins
	1234	Amide III; Phosphate from nucleic acids
	1165	C-O and C-OH stretching of proteins
	1084	Symmetric phosphate stretching from nucleic acids
	1057	C-O stretching of deoxyribose in DNA
	964	C-C or C-O of deoxyribose in DNA
Mallard	1744	C=O stretching of lipids, phospholipids and triglycerides
	1690	Amide I; β-sheet; Carbonyl stretching
	1639	Amide I
	1512	Amide II; CH in-plane bending
	1450	CH_3 deformation
	1393	Symmetric CH_3 bending of proteins
	1312	Amide III
	1238	Amide III; Phosphate from nucleic acids
	1161	C-O and C-OH stretching of proteins
	1080	Symmetric phosphate stretching from nucleic acids; Collagen

Table S1 – Top ten tentative wavenumber assignments derived from second derivative ATR-FTIR spectra of untreated MCF-7 and Mallard cells. Assignments derived from Movasaghi et al, 2008.

A

Wavenumber (cm^{-1})	Assignment
1724	Lipid
1636	Amide I; β-sheet
1555	Amide II
1520	Amide II
1219	Asymmetric phosphate (DNA)
1030	Glycogen; Collagen; C-O ribose

B

Wavenumber (cm^{-1})	Assignment
1767	*ν*(C=C) of lipids and fatty acids
1713	Lipids
1643	Amide I (C=O stretching)
1543	Amide II
1497	Amide II (C=C vibration)
1454	Asymmetric CH_3 deformation

Table S2 – Top six tentative wavenumber assignments derived from PCA-LDA loadings plots for MCF-7 and Mallard cells treated with PBDE 47. (**A**) MCF-7 cells treated with 10^{-8}, 10^{-10} and 10^{-12} M PBDE 47; (**B**) Mallard cells treated with 10^{-8}, 10^{-10} and 10^{-12} M PBDE 47. Assignments derived from Movasaghi et al, 2008.

A

Wavenumber (cm^{-1})	Assignment
1755	*v*(C=C) of lipids and fatty acids
1632	Amide I; C=O
1578	Amide II; C-C stretch of phenyl
1400	COO^- of fatty acids and proteins; CH_3 of protein
1076	CC vibration of DNA
1034	Collagen

B

Wavenumber (cm^{-1})	Assignment
1748	*v*(C=C) of lipids
1713	Lipids; C=O
1655	Amide I (C=O, C-N, N-H)
1597	C=N, NH_2 of adenine
1555	Amide II
1504	Amide II (CH Bending)

Table S3 – Top six tentative wavenumber assignments derived from PCA-LDA loadings plots for MCF-7 and Mallard cells treated with PBDE 99. (**A**) MCF-7 cells treated with 10^{-8}, 10^{-10} and 10^{-12} M PBDE 99; (**B**) Mallard cells treated with 10^{-8}, 10^{-10} and 10^{-12} M PBDE 99. Assignments derived from Movasaghi et al, 2008.

A

Wavenumber (cm^{-1})	Assignment
1697	Amide I; C=O
1651	Amide I (C=O Stretching)
1539	Amide II; β-sheet
1504	Amide II; CH bending of phenyl ring
1049	Glycogen; C-O stretch of carbohydrates
903	Phosphodiester stretching (DNA)

B

Wavenumber (cm^{-1})	Assignment
1728	Lipids (C=O Band)
1694	Amide I; C=O
1651	Amide I (C=O Stretching)
1524	Amide II (C=N and C=C Stretching)
1485	Amide II; C-H deformation
1450	CH_3 deformation

Table S4 – Top six tentative wavenumber assignments derived from PCA-LDA loadings plots for MCF-7 and Mallard cells treated with PBDE 153. (**A**) MCF-7 cells treated with 10^{-8}, 10^{-10} and 10^{-12} M PBDE 153; (**B**) Mallard cells treated with 10^{-8}, 10^{-10} and 10^{-12} M PBDE 153. Assignments derived from Movasaghi et al, 2008.

A

Wavenumber (cm^{-1})	Assignment
1732	Fatty acid esters
1697	Amide I; C=O
1643	Amide I (C=O stretching)
1597	C=N, NH_2 of adenine
1543	Amide II
1072	Symmetric Phosphate (DNA)

B

Wavenumber (cm^{-1})	Assignment
1709	Lipids; C=O thymine
1624	Amide I
1535	Amide II (C=N and C=C stretching)
1497	Amide II (C=C vibration)
1458	CH_3 of collagen
1030	Glycogen; Collagen; C-O ribose

Table S5 – Top six tentative wavenumber assignments derived from PCA-LDA loadings plots for MCF-7 and Mallard cells treated with PCB 153. (**A**) MCF-7 cells treated with 10^{-8}, 10^{-10} and 10^{-12} M PCB 153; (**B**) Mallard cells treated with 10^{-8}, 10^{-10} and 10^{-12} M PCB 153. Assignments derived from Movasaghi et al, 2008.

Figure	Cell Type	Experimental Treatment	No of PCs	Variance Captured (%)
2	Both	Untreated	3	95.44
4	MCF-7	PBDE 47	7	95.66
4	Mallard	PBDE 47	8	95.42
5	MCF-7	PBDE 99	9	95.8
5	Mallard	PBDE 99	8	95.11
6	MCF-7	PBDE 153	7	95.8
6	Mallard	PBDE 153	9	95.12
7	MCF-7	PCB 153	9	95.05
7	Mallard	PCB 153	11	95.17

Table S6 – The number of principal components (PCs) used in PCA-LDA and the percentage of variance the PCs captured.

Chapter 6. Identification and prediction of binary mixture effects from genotoxic and endocrine disrupting environmental contaminants using infrared spectroscopy

Kelly A. Heys, Richard F. Shore, M. Glória Pereira, Kevin C. Jones and Francis L. Martin.

Manuscript for submission

Contribution:

- I conducted all experiments for the study.
- I prepared, processed and acquired data for all samples including conducting computational analysis.
- I prepared the first draft of the manuscript.

…… ……

Kelly A. Heys Prof. Francis L. Martin

Identification and prediction of binary mixture effects from genotoxic and endocrine disrupting environmental contaminants using infrared spectroscopy

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Abstract

Benzo[*a*]pyrene (B[*a*]P), polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are persistent contaminants and concern has arisen over co-exposure of organisms when the chemicals exist in mixtures. Regulatory assessments use additive models to assess the effects of mixtures but interactions between chemicals can lead to over- or under-estimation of risk and leave environmental organisms vulnerable to unexpected toxicity. It is therefore essential to understand how chemicals in mixtures interact and to have suitable tools to analyse them. Here, attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy was used to identify biochemical alterations induced in cells by single and binary mixtures of environmental chemicals. It was also investigated as a method to identify if interactions are occurring in mixtures and as a possible tool to predict mixture effects. Mallard fibroblasts were treated with single B[*a*]P, PCB 126, PCB 153, PBDE 47 or PBDE 209 or binary mixtures of B[*a*]P with a PCB or PBDE congener. Comparison of observed spectra from cells treated with binary mixtures with predicted additive spectra which were created from individual exposure spectra indicated that in many areas of the spectrum, less-than-additive binary mixture effects may occur. However, possible greater-than-additive alterations were identified in the 1650-1750 cm^{-1} lipid region and may demonstrate a common toxicity mechanism of B[*a*]P and PCBs or PBDEs which can enhance toxicity in mixtures.

Introduction

There are many types of chemical contaminant that find their way into environmental compartments during their usage cycle or through accidental release. The past century has seen an increasing awareness of the potential risk such chemicals pose to the health of ecosystems and environmental organisms. Some pollutants are extremely persistent and bioaccumulate up food chains, giving rise to concern for top level predators such as predatory bird species (Gobas et al. 2009). Benzo[*a*]pyrene (B[*a*]P) is an example of a pervasive polycyclic aromatic hydrocarbon (PAH) contaminant that is abundantly found in the environment due to anthropogenic activity associated with partial combustion. B[*a*]P exposure has been largely associated with inhalation of cigarette smoke, car exhaust and industrial air pollution as well as via dietary intake (Samanta et al. 2002; Sinha et al. 2005). Exposure to B[*a*]P and other PAHs is considered a risk to humans and wildlife due to reported carcinogenic toxicity. B[*a*]P can bind to the aryl hydrocarbon receptor (AhR) and mediate the expression of cytochrome P450 enzymes including CYP1A1 which metabolises the chemical into its DNA binding, mutagenic form (Cella and Colonna 2015; Rojas et al. 2000). Other highly persistent chemicals include polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), some congeners of which are also reported to possess AhR binding abilities like B[*a*]P (Hestermann et al. 2000; Peters et al. 2006). PCBs and PBDEs have been used as additives in various consumer products such as paints, textiles and electronics to act as coolants and flame retardants (Talsness 2008). They have been found to exert toxicity on a number of biological systems including the endocrine, immune and nervous systems (Dingemans et al. 2011; Liu and Lewis 2014). Although PCBs and many PBDE congeners are now banned in the E.U. and other locations, they are still currently found in environmental matrices due to their persistent nature and are presently used in some developing countries (Crosse et al. 2012b; Hassanin et al. 2004; Lammel et al. 2015).

The concern related to these contaminants is not solely due to their potential toxicities alone but also due to the possibility that they exist as part of mixtures. Chemicals are often considered in isolation but in reality organisms in the environment are simultaneously and sequentially exposed to a wide range of contaminants, many of which have different toxic mechanisms. It is commonly assumed that the toxicity of a mixture can be predicted by adding the toxicities of the mixture components together, in what is known as an additive model of mixture effects (Bliss 1939; Loewe 1926). Although this leads to accurate mixture toxicity predictions in most instances, interactions may occur at the biological target sites or between chemicals which can cause unexpected mixture toxicity. Interactions can lead to two possible outcomes, a reduction in expected toxicity (antagonism) or a greater than expected toxicity (synergism) (Kortenkamp 2007). The additive approach is used for the majority of regulatory assessments regarding mixtures meaning that environmental organisms may be left vulnerable to the effects of interactive mixture effects (Kortenkamp et al. 2009a). It is not practically possible to test every single mixture that may occur in the wider environment and as some chemicals are not being actively released, they may not be incorporated into risk assessments. B[*a*]P along with PCBs and PBDEs are extremely abundant contaminants and therefore are highly like to occur together in mixtures. They also have at least one common toxicity pathway which may allow for interactions to arise. The possibility that synergy in mixtures, especially those including legacy contaminants, may go undetected is concerning. Therefore, we need to have efficient techniques to test for interactions in mixtures that can be used to guide risk assessments.

Vibrational spectroscopy techniques have proved to be valuable exploratory tools for various, diverse experimental purposes including cancer research (Hands et al. 2013; Theophilou et al. 2016), environmental monitoring (Obinaju et al. 2014; Wood et al. 2005) and quality assurance (Oca et al. 2012; Wood et al. 2005). These techniques offer a number of advantages such as being cost-effective, non-destructive to samples and high-throughput. Vibrational spectroscopy can be used to create a biochemical profile of samples by measuring the

absorption of light and changes in vibrational energy levels (Harz et al. 2009). Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy is a specific type of vibrational spectroscopy where the sample of choice is interrogated with polychromatic infrared (IR) light which is reflected within an internal reflective element (IRE) (Baker et al. 2014a). Biochemical bonds absorb photons at specific, characteristic wavelengths depending on the frequency required to for bonds to vibrate and have a change in dipole moment. The output from this is a spectrum showing the absorbance of infrared light at each of the measured wavelengths which characterises the molecular composition of the sample and can be used to infer structural and functional information (Kelly et al. 2011). Previously, ATR-FTIR has been used for environmental monitoring (Obinaju et al. 2014) and to study the effects of environmentally relevant chemicals in cells and tissues (Llabjani et al. 2010; Obinaju et al. 2015). This has led to consideration of the technique as a tool to analyse the biological effects of chemical mixtures. In this study, we aim to assess ATR-FTIR in this capacity by using it to characterise the cellular effects of exposure to single contaminants as well as binary mixtures of B[*a*]P with PCB or PBDE congeners in avian fibroblast cells. We also aim to determine whether IR spectroscopy can be used to identify when a binary mixture of dissimilarly acting agents leads to non-additive mixture effects and whether the effects of mixtures can be predicted by creating predictive spectra from cells treated with the individual component chemicals. In this manner, ATR-FTIR may represent an essential tool to quickly and cheaply analyse the effects of binary pairs of environmental pollutants, either as a screening tool before further analysis or to reduce the scale of mixture experiments by using single exposure data.

Methods and Materials

Test Agents

Stocks of PBDE congeners 47 and 209 were purchased, dissolved in nonane at a concentration of 50 µg/mL, from LGC standards (Teddington, UK). PCBs 153 and 126 were purchased as

powders from Greyhound Chromatography and Allied Chemicals (Birkenhead, UK) and made up in nonane (Sigma-Aldrich, Dorset, UK). B[*a*]P was purchased in powder form from Sigma and dissolved in DMSO. Stock solutions of treatment chemicals were made up to a concentration of 2 µM in DMSO and then serially diluted in DMSO to the required experimental concentrations. Vehicle controls consisted of the same amount of DMSO as used in chemical treatments, spiked with equal quantities of nonane.

Cell Culture

Mallard (*Anas platyrhynchos*) dermal fibroblasts were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2% chicken serum (Sigma-Aldrich), 1% non-essential amino acids (Thermo Fisher Scientific, Nottinghamshire, UK) and a penicillin and streptomycin mixture (100 U/mL and 100 µg/mL respectively). Cells were cultured in a humidified atmosphere with 5% CO_2 in air, at a temperature 37 °C. Sub-culture was performed twice weekly by disaggregation with trypsin (0.05%)/EDTA (0.02%) solution before spinning at 1000 x g for 5 minutes. The resultant cell pellet was then resuspended in fresh complete DMEM and seeded into T75 flasks for routine sub-culture or T25 for cell experiments. Unless stated otherwise, all cell culture consumables were purchased from Lonza (Verviers, Belgium).

Cell Experiments

After seeding into T25 flasks, cultures were left for 24 hours to allow cells to attach and enter into S phase. After 24 hours, cells were treated with either single agents of B[*a*]P, PBDE 47, PBDE 209, PCB 126 or PCB 153, or binary mixtures of 10^{-6} M or 10^{-10} M B[*a*]P with a PCB or PBDE congener at 10^{-8} M or 10^{-12} M. For single agent treatments, 25 µl of the appropriate treatment was added to each flask as well as 25 µl of DMSO so the effects could be compared to binary mixture exposures which involved treating with 25 µl each of two chemicals. Vehicle controls were treated with 50 µl of DMSO (with nonane). Total DMSO concentrations did not exceed 1% v/v. Cells were exposed to treatments for 24 hours before

they were disaggregated with trypsin, washed three times with 70% ethanol to remove residual media and then fixed for 24 hours in 70% ethanol. After fixation, cells were pipetted onto IR-reflective low-E glass slides (Kevley Technologies, Chesterland, OH, USA) and allowed to air dry before being placed in a desiccator for 24 hours to remove any remaining water. This procedure was repeated at five different points in time to give five technical replicates (n=5).

ATR-FTIR Spectroscopy

Five spectra per slide were acquired using a Bruker TENSOR 27 FTIR spectrometer with Helios ATR attachment which contained a diamond IRE with a sampling area of 250 µm x 250 µm (Bruker Optics, Coventry, UK). Spectra were acquired with an 8 cm^{-1} spectral resolution with 32 co-additions, giving rise to a 3.84 cm^{-1} spectral data spacing. A mirror velocity of 2.2 kHz was used. Before each sample, a background measurement was taken to account for atmospheric changes and the diamond was cleaned with distilled water between samples.

Spectral Processing and Computational Analysis

An in-house Matlab 2013a (The Maths Works, MA, USA) toolbox called IRootLab was used for pre-processing and computational analysis of the spectra (http://trevisanj.github.io/irootlab/). Raw spectra were cut to the fingerprint region, 900-1800 cm^{-1}. Spectra were 1st order differentiation baseline corrected, vector normalised and mean centred. Principal component analysis (PCA) was paired with linear discriminant analysis (LDA) to allow exploratory analysis of treatment-induced spectral alterations. PCA was used for data reduction purposes and was optimised, to incorporate the maximum amount of variance which minimised noise, using the PCA pareto tool. LDA is a supervised technique that was used to optimise inter-class separation. K-fold, leave-one-out cross-calculation was used to avoid overfitting. Significance was determined in GraphPad Prism 4 (GraphPad Software Inc., CA, USA) using one-way ANOVA followed by Dunnett's post-hoc test.

Significance testing was performed using sample means rather than all spectral data. To compare 'predicted' and 'observed' spectral datasets, raw spectra were pre-processed as above and control-subtracted class averages for individual data were added together to form a 'predicted' spectrum. Goodness of fit between the model and observed result was calculated by a Spearman's correlation.

Western Blotting Methodology

For western blot experiments, cells were seeded in 6-well culture plates at a density of 1 x 10^{-6} cells and allowed to grow for 24 hours before being exposed to treatments for 24 hours in the same manner as described for cell experiments. Cells were lysed using 300 µl of RIPA buffer (50 mM Tris, 150 mM NaCl with 0.5% sodium deoxycholate, 1% IGEPAL CA-630 and 0.1% SDS) supplemented with protease and phosphatase inhibitor cocktails (Sigma). Lysates were spun at 12000 rpm for 10 minutes and the supernatant was transferred into a clean, labelled tube. Samples were then mixed 1:3 with 4x Laemmli sample buffer with 10% β-mercaptoethanol and boiled for 2 minutes at 95 ˚C. After cooling, samples were run in a 12% acrylamide gel for 35 minutes at 180 V. Proteins were then transferred to a nitrocellulose membrane using the Bio-Rad Trans-Blot Turbo Transfer system. Membranes were blocked in a 3% solution of non-fat milk powder in Tris-buffered saline Tween-20 (TBST) for 1 hour at room temperature with constant shaking. Membranes were then incubated with 1:1000 rabbit anti-CYP1A1 antibody (Abcam, Cambridge, UK), overnight at 4 ˚C. The membranes were washed three times with TBST before incubation, at room temperature for 1 hour, with 1:10000 goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz, CA, USA) in TBST. Finally, membranes were washed three times with TBST and protein bands were quantified using the Clarity Western ELC substrate on the Bio-Rad ChemiDoc XRS imaging system. β-actin was used as a loading control. This procedure was repeated at three different points to give three replicates (n=3). Unless stated otherwise, western blotting equipment and consumables were from Bio-Rad (Hercules, CA, USA).

Results and Discussion

Biochemical alterations induced by contaminants

In order to verify if ATR-FTIR spectroscopy can be used as a tool to study mixtures, it was first established whether the technique could identify biochemical alterations associated with dose and treatment exposures. For visualisation, spectra were processed using PCA-LDA to produce one-dimensional (1D) scores plots that illustrate treatment-induced separation from control along with corresponding loadings plots to show biochemical alterations responsible for the separation. Tentative wavenumber alterations were assigned using spectral interpretations from Movasaghi et al, 2008 (Movasaghi et al. 2008). Loadings plots along linear discriminant 1 (LD1) from B[*a*]P treated mallard fibroblasts (Fig. 1B and S.I. Tab. S1) showed that the top six wavenumber associated alterations induced by B[*a*]P were seen in lipid (1709 cm^{-1}), Amide I (1647 cm^{-1}; 1612 cm^{-1}), Amide II (1547 cm^{-1}; 1504 cm^{-1}) and glycogen (1026 cm^{-1}) regions. As B[*a*]P is reported to be a genotoxin and potential carcinogen, some DNA alterations were expected. The loadings show that there are peaks in the DNA region indicating that alterations are occurring there, however other alterations outweigh those in magnitude. This indicates that alterations associated with different mechanisms of toxicity, or possibly those downstream of interactions with DNA, are affecting cells to a greater extent. It has been shown that in its parent form, B[*a*]P can also cause toxicity via non-genotoxic pathways (Luch 2005). The toxicity of B[*a*]P is known to be dependent on a number of factors including cell type, as the expression of CYP isoforms is necessary to metabolise it to a pro-carcinogenic, DNA-binding form (Hockley et al. 2006). Fewer DNA alterations than expected may be seen in mallard cells due to differential expression of CYP1A1 or differences in the AhR receptor which needs to be bound in order to instigate downstream responses leading to the expression of CYP enzymes (Hirano et al. 2015). Although overall results were not significant, Western blot analysis (S.I. Tab. S7) does appear to show a reduction in CYP1A1 producton in comparison to data available from MCF-7 cells (not presented here) which may explain this. As well as genotoxic mechanisms, some

metabolic intermediates of B[*a*]P have redox cycling capabilities which can cause oxidative stress (Burchiel et al. 2007). This may explain alterations seen in lipids and proteins due to damage by ROS as well as subsequent instigation of protein and carbohydrate remodelling pathways (Hockley et al. 2006; Yan et al. 2010).

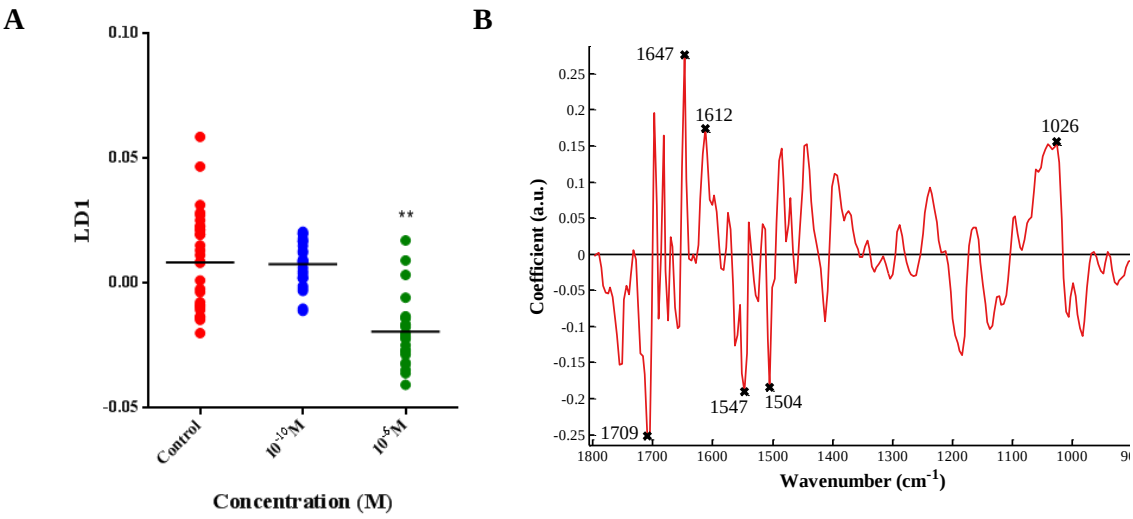

Figure 1 – PCA-LDA scores plot (**A**) and corresponding LD1 loadings plots with the top six wavenumbers highlighted (**B**) from mallard cells treated with 10^{-6} and 10^{-10} M B[*a*]P. Significance from control calculated using one-way ANOVA followed by Dunnett's post-hoc test ($P<0.05$ level (*) or $P<0.01$ level. (**)).

ATR-FTIR spectroscopy was able to detect different alteration profiles in cells exposed to PBDE congeners 47 and 209 (Fig. 2A and 2B). The six largest alterations in PBDE 47 treated mallard cells (Fig. 2A and S.I. Tab. S2) largely reflected protein biomolecule alterations including Amide I C=O stretching (1643 cm^{-1}), Amide II (1543 cm^{-1}; 1497 cm^{-1}) and methyl groups (1454 cm^{-1}) as well as some fatty acid lipid alterations (1767 cm^{-1}; 1713 cm^{-1}). Scores and loadings plots from mallard cells treated with PBDE 209 (Fig. 2B and S.I. Tab. S3) also highlight extensive protein alterations. Exposure is also associated with Amide I (1612 cm^{-1}), Amide II (1497 cm^{-1}) and Amide III (1234 cm^{-1}) changes as well as lipid (1717 cm^{-1}) and DNA (1088 cm^{-1}; 976 cm^{-1}) alterations. Although the toxicities of PBDE congeners such as 47 and 209 have been comparatively well studied in whole tissues or organisms (Viberg et al. 2004), there is less information on the underlying toxic cellular effects. It has been shown

that both these congeners, particularly PBDE 47, have neurological and developmental toxicity which is thought to be caused by contaminant-induced oxidative stress. Generation of ROS can occur even at low contaminant concentrations and lead to sublethal effects such as damage to protein secondary structure and lipids (He et al. 2009). Unlike PBDE 47, PBDE 209 also induced DNA alterations in the mallard cells. PBDE 209 is a much larger congener and has been associated with carcinogenic effects. It has been found to cause DNA damage via oxidative stress related pathways but it may also be able to induce epigenetic changes as well (Li et al. 2014; Pellacani et al. 2012).

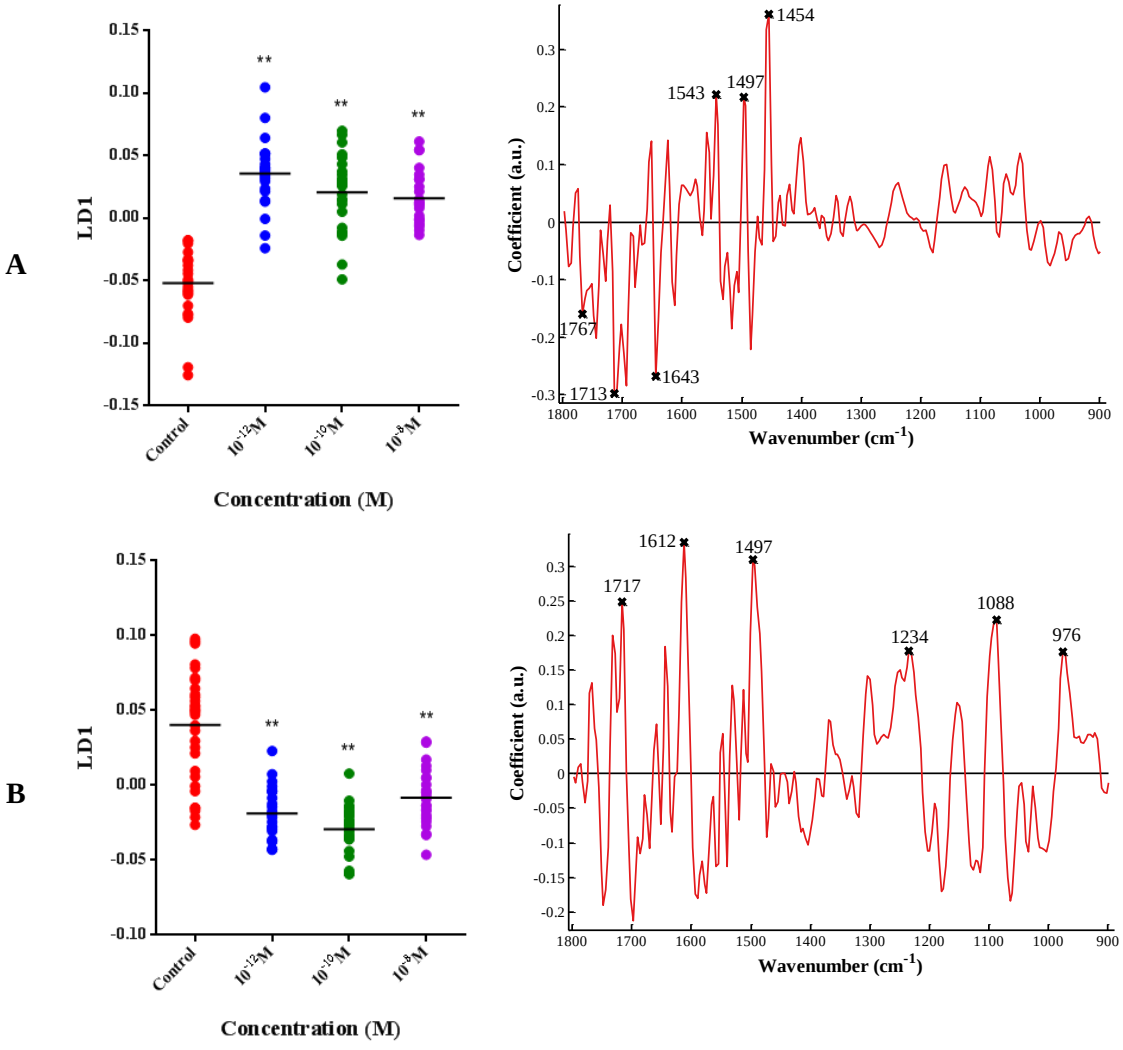

Figure 2 – PCA-LDA scores plots and corresponding LD1 loadings plots with the top six wavenumbers highlighted from mallard cells treated with PBDE 47 and PBDE 209. Significance from control calculated using one-way ANOVA followed by Dunnett's post-hoc test (P<0.05 level (*) or P<0.01 level. (**)). Mallard cells treated with (**A**) 10^{-8} M, 10^{-10} M and 10^{-12} M PBDE 47 and (**B**) 10^{-8} M, 10^{-10} M and 10^{-12} M PBDE 209.

Similar to the toxicity of PBDEs, PCBs can cause neurotoxicity, endocrine disruption and potentially carcinogenic changes depending on the congener involved. The position of chlorine molecule substitutions in each congener determines its ability to exert toxicity via AhR mediated pathways. Co-planar congeners such as PCB 126 have a much stronger affinity for the receptor than those that are planar such as PCB 153. One of each type of congener was investigated to determine whether ATR-FTIR could elucidate different mechanism of toxicity between the two. Fig. 3B (and S.I. Tab. S4) shows that in mallard cells, PCB 153 treatment was associated with alterations in lipids (1709 cm^{-1}), Amide I (1624 cm^{-1}) and Amide II (1535 cm^{-1}; 1497 cm^{-1}) as well as in regions associated with collagen (1458 cm^{-1}; 1030 cm^{-1}). Alterations in collagen related areas of the spectra were only induced by PCB 153 and were not seen in mallard fibroblasts treated with PCB 126. Previous study has shown that in fibroblast cells, PCB 153 can cause an increase in cellular levels of type I collagen (Diamond et al. 2008). This may represent an AhR- independent pathway that PCB 153 can mediate cellular effects through and that is detectable using ATR-FTIR. PCB 126 treated mallard cells (Fig. 3B and S.I. Tab. S5) showed alterations in lipids (1744 cm^{-1}; 1705 cm^{-1}), Amide I (1647 cm^{-1}; 1609 cm^{-1}), Amide II (1504 cm^{-1}) and cytosine and guanine in DNA (1369 cm^{-1}). Initially, the range of alterations induced appears quite similar but there are crucial differences between the alterations induced by the two congeners, such as collagen alterations in PCB 153 treated fibroblasts, which when supported by evidence from other studies could reveal key toxicity mechanisms. In cells treated with PCB 126, alterations in the DNA region of the spectra were more pronounced than in PCB 153 treated cells. This may be mediated by binding to the AhR and downstream gene transcription processes as PCB 126 is a co-planar congener and therefore a more potent AhR agonist. PCB 153 has a much weaker affinity for AhR binding and is hypothesised to exert toxicity via a number of other receptors (Al-Salman and Plant 2012).

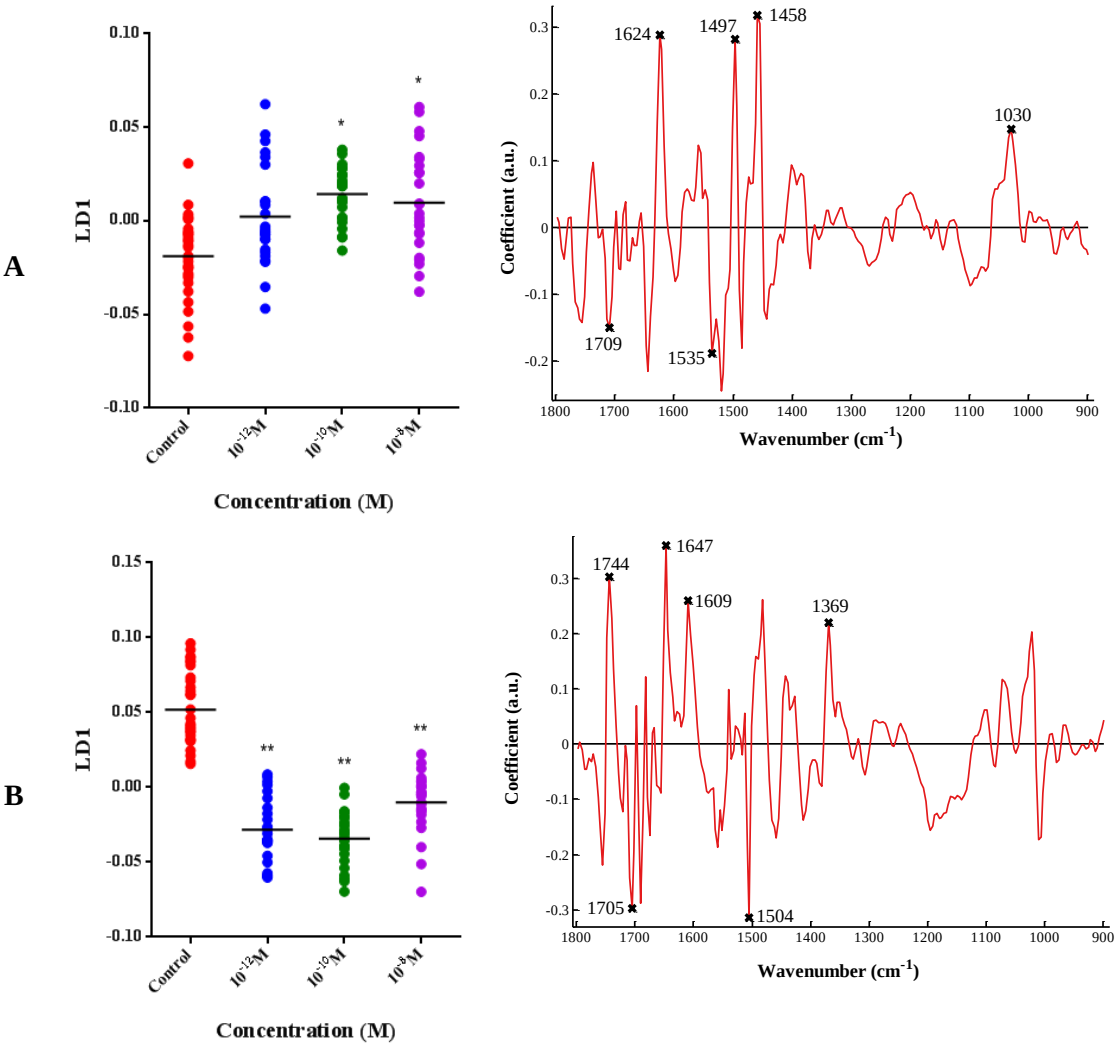

Figure 3 – PCA-LDA scores plots and corresponding LD1 loadings plots with the top six wavenumbers highlighted from mallard cells treated with PCB 153 and PCB 126. Significance from control calculated using one-way ANOVA followed by Dunnett's post-hoc test (P<0.05 level (*) or P<0.01 level. (**)). Mallard cells treated with (**A**) 10^{-8} M, 10^{-10} M and 10^{-12} M PCB 153 and (**B**) 10^{-8} M, 10^{-10} M and 10^{-12} M PCB 126.

Identification of interactions in binary mixtures

ATR-FTIR spectroscopy is a time and cost efficient technique that has been used to study the toxicity of environmental contaminants (Ahmad et al. 2008; Strong et al. 2016b) and may have potential as a tool to predict mixture toxicity. Spectral data from cells treated with individual component chemicals were added together (once background alterations were removed) to produce a 'predicted' spectrum which could be compared with the 'observed'

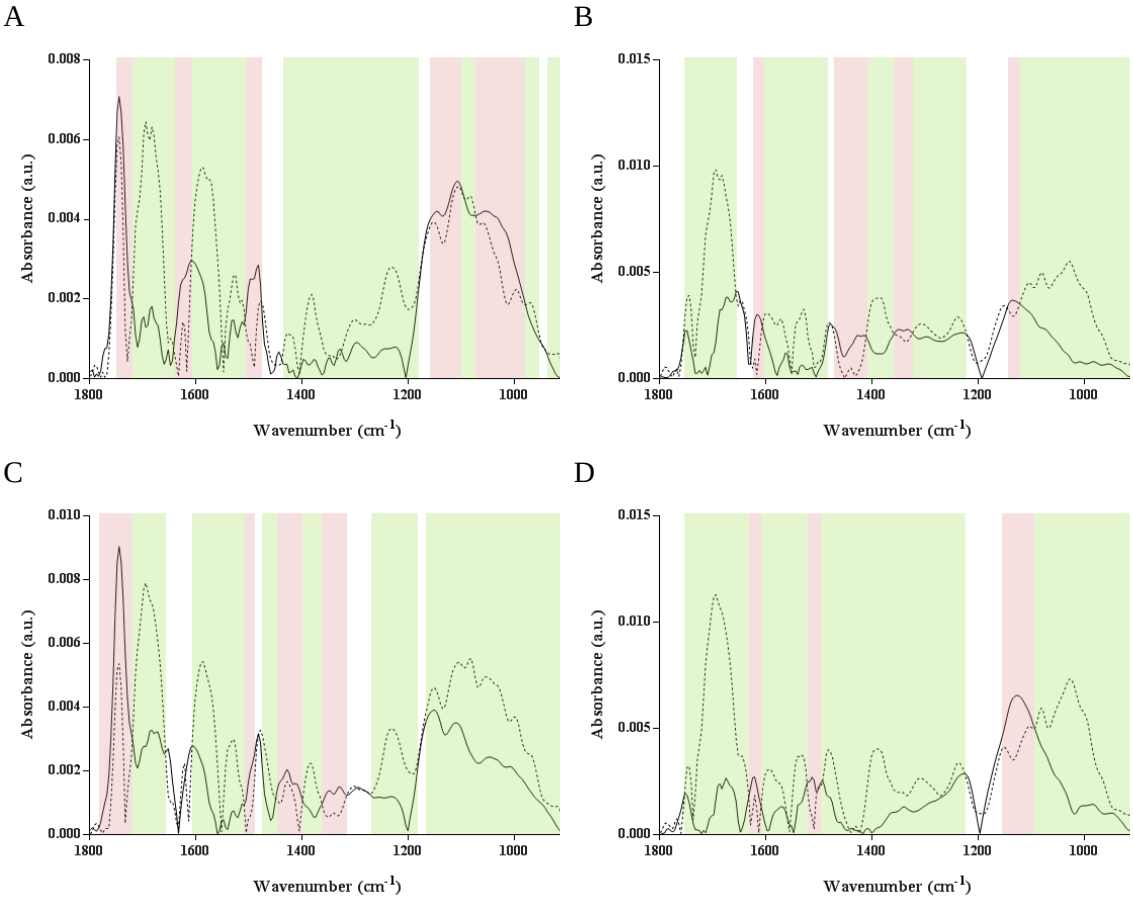

Figure 4 – Additive spectral models, showing predicted and observed spectra from Mallard cells treated with a binary mixture of B[*a*]P and PBDE 47. Predicted spectra are denoted by a dashed line and observed spectra are denoted by a solid line. Green areas represent where the observed spectrum is less than the predicted spectrum and red areas represent where the observed spectral result is greater than the predicted spectrum. (**A**) B[*a*]P 10^{-6} M and PBDE 47 10^{-8} M; (**B**) B[*a*]P 10^{-6} M and PBDE 47 10^{-12} M; (**C**) B[*a*]P 10^{-10} M and PBDE 47 10^{-8} M; (**D**) B[*a*]P 10^{-10} M and PBDE 47 10^{-12} M.

spectrum derived from cells treated with the actual binary mixture. In order to identify of areas of the spectrum where the observed and predicted spectra diverged, the plots were colour coded so that green areas represent where the observed spectrum is less than the predicted and red areas represent where the observed spectral result is greater than predicted. Theoretically, when interactions occur in a mixture, the predicted and observed spectrum will be significantly different and these areas need to be investigated as if the observed spectrum is greater than predicted, enhanced toxicity may occur (Cedergreen 2014a). As there were a number of predicted models tested, colour coding the spectra in this manner facilitates broad

identification of interactions for consideration before more detailed analysis. This allows the researcher to rapidly answer experimental questions such as in which binary mixture is an interaction most likely to be occurring, where enhanced chemical action is most likely to be occurring and which areas of the spectrum are most affected. Fig. 4, 5, 6 and 7 show plots of predicted and observed spectra for mallard cells treated with binary mixtures of B[*a*]P with PBDEs or PCBs. The observed spectrum is derived from cells treated with the actual binary mixture (denoted by a dashed line) and the predicted spectra are based on an additive prediction using cells treated with individual chemical components (denoted by a solid line).

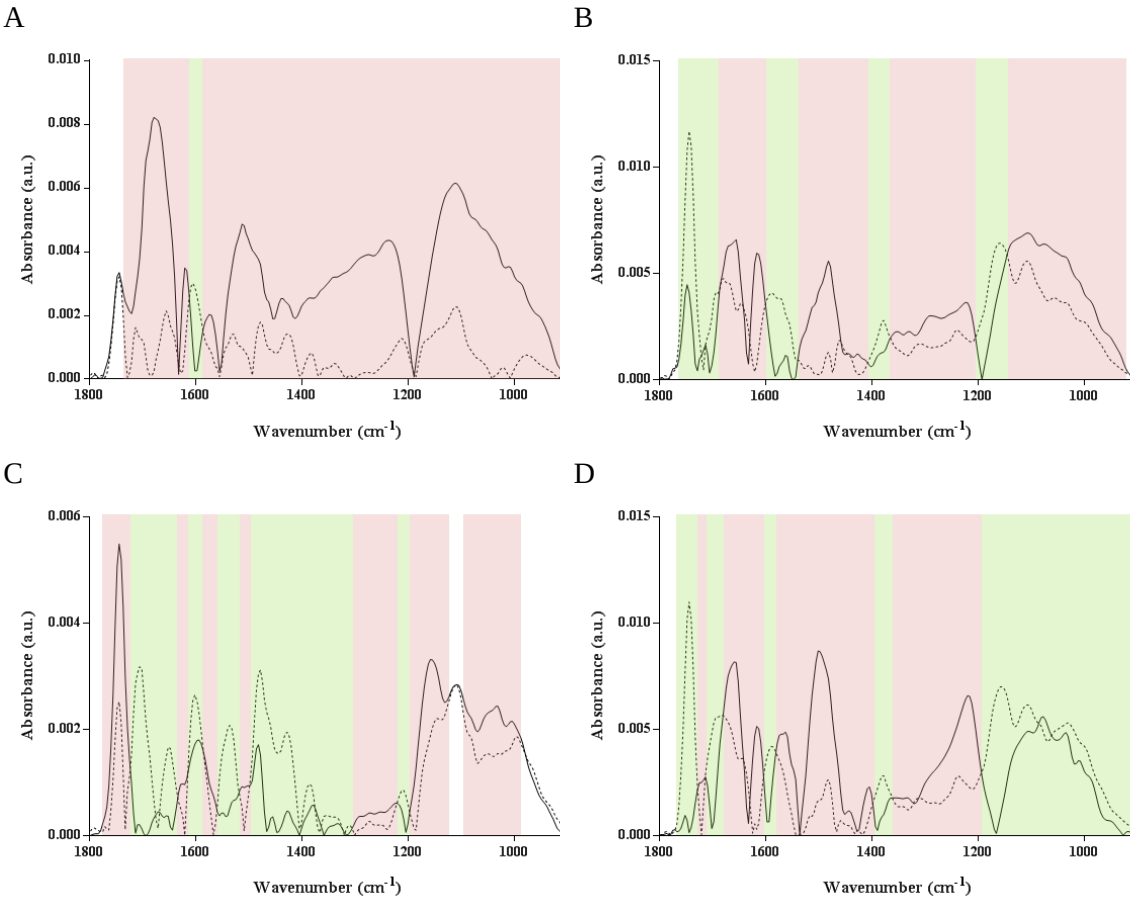

Figure 5 – Additive spectral models, showing predicted and observed spectra from Mallard cells treated with a binary mixture of B[*a*]P and PBDE 209. Predicted spectra are denoted by a dashed line and observed spectra are denoted by a solid line. Green areas represent where the observed spectrum is less than the predicted spectrum and red areas represent where the observed spectral result is greater than the predicted spectrum. (**A**) B[*a*]P 10^{-6} M and PBDE 209 10^{-8} M; (**B**) B[*a*]P 10^{-6} M and PBDE 209 10^{-12} M; (**C**) B[*a*]P 10^{-10} M and PBDE 209 10^{-8} M; (**D**) B[*a*]P 10^{-10} M and PBDE 209 10^{-12} M.

The majority of the plots showed more green or red coded areas than white areas which indicate that there is a match between the predicted and observed spectra. This appears to suggest that interactions could be occurring when cells are treated with most of the binary mixtures, however the extent of the difference between the predicted and observed spectra is likely to be an important factor.

Furthermore, spectral results can be more complex to interpret as the range of alterations measured encompasses many toxicological endpoints (Hu et al. 2016). For toxicological assessment, cases where the observed is less than the predicted are not as much of a concern as the prediction has been conservative. In the figures presented here these are denoted by green areas, where the observed absorbance of biomolecules in cells exposed to a binary mixture is less than predicted or expected. Overall, the spectra from cells treated with mixtures of B[*a*]P and PBDE 47 (Fig. 4) had the most green areas, potentially signifying that these compounds may instigate less than additive alterations when in a mixture. There were also some regions indicating less than additive alterations in spectra from cells treated with B[*a*]P and PBDE 209 (Fig. 5), mostly when exposed to 10^{-10} M B[*a*]P and 10^{-8} M PBDE 209 (Fig. 5C). However, some spectra were immediately noticeable as they had large red coded areas where the observed spectral alterations are greater than predicted. When assessing toxicity, these areas would warrant further investigation as they could represent possible biomolecular endpoints where greater than additive mixture effects may be occurring. Failure to predict and capture enhanced mixture toxicity could put the species under consideration at risk. Mallard cells exposed to mixtures of B[*a*]P and PBDE 209 (Fig. 5) had large spectral areas where greater than predicted alterations were occurring. In particular, the plot of cells treated with 10^{-6} M B[*a*]P and 10^{-8} M PBDE 209 (Fig. 5A) was more than 90 % red and the observed alterations were more than double those predicted in some areas. A similar effect was also seen in cells exposed to 10^{-6} M B[*a*]P and 10^{-8} M PCB 153 (Fig. 6A). The areas of the spectrum which were coded white, to indicate a match of observed alterations with those predicted, varied between treatments. The binary combination which resulted in the greatest

degree of match with the model was seen in cells treated with B[*a*]P and PBDE 47 (Fig. 4) as well as those treated with 10^{-6} M B[*a*]P and 10^{-12} M PCB 153 (Fig. 6B), implying that those mixtures induce spectral alterations which are closer to an additive mixture model.

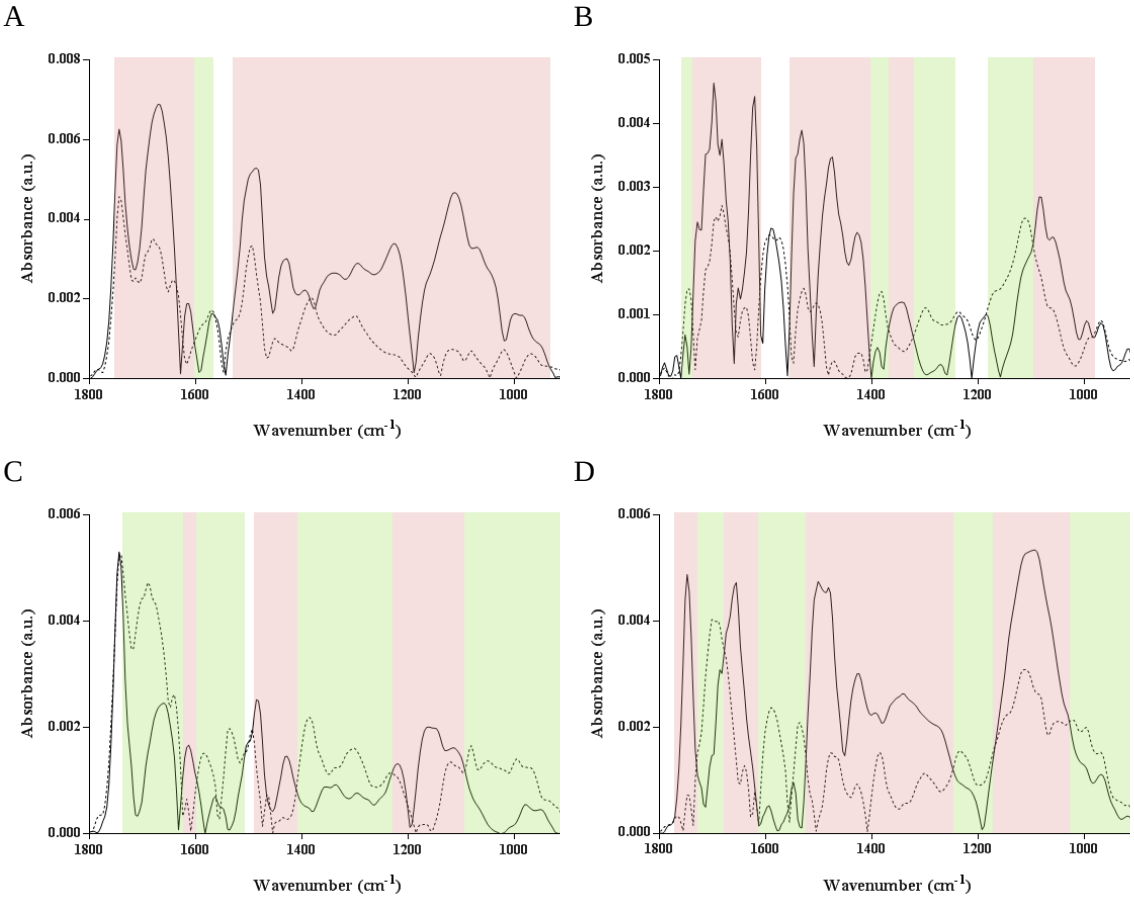

Figure 6 – Additive spectral models, showing predicted and observed spectra from Mallard cells treated with a binary mixture of B[*a*]P and PCB 153. Predicted spectra are denoted by a dashed line and observed spectra are denoted by a solid line. Green areas represent where the observed spectrum is less than the predicted spectrum and red areas represent where the observed spectral result is greater than the predicted spectrum. (**A**) B[*a*]P 10^{-6} M and PCB 153 10^{-8} M; (**B**) B[*a*]P 10^{-6} M and PCB 153 10^{-12} M; (**C**) B[*a*]P 10^{-10} M and PCB 153 10^{-8} M; (**D**) B[*a*]P 10^{-10} M and PCB 153 10^{-12} M.

Depending on the binary mixture and concentration, the biomolecules that were most affected varied which can occur as IR methods measure all biomolecules in a cell and thus all toxicological endpoints. This makes the technique more suited to broad assessment of trends between predicted and observed spectra. For example, in the ~900-1100 cm^{-1} region mixtures were most likely to generate less than additive alterations or mixture effects where the

predicted and observed spectra match well. Used in this manner, ATR-FTIR spectroscopy can provide a simple and fast tool to identify general areas of divergence between predicted and observed spectra, making it an ideal screening tool for mixture interactions. It may be used to identify mixture effect trends and direct further in-depth analysis.

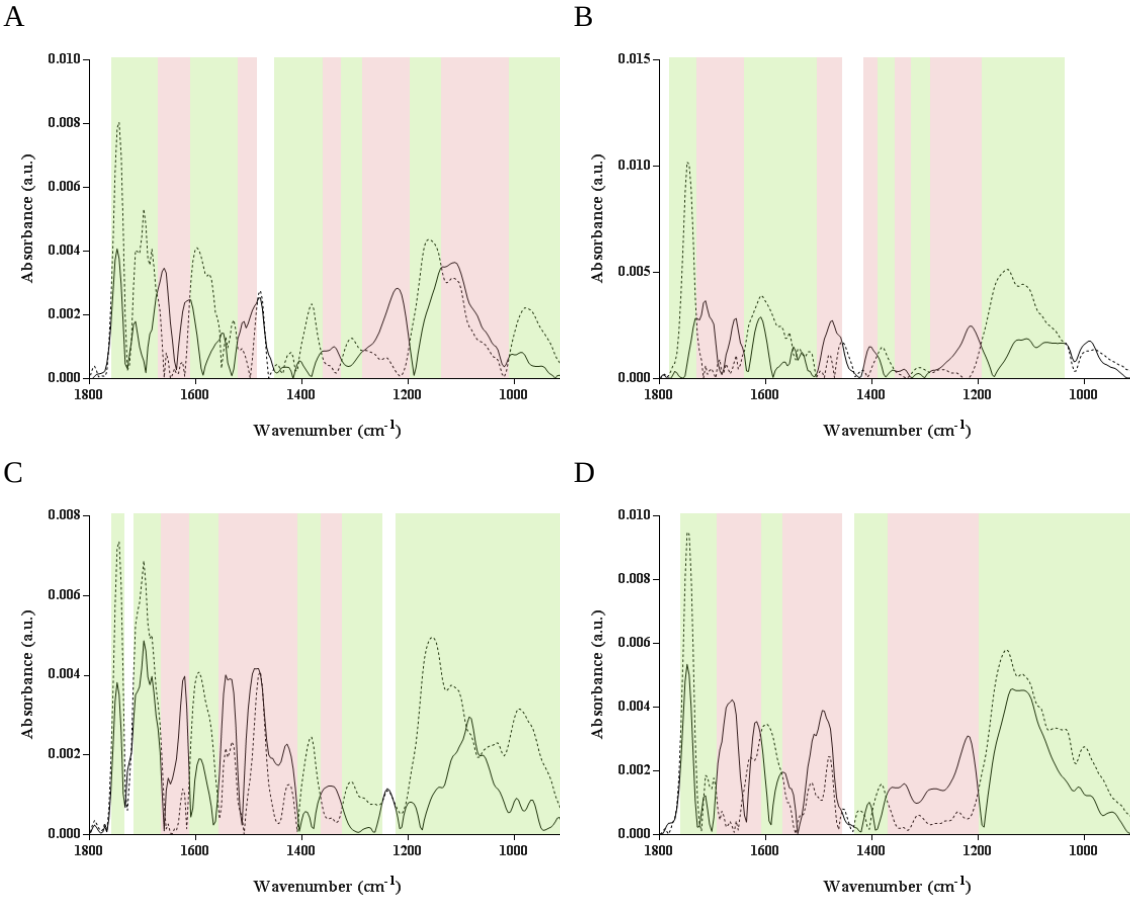

Figure 7 – Additive spectral models, showing predicted vs. observed spectra from Mallard cells treated with a binary mixture of B[*a*]P and PCB 126. Predicted spectra are denoted by a dashed line and observed spectra are denoted by a solid line. Green areas represent where the observed spectrum is less than the predicted spectrum and red areas represent where the observed spectral result is greater than the predicted spectrum. (**A**) B[*a*]P 10^{-6} M and PCB 126 10^{-8} M; (**B**) B[*a*]P 10^{-6} M and PCB 126 10^{-12} M; (**C**) B[*a*]P 10^{-10} M and PCB 126 10^{-8} M; (**D**) B[*a*]P 10^{-10} M and PCB 126 10^{-12} M.

Predicting effects of binary mixture using IR spectroscopy

An essential part of the study of mixtures is investigating if the effects of a chemical combination can be accurately predicted so that detrimental mixture toxicity can be

circumvented. As the majority of mixtures exhibit additive toxicity, regulatory assessments are commonly based on this assumption so in this study, a predictive pseudospectrum was created based on the model of additivity. The predicted and observed spectra were compared in order to understand how accurate the predictive model was as well as looking at how and why the two spectral results might differ. The use of a predictive peudospectra created from individual spectral data may be useful in reducing the scope of mixture toxicity investigations as it may not be practical to actually test all possible mixtures.

It was immediately visually evident that the majority of observed spectral alterations induced by binary mixtures of B[*a*]P and PBDE or PCB congeners did not match those predicted using component chemical data. This was also confirmed by a goodness of fit analysis (S.I. Tab. S6). In many areas, the observed alterations were greater than expected, as seen in mallard cells treated with binary mixtures of B[*a*]P and PBDE 209 (Fig. 5) where observed absorbances were actually much greater than the predicted absorbances in many spectral regions. This was particularly apparent in spectra of 10^{-6} M B[*a*]P and 10^{-8} M PBDE 209 (Fig. 5A) treated cells where the predicted and observed spectra followed the same general pattern but the observed was, in places, at least double the absorbance intensity. Spectra from other concentrations were more mixed with either observed or predicted being higher in different areas of the spectra but with no consistent alterations which might reveal a toxic mechanism. Dissimilar to cells treated with PBDE 209 containing binary mixtures, spectra from fibroblasts treated with binary mixtures of B[*a*]P and PBDE 47 (Fig. 4) revealed that across most regions of the spectrum, the observed absorbances were smaller than predicted. PBDE 209 and 47 have been reported as having many common toxicities but the main difference between the two types of PBDE-containing mixtures is that PBDE 209 is much larger and more brominated than the other congener (Darnerud et al. 2001). This physical difference could alter how the molecule interacts with targets and other chemicals and may explain differences in adherence to the additive model that can be seen between the two mixture types. Observed spectra from avian cells treated with binary mixtures, containing the highest concentration of

either PBDE congener with B[*a*]P (Fig. 4A and C and Fig. 5A and C), both showed consistent enhancement of a peak at 1750 cm^{-1} above that predicted. This is the major region associated with C=O vibrations of lipids and may denote greater-than-additive lipid damage which occurs when mallard cells are co-exposed to concentrations of 10^{-8} M PBDE congeners with B[*a*]P.

The observed spectral alterations in mallard fibroblasts treated with B[*a*]P and PCB 153 (Fig. 6) were typically greater than those predicted over most regions of the spectrum. This could be seen at all concentrations except B[*a*]P 10^{-10} M and PCB 153 10^{-8} M (Fig. 6C) when there were also quite a number of spectral areas where the observed absorbances were smaller than predicted. In those combinations that showed largely greater-than-additive observed alterations, the peaks in the ~1650-1750 cm^{-1} area were also noticeably enhanced. This was also observed in cells treated with binary mixtures of B[*a*]P with PBDE congeners. When treated with B[*a*]P and PCB 126 (Fig. 7), avian cells showed reduced observed alterations compared to binary mixtures which included PCB 153. In these mixtures the differences between the observed and predicted spectra were also smaller than those seen at in cells treated with PCB 153, possibly implying that the mixtures containing B[*a*]P and PCB 126 exhibit a closer approximation of additive toxicity. The general decrease in observed spectral alterations may be due to the enhanced AhR binding affinity of co-planar PCB 126 in comparison to the planar PCB 153 congener (Bemis et al. 2005). There may be binding competition of receptors between B[*a*]P and PCB 126 which led to a reduction in mixture toxicity. Further exploration by western blot analysis of CYP1A1 which is downstream of the receptor (S.I. Tab. S7) did show less production of CYP1A1 in mallard cells treated with binary mixtures containing PCB 126 compared to those containing PCB 153 but the result was not found to be significant. This may indicate that the overall mixture toxicity is occurring via AhR-independent mechanisms or that incorporation of all toxic endpoints across the spectrum may mask specific toxicities which need further testing for elucidation.

A number of chemical combinations were tested and the results have varied across the spectrum with less-than or more-than-additive alterations being observed compared to the result expected using predictive additive models. Only a very small proportion of the spectrum for each combination showed a good fit between the observed and predicted result. This may be caused by interactions in the mixtures indicating that an additive model is not appropriate or may be due to the scale of toxic endpoints incorporated into an IR spectrum. The AhR pathway, and induction of downstream expression of phase I and II metabolism enzymes, is known to be a common pathway involved in metabolism of the contaminants studied, some of which are reported to have AhR binding affinities. Although less-than-expected results are not concerning from a regulatory perspective as they represent less toxicity than predicted, these results also occurred in a large proportion of spectral areas. Activation of the AhR pathway and metabolism of B[*a*]P is essential for its toxicity, it may be that the presence of other PCB or PBDE contaminants shifts the pathway towards detoxification so that more B[*a*]P is fully detoxified than converted to the procarcinogen form. This has been observed in cells exposed to mixtures of B[*a*]P as well as other PAHs (Courter et al. 2007) (Tarantini et al. 2011). If IR spectroscopy was used as a predictive tool as described here, the mixtures that display greater than expected alterations according to an additive model would be those that represent the most risk to environmental organisms. The combinations that lead to the most greater-than-expected effects are seen in mallard cells treated with binary mixtures including B[*a*]P with PBDE 209 (Fig. 5) or PCB 153 (Fig. 6) and deviations in the region around ~1650-1750 cm^{-1} are notable. As a greater-than-additive effect in this area of the spectrum was induced by many of the combinations tested, it may represent a common mechanism for environmental binary mixtures of B[*a*]P with PBDEs or PCBs, which can lead to enhancement of toxicity. Further testing is needed to explore this effect but as a possible explanation, B[*a*]P, PBDEs and PCBs are able to cause lipid damage via ROS production which may be a means for the enhanced lipid alterations observed.

Conclusions

Evidence of potential greater than predicted alterations to some biomolecules represents a cause for further investigation, particularly as these effects were observed in mallard fibroblast cells, a species commonly found in the environment. The results also suggest that the effects of binary mixtures composed of B[*a*]P with PCBs or PBDEs are contaminant and dose dependant. This lends evidence for the rationale that all possible mixtures need to be considered during regulatory decisions as interactions between components or at biological target sites can lead to deviations from the additive model. Specific toxicology testing of mixtures on this scale would be daunting but we have shown that a panel of binary mixtures, composed of various chemicals at difference concentrations, can be studied in a high-throughput manner using ATR-FTIR spectroscopy. Further testing is needed to understand why so much of the observed spectral alterations deviate away from the predictive additive model but IR spectroscopy is a unique approach which can study the effects of binary mixtures at the biomolecular level. It may have application as a tool to screen chemical mixture induced alterations for non-conformance to additivity and to direct further toxicology testing. This would be particularly effective when paired with colour coding of the spectra to indicate where deviation from the additive model and possible interaction occurs.

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Supplementary Information:

Wavenumber (cm^{-1})	Assignment
1709	Lipids
1647	Amide I
1612	Amide I
1547	Amide II
1504	Amide II (CH Bending)
1026	Carbohydrate (Glycogen)

Table S1 – Top six tentative wavenumber assignments derived from PCA-LDA LD1 loadings plots for mallard cells treated with 10^{-6} M and 10^{-10} M B[*a*]P.

Wavenumber (cm^{-1})	Assignment
1767	Lipids (Fatty Acids)
1713	Lipids (Fatty Acids)
1643	Amide I (C=O Stretching)
1543	Amide II
1497	Amide II (C=C Vibration)
1454	Methyl group vibration of protein

Table S2 – Top six tentative wavenumber assignments derived from PCA-LDA LD1 loadings plots from MCF-7 and mallard cells treated with 10^{-8} M, 10^{-10} M and 10^{-12} M PBDE 47.

Wavenumber (cm^{-1})	Assignment
1717	Lipids
1612	Amide I
1497	Amide II (C=C Vibration)
1234	Amide III
1088	Symmetric Phosphate (DNA)
976	Symmetric Phosphate (DNA)

Table S3 – Top six tentative wavenumber assignments derived from PCA-LDA LD1 loadings plots from mallard cells treated with 10^{-8} M, 10^{-10} M and 10^{-12} M PBDE 209.

Wavenumber (cm^{-1})	Assignment
1709	Lipids
1624	Amide I
1535	Amide II (C=N Stretching)
1497	Amide II (C=C Vibration)
1458	CH3 group of collagen
1030	Collagen

Table S4 – Top six tentative wavenumber assignments derived from PCA-LDA LD1 loadings plots from mallard cells treated with 10^{-8} M, 10^{-10} M and 10^{-12} M PCB 153.

Wavenumber (cm^{-1})	Assignment
1744	Lipids (C=O stretching)
1705	Lipids (Fatty acids)
1647	Amide I
1609	Amide I
1504	Amide II (CH Bending)
1369	C-N stretching in guanine and cytosine

Table S5 – Top six tentative wavenumber assignments derived from PCA-LDA LD1 loadings plots from mallard cells treated with 10^{-8} M, 10^{-10} M and 10^{-12} M PCB 126.

Mixture	R^2	$P < 0.01$
B[*a*]P 10^{-10} M + PBDE 209 10^{-8} M	0.257	Yes
B[*a*]P 10^{-10} M + PBDE 209 10^{-12} M	0.041	Yes
B[*a*]P 10^{-10} M + PBDE 47 10^{-8} M	0.286	Yes
B[*a*]P 10^{-10} M + PBDE 47 10^{-12} M	0.090	Yes
B[*a*]P 10^{-10} M + PCB 126 10^{-8} M	0.221	Yes
B[*a*]P 10^{-10} M + PCB 126 10^{-12} M	0.374	Yes
B[*a*]P 10^{-10} M + PCB 153 10^{-8} M	0.228	Yes
B[*a*]P 10^{-10} M + PCB 153 10^{-12} M	0.083	Yes
B[*a*]P 10^{-6} M + PBDE 209 10^{-8} M	0.046	Yes
B[*a*]P 10^{-6} M + PBDE 209 10^{-12} M	0.225	Yes
B[*a*]P 10^{-6} M + PBDE 47 10^{-8} M	0.369	Yes
B[*a*]P 10^{-6} M + PBDE 47 10^{-12} M	0.149	Yes
B[*a*]P 10^{-6} M + PCB 126 10^{-8} M	0.174	Yes
B[*a*]P 10^{-6} M + PCB 126 10^{-12} M	0.017	No
B[*a*]P 10^{-6} M + PCB 153 10^{-8} M	0.453	Yes
B[*a*]P 10^{-6} M + PCB 153 10^{-12} M	0.079	Yes

Table S6 – Analysis of goodness of fit between predicted and observed binary mixture spectra, for mallard cells, as determined by Pearson's correlation coefficient and accompanying *P* value.

Mixture	MCF-7	Avian
Control	1.000	1.000
B[*a*]P 10^{-6} M	1.555	0.548
PBDE 209 10^{-8} M	1.903	0.883
PBDE 47 10^{-8} M	8.601	0.932
PCB 126 10^{-8} M	2.313	0.389
PCB 153 10^{-8} M	1.783	0.442
B[*a*]P 10^{-6} M + PBDE 209 10^{-8} M	1.673	0.292
B[*a*]P 10^{-6} M + PBDE 47 10^{-8} M	2.109	0.439
B[*a*]P 10^{-6} M + PCB 126 10^{-8} M	1.483	0.365
B[*a*]P 10^{-6} M + PCB 153 10^{-8} M	1.576	0.370

Table S7 – CYP1A1 protein band intensity as assessed by western blot in MCF-7 and Mallard cells, as induced by treatment with binary mixtures of 10^{-6} M B[*a*]P with 10^{-8} M PBDE 47, PBDE 99, PBDE 153, PCB 153 or single treatments of these. Intensity expressed relative to expression in control to give a 'fold' increase or decrease and adjusted to background and the β-actin loading control. A value of more than 1 indicates an increase in production and a value of less than 1 indicates a decrease.

Chapter 7. General Discussion

Of all the types of bird, predatory bird species are particularly vulnerable to the presence of contaminants in their environment. Numerous studies have shown the detrimental consequences that this can have on population numbers, the most well-known examples being the reduced reproduction rate of many species caused by DDT-mediated eggshell thinning (Blus et al. 1971; Lincer 1975) and the lethal toxicity of cyclodeine insecticides to peregrine falcons and sparrowhawks (Newton 1988; Sibly et al. 2000). These two examples clearly demonstrate the need to monitor environmental contaminants which can have critical effects on predatory bird populations. Predatory birds are especially sensitive to compounds that accumulate in fat tissues and have long half-lives such as OC pesticides and PCBs. These compounds can bioaccumulate upwards through food chains and the most damaging toxicities are often seen in apex predators such as predatory birds (Armitage and Gobas 2007). These species of birds are also relatively long-lived which adds a greater temporal factor into contaminant accumulation. The features which make them vulnerable to contaminants also make predatory birds ideal sentinels in which to monitor and study environmental pollution. Predatory birds have multiple, essential roles in ecosystems including trophic processing and regulatory functions and protection at this level, although beneficial to the birds themselves, also confers protection to other organisms in the food chain or web (Sekercioglu 2006).

The recognition of predatory birds as sentinel species has led to the development of multiple schemes that use them to monitor environmental contaminants in a number of ways including using tissue, egg and feather samples (Walker et al. 2008). Collection of the samples is often paired with analytical chemistry techniques which determine absolute concentrations of contaminants in the samples. These techniques are essential in many experimental and monitoring circumstances but may also be expensive, time-consuming and using many reagents. Additionally, there are far fewer studies which focus on specific toxicological endpoints such as genotoxicity or enzyme induction. Therefore alternative technologies such as vibrational spectroscopy techniques are being implemented more commonly as although

they do not necessarily provide absolute chemical values, they allow quick, cost-efficient and non-destructive analysis of samples (Baker et al. 2014a). Although vibrational spectroscopic technologies such as ATR-FTIR and Raman spectroscopy are fairly well utilised in laboratory and clinical biomedical settings (Gajjar et al. 2013a; Wang and Mizaikoff 2008; Wolf et al. 2007), they are comparatively new to environmental studies but can be highly useful as they are able to detect subtle changes in composition at the biomolecular level. By measuring absorbance of IR light by biomolecules in a sample such as lipids, proteins, carbohydrates and nucleic acids, a profile of lethal or sublethal alterations induced by environmental contaminants can be developed and potentially biomarkers of exposure can be identified (Kelly et al. 2011). The measured spectrum simultaneously incorporates all cellular biomolecules and many toxicological endpoints thus providing a complete metabolic profile of the sample under investigation (Ellis and Goodacre 2006). Vibrational spectroscopy has been used to monitor and investigate potentially harmful environmental chemicals in species such as fish, amphibians, mammals and even micro-organisms (Ahmed and Focht 1973; Malins et al. 2004; Strong et al. 2016a) but currently, few studies have applied this technology to ecotoxicity studies using predatory birds (Llabjani et al. 2012). Such studies are often limited by the accessibility of samples due to the protected status of many predatory bird species or lack of availability of post-mortem tissue, so using a non-destructive technique which allows samples to be re-used is highly desirable.

The overall aim of this work was to investigate the use of vibrational spectroscopy techniques with multivariate analysis as a novel tool to analyse the effects of environmental contaminants in predatory birds. In order to achieve this, predatory bird body tissues and avian cells, both untreated and those exposed to common environmental contaminants, were analysed. In this section of the thesis, the overall findings from each experimental chapter are discussed as well as how this fits into our framework of current knowledge on vibrational spectroscopy as a tool to investigate environmental pollution, particularly in predatory birds. A flowchart of general experimental questions asked throughout the thesis is presented in Fig. 1.

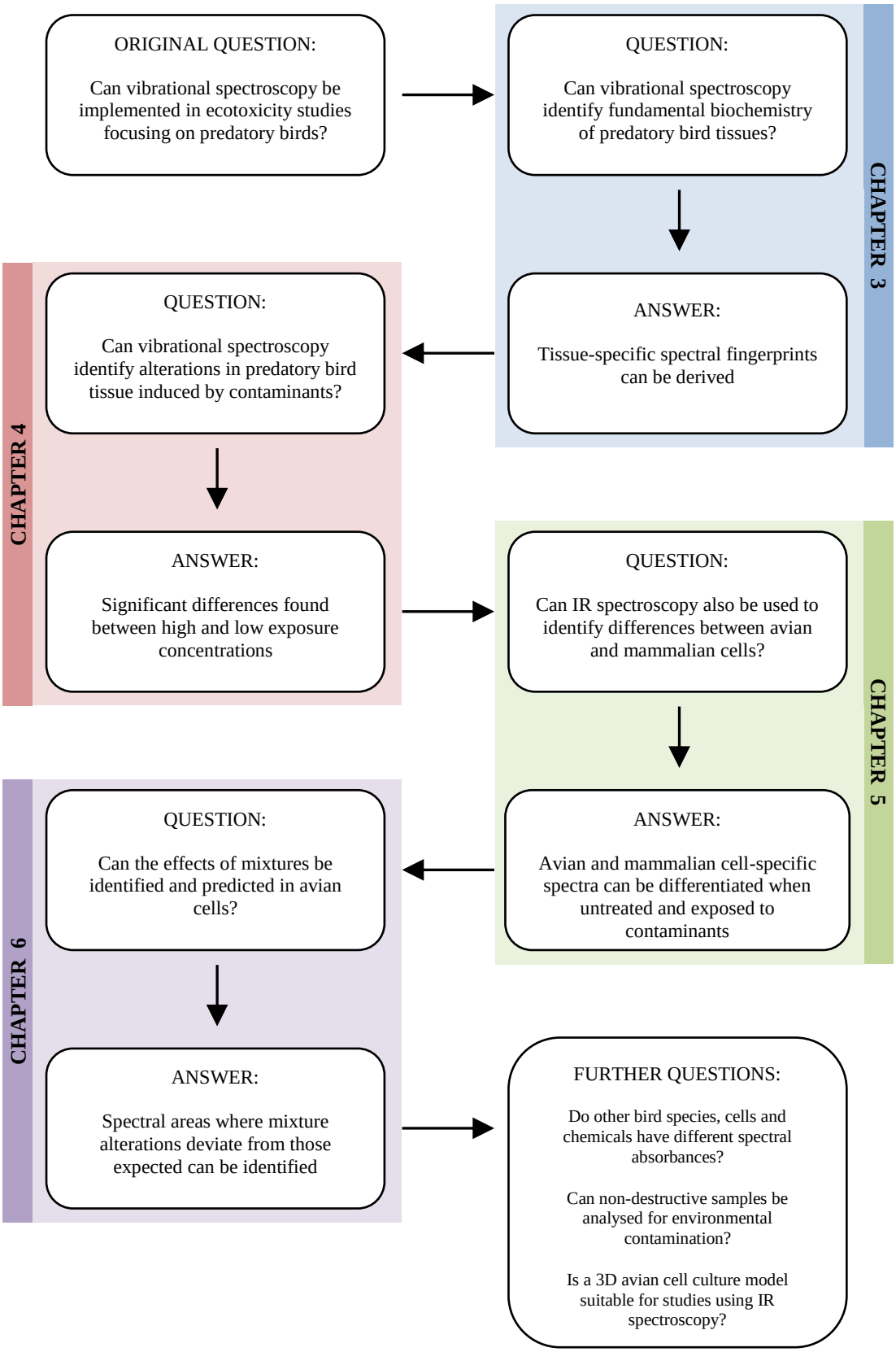

Figure 1 – Flowchart of general experimental questions asked in throughout the thesis chapters.

7.1 Identifying the underlying biochemistry of predatory bird tissues

To our knowledge, vibrational spectroscopy had not been used to analyse the body tissues and organs of predatory birds or to study them in the context of environmental exposure effects. Therefore, as presented in Chapter 3, a baseline study analysing untreated predatory bird tissues was performed, particularly as vibrational spectroscopy is also an emerging technology and not yet fully validated in the ecotoxicology field. The main aim of this study was to determine the fundamental biochemical and biomolecular composition of the tissues using ATR-FTIR and Raman spectroscopy. This is an area that has historically received little focus but the underlying composition and structure of tissues can be partly responsible for the differential effects of contaminants on various body tissues and should not be neglected when considering the toxicity of compounds. Furthermore, as there is little data available on the application of vibrational spectroscopy to the analysis of such tissues in any species, the baseline study had the benefit of verifying that the techniques are sensitive enough to derive tissue-specific spectral profiles. This is important for the wider field as validation of the techniques is needed for them to be successfully implemented in environmental research.

Fixed tissues from captive-bred American kestrels (*Falco sparverius*), which had not be subject to any chemical treatment, were analysed using ATR-FTIR and Raman spectroscopy with multivariate analysis. In particular, second derivatives of spectra from tissues were analysed as this method resolves overlapping bands allowing full identification of biomolecular composition (Staniszewska et al. 2014). The results demonstrated that in the fingerprint region, the measured absorbances were largely due to macromolecules such as proteins and lipids. However, examination of peak assignments from the second derivative spectra revealed a number of tissue specific peaks that were related to structure, function or metabolism of the particular tissue. The spectra from lipid-rich brain contained larger lipid absorbances, muscle tissues had peaks related to collagen in connective tissue, the liver which is known to be the body's glycogen storage organ was characterised by multiple glycogen assignments and the gonads were distinguished by increased absorbances related to nucleic acids from genetic material. These results agree with what we know about the biology of these

tissues (Berg 2002). This clearly demonstrates that the two spectroscopy techniques are able to identify tissue-specific spectral absorbances related to the underlying biochemistry which distinguish the tissues. Furthermore, sex-related tissue differences were also identified by ATR-FTIR and Raman spectroscopy. Significant differences were found between male and female brain, heart, kidney, skeletal muscle and gonad tissues. The ability to distinguish sex-specific spectral variation is valuable in ecotoxicity studies as the sex of the bird is a factor which can influence the fate and toxicity of environmental contaminants (Costantini et al. 2014; Robinson et al. 2012).

The results demonstrate that tissue specific spectral profiles and sex-specific alterations can be elucidated using vibrational spectroscopy in predatory birds. Studies such as this are key to our understanding of how tissue biochemistry can present in spectral results and how tissue composition can influence how an organism is affected by a contaminant. However, this was only investigated in one species of predatory bird and analysis of more species may be necessary to confirm that vibrational spectroscopy can obtain tissue-specific spectra from other predatory birds.

7.2 Investigating the effects of OC pesticide exposure on predatory bird brains

In the environment, predatory birds are exposed to many contaminants with a variety of toxic mechanisms. Although laboratory studies aim to replicate such exposures, the analysis of samples from free-flying birds often best reflects 'real' exposure concentrations and effects. With this in mind, brain samples from 58 wild sparrowhawks (*Accipiter nisus*), which had previously been analysed for liver concentrations of OC pesticide contaminants, were further investigated using vibrational spectroscopy. By coupling chemical and spectral data, the main aims of this study were to determine the spectral alterations induced in brain tissue by exposure to high and low concentrations of OC pesticides, including those influenced by sex and age of the bird. It also aimed to investigate whether exposure to OC pesticides is

associated with aggregation of cerebral amyloid by using spectroscopy, staining and immunoassay techniques.

The results, presented in Chapter 4, showed that the effects of OC pesticide exposures were largely seen in spectral alterations associated with lipids and the secondary structure of proteins. The effects were also found to be dependent on the sex and the age of the bird. In order to investigate the possible aggregation of amyloid caused by OC exposures, the secondary structure of proteins within the brain samples was further investigated. It was found that a shift of protein conformation from α-helix to β-sheet occurred in brains exposed to OC pesticide concentrations higher than 7.18 μg/g. A protein secondary structure shift of this manner is seen in the amyloid protein when it aggregates (Ding et al. 2003) which may indicate that cerebral amyloid aggregation is, at least partially, influenced by exposure to OC pesticides.

This study is novel in a number of respects as it is the first to spectroscopically analyse brain samples from wild predatory birds and to try to relate the spectral results with chemical data from the same birds. The interpretation of the results has important implications not only for the birds but also for humans. In birds, OC chemicals are known to have a number of toxic mechanisms (Fry 1995; Ratcliffe 1970) and although cerebral effects have not been well studied, if OCs impair brain function it could particularly impact predatory birds which utilize specialised regions for prey hunting behaviours (Garamszegi et al. 2002). However in humans, these compounds have been directly linked to the development of neurodegenerative diseases such as Alzheimer's disease (AD) (Singh et al. 2013), possibly due to oxidative stress-mediated aggregation of proteins like amyloid (Cannon and Greenamyre 2011). As long-lived apex predators, predatory birds represent sentinel species which occupy a trophic niche similar to humans and recent research has also shown that avian brains are not as different from human brains as previously thought (Clayton and Emery 2015). Therefore the results of this study are not only essential for our understanding of how OC pesticides can

affect predatory birds but also may extend our knowledge of how these environmental contaminants are involved in AD development. However, as the tissues in this study were obtained from wild birds found dead, the brain samples could not be fixed in their original state. This study could be strengthened by full histological staining of brain sections which may allow localisation of potential amyloid aggregations.

7.3 Characterising an avian cell line using biospectroscopy

Although the use of tissues from avian laboratory models or wild birds found dead in the field are often considered the best matrices in which to study toxicology, post-mortem tissue may not be readily available and as defined by the NC3Rs, we must aim to reduce the number of vertebrates used in scientific procedures (Kilkenny et al. 2010). Due to this, many toxicology studies now attempt to analyse chemicals using cell-based assays and the choice of cell line is a critical experimental factor. This can be particularly important in environmental studies as commonly used laboratory cell lines may be physiologically and biochemically diverse from cells and organisms found in the environment. Predatory birds are known to suffer detrimental effects from exposure to some contaminants and have distinct biology from organisms in other taxa and yet few studies have used avian cell lines (Allen et al. 2005).

Avian dermal fibroblasts and MCF-7 cells, from a commonly used laboratory cell line, were analysed using ATR-FTIR spectroscopy. Cells were analysed when not exposed to any treatment and also after exposure to single agents. The overall aims were firstly to establish whether IR spectroscopy could discriminate between the two cell types and to identify any structural or compositional biochemical differences between the cells which would warrant investigation of avian cell lines in toxicity studies. As presented in Chapter 5, ATR-FTIR spectroscopy with multivariate analysis was able to discriminate between the cell types with a classification rate of 100%. This confirms that, as demonstrated in other species, IR spectroscopy is a sensitive technique for *in vitro* studies (Ahmadzai et al. 2015; Gorrochategui et al. 2016). The results also showed that most of the spectral peaks in common between the

two cell types when untreated were associated with cellular macromolecules such as proteins. However, spectra also showed cell-specific absorbances; Mallard cell spectra contained pronounced lipid peaks whereas MCF-7 cell spectra had larger absorbances associated with DNA and nucleic acid content. These differences were sites of cell-specific spectral alterations when cells were exposed to PCB and PBDE contaminants. The findings from this study are important for the use of biologically appropriate cell lines in experimental settings and to prevent erroneous extrapolation of results from such experiments. Biochemical differences may influence species susceptibility to the effects of environmental exposures and these variations should be taken into account when cell-based assays are used (Riss et al. 2005).

7.4 Determining and predicting the effect of binary mixtures in cells

In many studies, compounds are examined in isolation but in the environment organisms are exposed to multiple contaminants at the same time. This is important for understanding how a chemical will exert toxicity in the wider environment as depending on interactions in a mixture, the toxicity can be greater or less than expected (Backhaus and Faust 2012). Thus, a large part of ecotoxicity work focuses on predicting the effect of chemical mixtures in order to prevent unexpected, enhanced mixture toxicity which may be harmful to humans and wildlife (Backhaus et al. 2003; Belden et al. 2007). As established in Chapter 5, ATR-FTIR spectroscopy is a sensitive technique for analysing cell samples and so the study presented in Chapter 6 aimed to determine whether the effects of single agents and binary mixtures of common environmental contaminants could be characterised using this technique. Further to this, it aimed to assess whether the technique could be used as a rapid method to identify when interactions in a mixture occur and if the spectral effects of a chemical mixture could be predicted using spectral data from component compounds. In order to achieve this, mallard cells were exposed to individual B[a]P, PCB or PBDE agents or to binary mixture of B[a]P with a PCB or PBDE congener.

The results indicated that ATR-FTIR spectroscopy could identify differential spectral alterations induced by single and combined exposures. Spectra of cells treated with actual binary mixtures were compared to predictive pseudospectra, created by summing spectral absorbances from cells treated with component chemicals. This demonstrated the use of IR spectroscopy as a rapid screening tool to identify when binary mixtures induce alterations which deviate from those expected using the additive mixture toxicity model. Although many spectral areas were identified as showing alterations that were less than predicted, some areas did exhibit greater than expected alterations. This was seen particularly in lipid spectral regions, possibly due to enhanced cellular oxidative stress induced by the binary mixtures. The additive model employed here is also used in regulatory frameworks to estimate how toxic a contaminant will be when included in mixtures in the environment and thus to inform usage policies (Safe 1998). Therefore, areas of greater than additive alterations represent a significant concern that mixture toxicity may be underestimated and environmental organisms put at risk (Laetz et al. 2009). This emphasises the need for methodologies and tools to accurately predict the toxicity of environmental contaminants when in mixtures. However, it is possible that the amount of mixtures that would need to be tested may become impractical and so IR spectroscopy methods may be useful to efficiently screen panels of chemical mixtures for interactions. Use in this way, deviations from the additive model can be rapidly identified and direct further toxicology testing. Although this would need to be verified using different binary combinations to determine if it works for all types of agent mixtures, it represents an exciting potential application of this technique. Biospectroscopy approaches such as ATR-FTIR spectroscopy are also beneficial as they incorporate many toxicological endpoints within the measured spectrum so an integrated cell metabolism fingerprint can be acquired.

7.5 General conclusions and future work

Vibrational spectroscopy is a highly useful technique which can be used to obtain information on the biomolecular composition and structure of many biological samples. Although the

implementation of this technique is fairly new in the field of ecotoxicology, it has great potential as it can detect such small alterations in samples. This thesis has demonstrated that ATR-FTIR and Raman spectroscopy are powerful and valuable tools for detecting subtle differences in predatory bird tissues and avian cells when paired with multivariate analysis. Vibrational spectroscopy has been shown to have a diverse array of applications including spectral profiling of tissue biochemistry, investigation of exposure to contaminants at environmental concentrations, discrimination of cell types and prediction of non-additive mixture effects.

As described, the use of vibrational spectroscopy in environmental research is somewhat in its infancy and so this thesis aims to provide a foundation for investigation of ecotoxicity in predatory birds using IR spectroscopy. When considering the conclusions from the experiments presented here, the limitations of the studies must also be taken into account and used to guide work that should follow on from this thesis. For example, in Chapter 4, the tissue alterations induced by only one type of environmental contaminant and in one tissue type were spectrally analysed. To provide more conclusive results, other tissues exposed to OC pesticides should be analysed to determine if exposure induces similar alterations. Additionally, tissues from birds exposed to other contaminants such as PBDEs and PAHs should be spectrally analysed to build up a profile of contaminant-induced alterations. In such experiments, untreated control tissue should be obtained if possible to strengthen the interpretation of results. The tissues analysed here, using vibrational spectroscopy, were from birds which were found dead in the field or were control birds which were sacrificed for experimental use. The lack of abundant post-mortem tissue and the aim to reduce the number of animals used in such experiments necessitates the use of other samples types. Samples from wild birds may provide the most environmentally realistic exposure profiles and given the protected, and in some cases endangered, status of predatory birds, the development of protocols using non-destructive samples would be greatly beneficial (Eulaers et al. 2011b; Van den Steen et al. 2009). Non-destructive samples such as feathers, abandoned or addled

eggs and preen oil should be investigated to determine if they are suitable samples for ecotoxic studies which employ IR spectroscopy.

In a similar manner, an avian cell line was analysed with the aim that it may provide an environmentally relevant assay of contaminant exposure which does not require the use of whole birds. ATR-FTIR spectroscopy was able to detect cell-specific differences demonstrating that it has potential but it cannot be denied that there are problems extrapolating the results of cell experiments to the whole organism (Schirmer 2006; Yoon et al. 2012). For this reason, the development of an avian 3D culture system may improve the interpretation of results by providing a model that is biologically closer to the whole bird (Mazzoleni et al. 2009). This may be particularly useful using cell lines such as the mallard dermal fibroblasts as skin represents an important chemical exposure route and has been successfully replicated in human cell cultures (Götz et al. 2012). The development of cell culture from other avian tissues would also be useful in order to model the exposure and fate of other contaminants. Although the closest cell line to predatory bird cells was utilised, there may be fundamental differences between mallard and predatory bird cells. Therefore, if the results of cell experiments are to be directly extrapolated to them, predatory bird cells should be analysed and compared to the mallard fibroblasts using IR spectroscopy. This would determine if there are significant biochemical differences that should be taken into account when interpreting results or whether culturing a predatory bird cell line may be advantageous.

On a broader scale, application of vibrational spectroscopy to the study of predatory birds does not have to be limited to contaminant exposure. As pioneered by the use of IR spectroscopy in human biomedicine (Choo-Smith et al. 2002), with development these techniques could be used to identify biomarkers of disease which can threaten wild predatory bird populations. This would also be advanced by the use of non-destructive samples such as feathers.

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Appendices

Appendix 1. Mid-infrared spectroscopic assessment of nanotoxicity in Gram-negative vs. Gram-positive bacteria

Kelly A. Heys, Matthew J. Riding, Rebecca J. Strong, Richard F. Shore, M. Glória Pereira, Kevin C. Jones, Kirk T. Semple and Francis L. Martin.

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Contribution:

- Experiments were conducted by Matthew J. Riding.
- Data was acquired data from samples by Matthew J. Riding.
- I performed the computational and statistical analysis.
- I prepared the first draft of the manuscript.

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Kelly A. Heys Prof. Francis L. Martin

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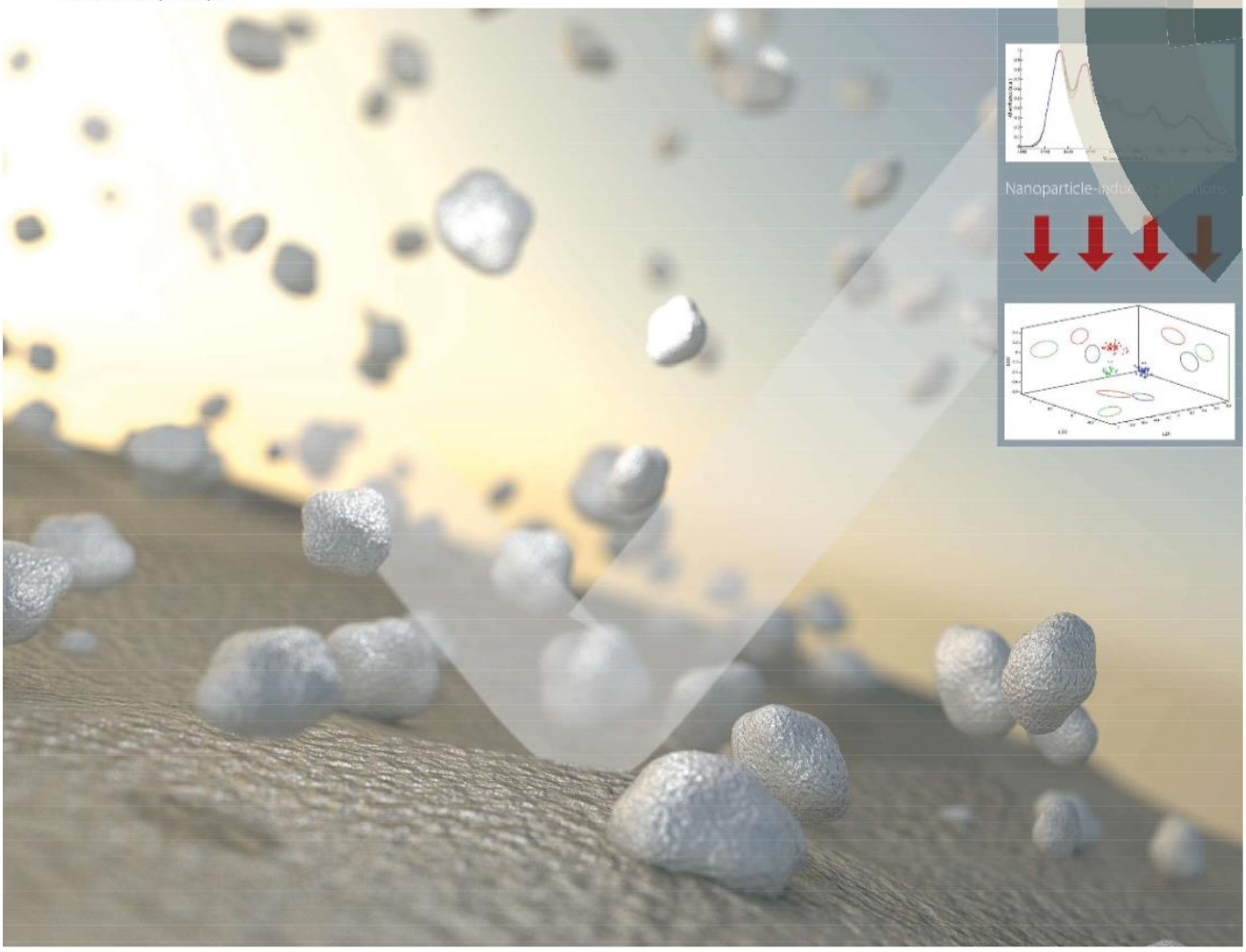

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Mid-infrared spectroscopic assessment of nanotoxicity in Gram-negative *vs.* Gram-positive bacteria†

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Nanoparticles appear to induce toxic effects through a variety of mechanisms including generation of reactive oxygen species (ROS), physical contact with the cell membrane and indirect catalysis due to remnants from manufacture. The development and subsequent increasing usage of nanomaterials has highlighted a growing need to characterize and assess the toxicity of nanoparticles, particularly those that may have detrimental health effects such as carbon-based nanomaterials (CBNs). Due to interactions of nanoparticles with some reagents, many traditional toxicity tests are unsuitable for use with CBNs. Infrared (IR) spectroscopy is a non-destructive, high throughput technique, which is unhindered by such problems. We explored the application of IR spectroscopy to investigate the effects of CBNs on Gram-negative (*Pseudomonas fluorescens*) and Gram-positive (*Mycobacterium vanbaalenii* PYR-1) bacteria. Two types of IR spectroscopy were compared: attenuated total reflection Fourier-transform infrared (ATR-FTIR) and synchrotron radiation-based FTIR (SR-FTIR) spectroscopy. This showed that Gram-positive and Gram-negative bacteria exhibit differing alterations when exposed to CBNs. Gram-positive bacteria appear more resistant to these agents and this may be due to the protection afforded by their more sturdy cell wall. Markers of exposure also vary according to Gram status; Amide II was consistently altered in Gram-negative bacteria and carbohydrate altered in Gram-positive bacteria. ATR-FTIR and SR-FTIR spectroscopy could both be applied to extract biochemical alterations induced by each CBN that were consistent across the two bacterial species; these may represent potential biomarkers of nanoparticle-induced alterations. Vibrational spectroscopy approaches may provide a novel means of fingerprinting the effects of CBNs in target cells.

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Introduction

Nanoparticles have unique physical, electrical and thermal properties, which make them useful in a wide range of applications in various industries including electronics and medicine.[1] A nanomaterial is defined as any material, whether natural or man-made, that has at least one external dimension <100 nm. Of the many materials associated with the nanotechnology revolution, carbon-based nanoparticles (CBNs) are thought to have some of the most diverse and distinct uses.[2] The first CBN, C_{60}, also known as Buckminsterfullerene was discovered in 1985. C_{60} has carbon atoms laid out in a geodesic dome arrangement, giving it a spherical shape.[3] It was subsequently discovered that the process which yields fullerenes could also be used to generate multi-walled carbon nanotubes (MWCNTs); these are constructed of multiple layers of rolled, graphene sheets of varying diameters.[4] Manufacture of single-walled carbon nanotubes (SWCNT) was achieved in 1993. SWCNTs consist of a single tube, which looks similar to a rolled sheet of graphene; the carbon atoms form this structure by bonding in a hexagonal pattern.[5]

The growing usage of carbon-based nanomaterials (CBNs) has led to concern over their potential release into and effects on the environment. There are various routes by which nanomaterials can be released including liberation from nanocomposite polymers during their usage cycle, incineration, and during processing at wastewater treatment plants.[6] CBNs can exert toxic effects at the cellular level, primarily *via* generation of reactive oxygen species (ROS) and subsequent cellular oxidative stress.[7] Studies have shown that their ability to aggregate and physical contact of these particles with cell membranes play a role in this toxicity.[8,9] In addition, due to methods of manufacture, there are often metal impurities (remnants of catalysis) that can cause adverse effects.[10] CBNs have been shown to have

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deleterious effects on a range of organisms such as algae, protozoa, guinea pigs and humans.[11–14]

Bacteria have essential functions in many ecosystems. CBNs can be toxic to bacteria causing loss of viability in *Escherichia coli*[8,15,16] and in also more environmentally-relevant species such as *Bacillus subtilis* and *Pseudomonas aeruginosa*.[17,18] A wide range of factors can affect the extent of toxicity such as size, surface area and purity of the nanoparticle,[19] cell membrane characteristics (whether the bacteria are Gram-negative or -positive),[20] and even the cell media used.[21] However, research into the effects of nanomaterials on bacteria has been hindered by the unsuitability of traditional cytotoxicity assays for use with nanoparticles. CBNs can interact with colorimetric reagent components of tests such as the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), neutral red and other assays.[22,23] CBNs commonly have large surface areas with hydrophobic properties, which are ideal for adsorption of dyes and many other molecules; this can invalidate the results of assays.[24] Such effects highlight the need to find better methods to assay nanoparticle effects and/or toxicity.

Infrared (IR) spectroscopy is a non-destructive, high throughput tool allowing analysis of biological samples. It exploits the principal that biochemical bonds can show some degree of movement such as stretching, bending, scissoring or twisting after absorption of energy from IR at specific wavelengths.[25–27] This absorbance is measured and generates spectra where peaks directly correlate to the structure of the material being investigated. The mid-IR region, known as the "biochemical-cell fingerprint" region, is where the majority of biochemical structures absorb IR energy and vibrate.[28,29] This technique, coupled with multivariate analysis, allows identification of biochemical alterations induced by specific treatments.

Previously, we have used IR spectroscopy, specifically multi-beam synchrotron radiation-based Fourier-transform IR (SR-FTIR) spectroscopy, to investigate the effects of CBNs on Gram-negative and -positive bacteria.[30] Whilst the average, laboratory-based spectrometer uses a globar IR source, SR sources produce much more brilliant light giving higher resolution due to the use of an accelerated electron beam.[29] This can be extremely advantageous but synchrotron facilities are large, expensive and accessibility is often limited. The aim of the current paper was to use attenuated total reflection FTIR (ATR-FTIR) spectroscopy, which uses a globar light source, with multivariate analysis to study the effects of CBNs on Gram-negative bacteria *Pseudomonas fluorescens* and Gram-positive bacteria *Mycobacterium vanbaalenii* PYR-1. Our specific aims were to explore CBN interactions with bacterial cells, as revealed by ATR-FTIR spectroscopy, and to compare these results with those generated by SR-FTIR spectroscopy.

Experimental methods

Preparation of monocultures

Gram-negative *P. fluorescens* and Gram-positive *M. vanbaalenii* PYR-1 were grown in an aqueous solution of mineral basal salts (MBS) with a phenanthrene growth substrate delivered using dimethylformamide (DMF) as a carrier solvent. Incubation of the cultures was undertaken in the dark at 21 ± 2 °C.

Experimental and control treatments

Cell cultures were tested with one of four treatments: long multi-walled carbon nanotubes (MWCNTs; 110–170 nm diameter, 5–9 μm length, >90% purity), short MWCNTs (10–15 nm diameter, 0.1–10 μm length, >90% purity), fullerene soot and C_{60} fullerene (1 nm, >99.5% purity, hereafter referred to as C_{60}). All test agents were sourced from Sigma Aldrich Co. (Dorset, UK). We used 10^7 cells at the late exponential growth-phase of development (4 days for *P. fluorescens* and 5 days for *M. vanbaalenii*) in each treatment; this standardisation being designed to avoid introduction of any bias associated with culture status, cell concentration or proportion of live/dead cells. Cells were harvested from liquid culture by centrifugation (3000 *g* for 5 min) and subsequent washing (three times) with sterile deionised water to remove growth media. Nanoparticles suspended in a 1% bovine serum albumin (BSA – 98% purity; Sigma Aldrich Co.) were diluted from a concentrated stock to deliver a concentration of 0.01 $mg \cdot L^{-1}$ in 1 mL BSA to the live cell pellet. The BSA–nanoparticle mixture was vortex shaken for 1 min to disperse the cell pellet, then end-over-end shaken to prevent gravitational settling during a 2 h incubation period in the dark. Following incubation, bacterial cells were centrifuge-washed five times with 70% ethanol to thoroughly remove residual traces of BSA and fix the cells. The resulting cellular material was then applied to 1 cm × 1 cm low-E reflective glass slides (Kevley Technologies, Chesterland, OH, USA), air-dried and stored in desiccators for at least 8 h prior to ATR-FTIR spectroscopy measurements.

Negative control samples of cells incubated with 1% BSA without CBNs were generated employing the same preparation protocol. Generation of ROS appears to be a major mechanism of nanotoxicity[31] and we generated positive control samples by exposing cells to ultraviolet (UV)-A radiation, a ROS-generating agent that is a suitable mediator of oxidative stress (OS) and outside the absorbance range of cellular components.[32]

Cultures for positive control experiments were grown, harvested and prepared for ATR-FTIR spectroscopy in exactly the same way as those tested with CBNs. Bacterial cells were re-suspended in 10 mL of 1% BSA and placed into T25 flasks. These positive control cultures were irradiated with UV-A delivered at a fluence rate of 50 W m^{-2} for 45 min (total dose = 135 kJ m^{-2}) under four 36 Watt UV-A bulbs with emission peaks at 371 nm: conditions which have previously generated an ROS-stimulated response in bacteria.[33] Flasks were agitated after 20 min UV-A exposure to prevent the depletion of oxygen within the media and re-distribute bacteria. The temperature within T25 flasks was continuously monitored to ensure no excessive thermal accumulation was caused by UV-A treatment and that temperatures did not rise >27 °C. Non-UV-A irradiated positive control samples, wrapped in aluminium foil to block all light from reaching cells, were placed under UV-A lamps alongside the UV-A irradiated samples to ensure an equivalence of conditions. All treatments and controls were conducted in triplicate.

ATR-FTIR spectroscopy

A Bruker TENSOR 27 FTIR spectrometer equipped with a Helios ATR diamond attachment (Bruker Optics Ltd, Coventry, UK) was

used to acquire IR spectra. Spectra were acquired at 3.84 cm^{-1} resolution, 2.2 kHz mirror velocity and 32 co-additions. A total of 10 spectra were acquired per slide, specifically from areas containing agglomerates of CBNs visible through the ATR magnification-limited viewfinder camera. The crystal was cleaned with deionised water and background readings re-taken prior to measurement of each new sample. Spectra acquired from nanoparticles alone did not show any peaks in the biochemical-cell fingerprint range (1800–900 cm^{-1}); hence, acquired spectra reveal the effects of CBNs rather than the nanoparticles themselves.

Pre-processing of spectra and PCA-LDA

All data processing was carried out using MATLAB r2012b (The MathWorks Inc., US) with our in-house toolbox (http://bioph.lancs.ac.uk/iroot). Each of the acquired IR spectra were cut to the biochemical-cell fingerprint region (1800–900 cm^{-1}), baseline corrected by 1st order differentiation and then vector normalised. Spectra were acquired at 3.84 cm^{-1} resolution giving rise to 235 absorbance intensities per IR spectrum. The optimum number of PC factors for subsequent input into LDA was calculated for each dataset separately through an optimization procedure using classification [see ESI Fig. S1 and Table S1†]. Cross-calculated principal component analysis (PCA)-linear discriminant analysis (LDA) was then applied to each dataset where appropriate. The leave-one-out cross-validation method uses a small portion of the dataset to train the model in order to prevent LDA overfitting. LDA applied to each of the selected PCs maximizes inter-class variance relative to intra-class variance, allowing maximum separation of PCA-LDA scores between CBN treatments, and subsequently allows the wavenumbers responsible for the separation of the scores to be determined.[28,34]

PCA-LDA cluster vector

To highlight important biomarkers related to each class of data, the cluster vectors approach is employed. Simplification of agent-induced biochemical alterations relative to the corresponding vehicle control is achieved by moving the centre of the control cluster itself to the origin of the PCA-LDA factor space, hence making the control cluster vector, which represents no biochemical alteration, the zero vector.[35] The extent of peak deviation away from the origin of the factor space then occurs proportional to the extent of biochemical alteration according to the centre of each corresponding agent-induced cluster.

Statistical analysis

For the purposes of statistical analysis, the spectra from each treatment class were pooled and each IR spectrum was treated as a replicate, as previously described.[36] One-way analysis of variance (ANOVA) with Tukey's post hoc test was employed to test the differences in scores between all CBN treatment classes. Unpaired two-tailed *t*-tests were used to test the differences between scores from UV-A irradiated and non-UV-A irradiated positive control samples. All statistical analysis was carried out in GraphPad Prism 4.

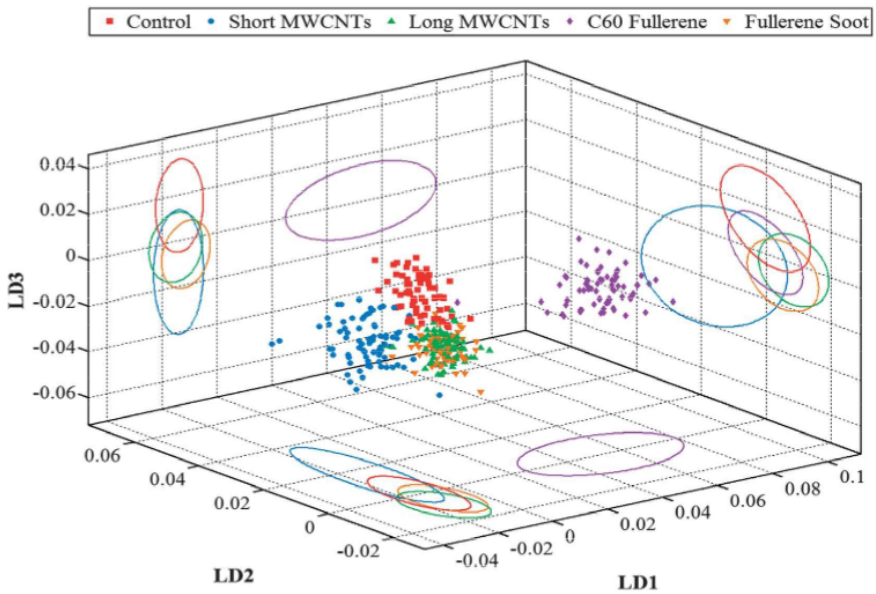

Fig. 1 Three-dimensional (3-D) scatter plot, derived from cross-validated principal component analysis-linear discriminant analysis (PCA-LDA), for *P. fluorescens* treated with 0.01 mg L^{-1} of carbon-based nanoparticles (CBNs). Infrared (IR) spectra are reduced to single points with PCA-LDA and subsequently plotted in 3-D. For clarity, 95% confidence intervals have been plotted on each axis.

Table 1 *P*-values, calculated by one-way ANOVA with Tukey's *post hoc* test, for (A) *P. fluorescens* and (B) *M. vanbaalenii* treated with 0.01 mg L^{-1} of CBNs. Bold italics indicates results that are not significant

(A) Treatment comparisons	LD1	LD2	LD3
Control *vs.* short MWCNTs	***P > 0.05***	$P < 0.001$	$P < 0.001$
Control *vs.* long MWCNTs	***P > 0.05***	$P < 0.001$	$P < 0.001$
Control *vs.* C_{60} fullerene	$P < 0.001$	***P > 0.05***	$P < 0.001$
Control *vs.* fullerene soot	***P > 0.05***	$P < 0.001$	$P < 0.001$
Short MWCNTs *vs.* long MWCNTs	***P > 0.05***	$P < 0.001$	$P < 0.001$
Short MWCNTs *vs.* C_{60} fullerene	$P < 0.001$	$P < 0.001$	$P < 0.001$
Short MWCNTs *vs.* fullerene soot	$P < 0.05$	$P < 0.001$	$P < 0.001$
Long MWCNTs *vs.* C_{60} fullerene	$P < 0.001$	$P < 0.001$	***P > 0.05***
Long MWCNTs *vs.* fullerene soot	$P < 0.001$	$P < 0.01$	$P < 0.01$
C_{60} fullerene *vs.* fullerene soot	$P < 0.001$	$P < 0.001$	$P < 0.001$
(B) Treatment comparisons	**LD1**	**LD2**	**LD3**
Control *vs.* short MWCNTs	$P < 0.001$	***P > 0.05***	$P < 0.001$
Control *vs.* long MWCNTs	$P < 0.001$	$P < 0.001$	$P < 0.05$
Control *vs.* C_{60} fullerene	$P < 0.001$	$P < 0.001$	$P < 0.001$
Control *vs.* fullerene soot	$P < 0.001$	$P < 0.001$	$P < 0.001$
Short MWCNTs *vs.* long MWCNTs	***P > 0.05***	$P < 0.001$	$P < 0.001$
Short MWCNTs *vs.* C_{60} fullerene	$P < 0.001$	$P < 0.001$	$P < 0.001$
Short MWCNTs *vs.* fullerene soot	$P < 0.001$	$P < 0.001$	$P < 0.001$
Long MWCNTs *vs.* C_{60} fullerene	$P < 0.001$	$P < 0.001$	$P < 0.001$
Long MWCNTs *vs.* fullerene soot	$P < 0.001$	$P < 0.001$	$P < 0.001$
C_{60} fullerene *vs.* fullerene soot	$P < 0.001$	***P > 0.05***	$P < 0.001$

Results and discussion

Effects of CBNs on bacteria

A scores plot for *P. fluorescens* (Fig. 1) shows distinct clustering for all treatment categories away from the control cluster of spectral points with the most profound being associated with cells treated with C_{60}. The clusters for short or long MWCNTs, fullerene soot and the control are all relatively close to each other along LD1 and LD2 whereas, for C_{60}, there is very clear separation along LD1 and LD3. C_{60} induces segregation that is much further away from the control category compared to other CBN treatments and a one-way ANOVA with Tukey's *post hoc* test (Table 1, (A)) indicates that although all CBNs are significantly different from each other along LD1, C_{60} is the only treatment to differ significantly from the control category along this axis. The *M. vanbaalenii* PCA-LDA scores plot (Fig. 2) indicates that CBN treatments resulted in obvious cluster segregation. Long MWCNTs and C_{60} treatment resulted in the most separation and distance away from the corresponding control but all CBN-treated cell clusters are to some extent segregated. All treatment categories are significantly different from the control category along LDs 1, 2 and 3 except for short MWCNTs on LD2 (Table 1, (B)). All treatment categories also differ from each other except long and short MWCNTs on LD1. Short MWCTs and fullerene soot are the closest to the control category suggesting that these two treatments induce the least marked bacterial-cell alterations.

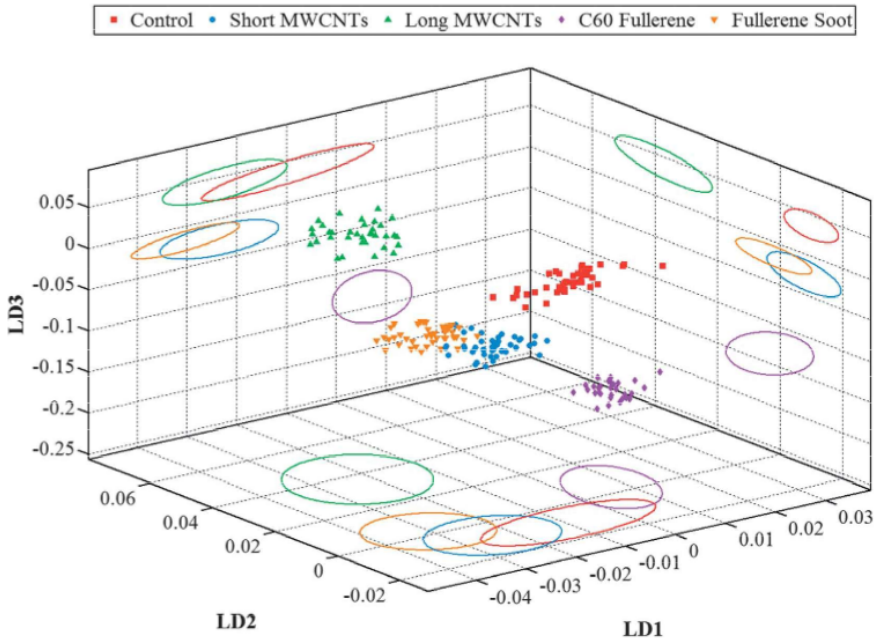

Fig. 2 Three-dimensional (3-D) scatter plot, derived from cross-validated principal component analysis-linear discriminant analysis (PCA-LDA), for *M. vanbaalenii* treated with 0.01 mg L^{-1} of carbon-based nanoparticles (CBNs). Infrared (IR) spectra are reduced to single points with PCA-LDA and are plotted in 3-D. For clarity, 95% confidence intervals have been plotted on each axis.

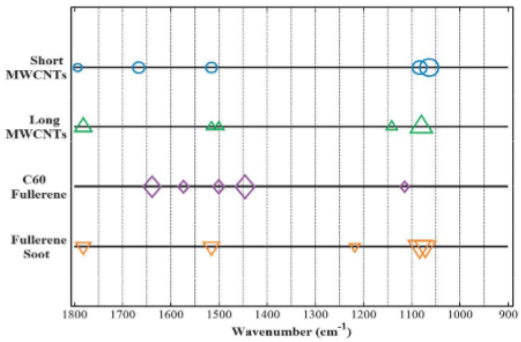

Fig. 3 One-dimensional, peak detection cluster vector plot, for *P. fluorescens* treated with 0.01 mg L^{-1} of CBNs.

Table 2 Tentative wavenumber assignments for the top five peaks (in order of magnitude) from the cluster vector for *P. fluorescens* treated with 0.01 mg L^{-1} of (A) short MWCNTs; (B) long MWCNTs; (C) C_{60} fullerene and (D) fullerene soot

(A) Short MWCNTs	
Wavenumber (cm^{-1})	Assignment
1065	C–O stretching of DNA
1084	$\nu_s PO_2^-$
1666	Amide I
1516	Amide II
1794	Lipid
(B) Long MWCNTs	
Wavenumber (cm^{-1})	Assignment
1080	$\nu_s PO_2^-$
1782	Lipid
1142	C–O stretching of carbohydrate
1516	Amide II
1501	Amide II
(C) C_{60} fullerene	
Wavenumber (cm^{-1})	Assignment
1447	CH_2 bending of proteins
1639	Amide I
1501	Amide II
1574	Amide II
1115	Carbohydrate
(D) Fullerene soot	
Wavenumber (cm^{-1})	Assignment
1084	$\nu_s PO_2^-$
1072	C–O vibration of DNA
1516	Amide II
1782	Lipid
1219	$\nu_{as} PO_2^-$

Cluster vectors plots separate categories from the corresponding control spectral cluster based on wavenumber, thus allowing identification of biomarkers of exposure to CBNs. The five largest peaks for *P. fluorescens* were picked from cluster vectors plots using a peak detection algorithm (Fig. 3) and tentative wavenumber alterations assigned (Table 2). Short MWCNTs (Table 2, (A)) induced the most marked alterations in DNA, protein (Amide I and II) and lipid regions whereas long MWCNTs (Table 2, (B)) caused alterations to symmetric phosphate stretching vibrations ($\nu_s PO_2^-$), lipid, carbohydrate and protein (Amide II). Fullerene soot exposure (Table 2, (D)) also generated a similar profile of alterations, associated with asymmetric phosphate stretching vibrations ($\nu_{as} PO_2^-$) and $\nu_s PO_2^-$, DNA, protein (Amide II) and lipid. C_{60} (Table 2, (C)) induced more extensive protein alterations. The top four peak assignments are in the protein region with the fifth associated with alterations to carbohydrates. Thus, short MWCNTs, long MWCNTs and fullerene soot induce fairly similar patterns of spectral alterations whereas C_{60} is distinctly different. This reflects the extent of dissimilarity in the PCA-LDA scores plot (Fig. 1) and may be due to a relationship between size and toxicity, as toxic effects of nanoparticles are related to particle size due to an increase in surface area to volume ratio.[8,37] C_{60} is the smallest CBN tested and it also caused the most distinct and extensive alterations whereas long MWCNTs (the largest CBN) induced the least marked alterations.

A cluster vectors peak detection plot (Fig. 4) and tentative peak assignments (Table 3) shows that in *M. vanbaalenii*, short MWCNTs (Table 3, (A)) cause extensive alterations in carbohydrate, proteins and DNA. Long MWCNTs (Table 3, (B)) induce protein (Amide II), carbohydrate and DNA alterations with C_{60} (Table 3, (C)) inducing changes in polysaccharides, other carbohydrates and $\nu_{as} PO_2^-$. Fullerene soot (Table 3, (D)) also affects carbohydrates including polysaccharides as well as lipid and protein (Amide II). Both short and long MWCNTs induce similar alterations in these bacteria and fullerene soot shares some of these characteristics. However, as with *P. fluorescens*, C_{60} induced a different spectral profile in *M. vanbaalenii* more associated with carbohydrate alterations.

Differences between Gram-negative and Gram-positive bacteria

There are many factors that can affect the toxicity that nanoparticles exert on bacteria, one of which is the species of bacteria and their associated membrane characteristics.[38] Gram-negative and Gram-positive bacteria have been shown to exhibit different responses to nanoparticles.[17] Gram-positive *M. vanbaalenii* display a much greater separation between treatment category clusters and from the control (Fig. 2) than that seen in Gram-negative *P. fluorescens*, which exhibits more overlap (Fig. 1). This overlap of CBN-treatment categories suggests that these bacteria are fairly equally affected (or unaffected) by the nanoparticles. The enhanced separation seen in *M. vanbaalenii* category clusters could indicate that it is

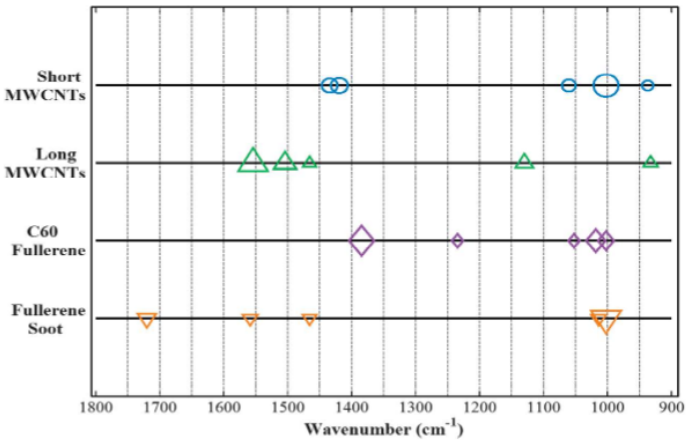

Fig. 4 One-dimensional, peak detection cluster vectors plot, for *M. vanbaalenii* treated with 0.01 mg L^{-1} of CBNs.

affected differently by the various CBN types and to differing extents. Gram-positive bacteria such as *M. vanbaalenii* have a thick ring of peptidoglycan and teichoic acid around their cell wall which increases structural integrity[39] and may be protective, thereby increasing robustness against some types of CBN.[17] The peptidoglycan layer in Gram-negative bacteria is thinner and also overlaid by a membrane layer meaning that these bacteria may be unable to withstand nanotoxic assault in the same way.

Despite differences in membrane structures and the potential variation this can cause, C_{60} (Tables 2, (C) and 3, (C)) caused the most extensive and marked alterations in both bacteria, perhaps due to its small size and relatively large surface area. However, the alterations induced in each species were not the same. In *P. fluorescens*, the alterations were mainly in proteins particularly Amide I and II (Fig. 3) but in *M. vanbaalenii*, there were more carbohydrate changes (Table 3, (C)). C_{60} has been found to cause toxic effects by direct contact with cells[40] and as peptidoglycan has a carbohydrate backbone, this may explain why carbohydrate changes were so predominant in the Gram-positive bacteria. In *P. fluorescens* treated with long or short MWCNTs or fullerene soot, lipid alterations were in the top five peaks detected whereas these were not present in any of the top peak assignments for *M. vanbaalenii*. Gram-negative bacteria have a membrane layer on their outermost surface of the cell so increased lipid alterations could be expected without a strong peptidoglycan layer for protection. In *P. fluorescens*, the only biomolecule that was consistently affected by CBNs was Amide II and in *M. vanbaalenii*, carbohydrates were altered. These biomarker effects may represent key biochemical changes, which signature nanotoxicity in these different bacterial species.

Positive controls

There are many hypotheses regarding the exact mechanisms of toxicity employed by carbon nanoparticles, one of which is that nanotoxicity is caused by the generation of ROS.[7] With this in mind, UV-A was used as a positive control for ROS generation. In both *P. fluorescens* (Fig. 5A) and *M. vanbaalenii* (Fig. 5B), UV-A irradiation induced alterations in lipids and proteins (Amide I) (Fig. 5; Table 4), which are consistent with ROS-induced damage such as lipid peroxidation. Two-tailed, unpaired *t*-tests (Table 4) show that UV-A-treated bacterial cells are significantly different from the non-UV-A exposed corresponding negative controls.

As UV-A is considered to be an independent oxidative stress-inducing mechanism; employing this treatment as a positive control allowed us to assess whether any of the CBNs tested generate ROS. In *P. fluorescens*, some of the CBNs did induce alterations in lipid and Amide I in the top five alterations, although not to the same extent as observed in UV-A-treated cells. Fullerene soot and long MWCNTs induced lipid alterations and C_{60} caused extensive Amide I alterations. However, exposure with short MWCNTs generated the most ROS-like spectral profile, causing significant alterations in both Amide I and lipid. Alterations induced by CBNs in *M. vanbaalenii* were less like those caused by UV-A. Only fullerene soot generated lipid alterations to such an extent that it appears in the top five peaks in cluster vectors plots and no CBN caused Amide I changes. These results suggest that although ROS generation may not appear to be the primary mechanism of nanotoxicity, it may have a role to play particularly in *P. fluorescens* treated with short MWCNTs. Other studies have shown that Gram-negative bacteria are more susceptible to nanoparticles than Gram-positive bacteria due to the lipopolysaccharide in the membrane facilitating better interaction between the cell and nanoparticle.[41,42] More ROS-like activity may be seen in *P. fluorescens* than in *M. vanbaalenii* as its Gram-negative status allows CBNs to cluster onto the surface of the cells and generate the molecules that cause oxidative stress.

Table 3 Tentative wavenumber assignments for the top five peaks (in order of magnitude) from the cluster vector for *M. vanbaalenii* treated with 0.01 mg L^{-1} of (A) short MWCNTs; (B) long MWCNTs; (C) C_{60} fullerene and (D) fullerene soot

(A) Short MWCNTs	
Wavenumber (cm^{-1})	Assignment
1003	Carbohydrate
1420	Proteins
1435	Proteins
1060	C–O stretching of DNA
937	DNA
(B) Long MWCNTs	
Wavenumber (cm^{-1})	Assignment
1555	Amide II
1504	Amide II
1130	C–O stretching of carbohydrate
934	DNA
1466	Amide II
(C) C_{60} fullerene	
Wavenumber (cm^{-1})	Assignment
1385	COO– stretching
1018	CO vibration of polysaccharides
1003	Carbohydrate
1234	$\nu_{as}PO_2$
1053	C–O stretching and C–O bending of carbohydrate
(D) Fullerene soot	
Wavenumber (cm^{-1})	Assignment
1003	Carbohydrate
1720	C=O stretching of lipid
1558	Amide II
1015	CO vibration of polysaccharides
1466	Amide II

Comparison to SR-FTIR spectroscopy

Our previous work in this area has utilised SR-FTIR spectroscopy to analyse the effect of CBNs on bacteria and other cells.[30] By comparing our results from this present study with those collected using the exact same experimental procedures but analysed by SR-FTIR spectroscopy (see ESI, Tables S2–S4†), we can assess how comparable both techniques are towards detecting CBN-induced changes in bacteria.[43,44] Pre-processing methods for both datasets were kept as similar as possible, providing they were appropriate, in order to prevent unnecessary variance. Both SR-FTIR and ATR-FTIR spectroscopy picked out the major trends in cluster separation in both bacterial species. They both showed that *M. vanbaalenii* exhibited better clustering and separation of category clusters whereas *P. fluorescens* had less defined clusters with much more overlap between categories. C_{60} was consistently detected as the CBN whose exposure resulted in spectral clusters that are furthest away from the control, with the most extensive alterations. The category cluster distance from the corresponding control in both bacterial species followed the order of C_{60} (the furthest), then MWCNTs and then fullerene soot; this was observed in both SR-FTIR and ATR-FTIR spectrochemical data.

In terms of biochemical alterations, data for *P. fluorescens* (see ESI, Tables S2 and S3†) from both spectroscopy techniques was fairly comparable. The top five peaks from each spectrochemical dataset did not match to exact wavenumbers and magnitude order but the overall trends in alterations were the same. With both techniques, short MWCNTs caused changes in DNA, protein and lipid, long MWCNTs altered protein and DNA, and fullerene soot caused alterations in protein, carbohydrate and lipid regions. C_{60} is a good example of where both techniques showed an alteration in the same top five peaks in cluster vectors plots but the magnitude of individual ones differed. Both techniques showed carbohydrate as being one of the most extensively altered biomolecules but SR-FTIR spectroscopy showed it to be the most altered whereas ATR-FTIR spectroscopy ranked it as fifth. Alterations caused by CBNs in *M. vanbaalenii* (Table 3) did not compare across both techniques as the top peaks were very different. This may have been because it is a Gram-positive bacterium and as the peptidoglycan could offer protection against nanotoxicity, we saw a greater range of alteration caused by the CBNs. In Gram-negative bacteria, CBNs come up against minimal buffers to their toxic assault and so all cause a similar extent of alteration. This coupled with instrumental and sample differences could have influenced the consistency of biochemical alterations across the two techniques. A major difference would be the spatial resolution of both methods employed with that of SR-FTIR spectroscopy being much greater compared to that of ATR-FTIR spectroscopy; this could explain why the former technique isolated carbohydrate alterations as being major because it could focus better on cell membranes.

ATR-FTIR and SR-FTIR spectroscopy were could both be applied to extract biochemical alterations induced by each CBN that were constant across the two bacterial species; these may represent potential biomarkers. Both techniques detected the same biomarkers but SR-FTIR seemed able to identify more. For example, for short MWCNTs, ATR-FTIR spectroscopy (Tables S3 and S5†) showed Amide I as a consistent biomarker which SR-FTIR spectroscopy was able to detect (see ESI, Table S2 and S4†) but it also showed that lipid and DNA were reproducibly altered. SR-FTIR spectroscopy detected more biomarkers than ATR-FTIR spectroscopy following exposure to all CBNs except in fullerene soot where the biomarkers (alterations in Amide II, lipids and carbohydrates) were exactly the same when extracted by either technique. There were also some areas where the two spectroscopic techniques were not

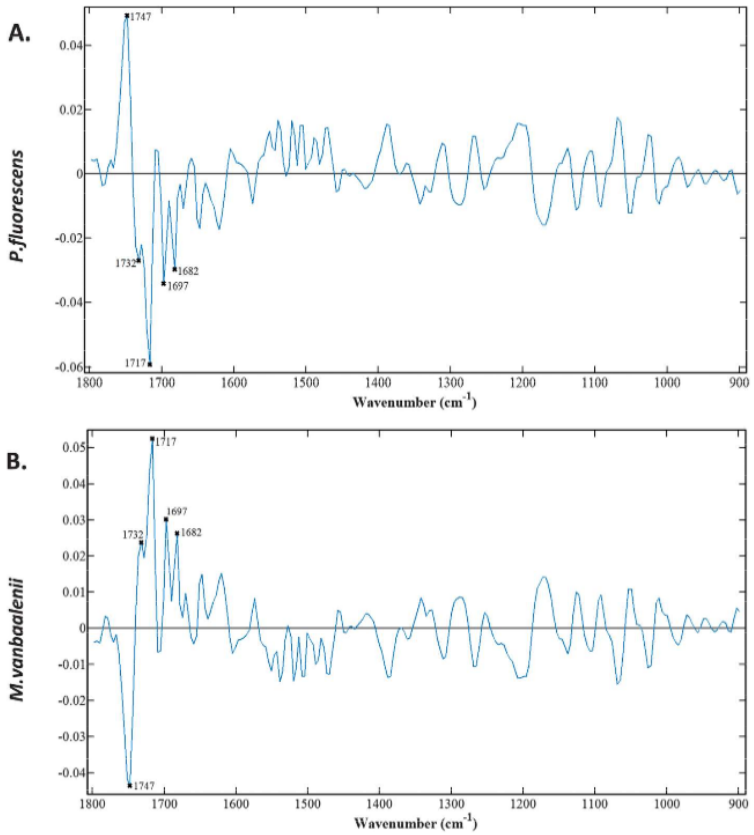

Fig. 5 Cross-validated principal component analysis-linear discriminant analysis (PCA-LDA) loadings plots of positive control samples for (A) *P. fluorescens* and (B) *M. vanbaalenii* irradiated with ultraviolet (UV)-A for 45 minutes.

comparable at all. SR-FTIR data showed that Amide I was altered following exposure to all CBNs tested irrespective of bacterial-cell types, which indicates that this might be an overall marker of nanotoxicity. It also detected that lipid alterations were significant. This was less apparent using ATR-FTIR spectroscopy.

ATR-FTIR and SR-FTIR spectroscopy did appear to generate comparable data but there are limitations to how interchangeable the two techniques are. Generally, ATR-FTIR spectroscopy did reveal the same information as SR-FTIR spectroscopy but in less detail; it seemed to analyse the overall trends in alterations in comparison to the detail that was revealed by SR-FTIR spectroscopy. However, given that the value of SR-FTIR spectroscopy is its superior resolution, it was not surprising that more could be elucidated about the dataset.

Conclusions

IR spectroscopy with multivariate analysis is a robust tool for the investigation of CBN–cell interactions.[45] This study has shown that CBNs induced a profile of alterations in Gram-negative and Gram-positive bacteria. Gram-positive bacteria exhibit more variance in the extent of these alterations, possibly due to the protective effect of their thick peptidoglycan layer, which potentially gives these cells greater structural integrity against CBN-mediated damage such as ROS generation. Potential biomarkers of exposure to CBNs also varied with membrane characteristics; in Gram-negative *P. fluorescens*, Amide II alterations were seen consistently across all nanoparticle types and in Gram-positive *M. vanbaalenii*, carbohydrate was the potential marker. In both bacteria, the nanoparticles induced a similar ranking of alteration extent with C_{60} causing the most significant differences. We also compared ATR-FTIR and SR-FTIR spectroscopy and found that, although there were some differences between the two methods, overall, the information retrieved was largely comparable. SR-FTIR spectroscopy provided detailed, in-depth information on nanotoxic alterations due to its superior resolution whereas ATR-FTIR spectroscopy was less exhaustive, pulling out fewer biomarkers, but it provided an excellent overview and reasonable detail of alterations induced by CBNs.

Table 4 Tentative wavenumber assignments for the top five peaks (in order of magnitude) of positive control (UV-A exposed) for (A) *P. fluorescens* and (B) *M. vanbaalenii* cells with unpaired *t*-tests to show significance

(A) *P. fluorescens* treated with 45 minute UV-A	
Wavenumber (cm^{-1})	Assignment
1717	C=O stretching of lipid
1747	C=C vibration of lipids
1697	Amide I
1682	Amide I
1732	Fatty acids
Unpaired *t*-test: UV-A treated *P. fluorescens*	
P-value	$P < 0.0001$
Are means signif. different? ($P < 0.05$)	Yes
One- or two-tailed *P*-value?	Two-tailed

(B) *M. vanbaalenii* treated with 45 minute UV-A	
Wavenumber (cm^{-1})	Assignment
1717	C=O stretching of lipid
1747	C=C vibration of lipids
1697	Amide I
1682	Amide I
1732	Fatty acids
Unpaired *t*-test: UV-A-treated *M. vanbaalenii*	
P-value	$P < 0.0001$
Are means signif. different? ($P < 0.05$)	Yes
One- or two-tailed *P*-value?	Two-tailed

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Appendix 2. List of publications from collaborative research

1. Baker, M.J., Trevisan, J., Bassan, P., Bhargava, R., Butler, H.J., Dorling, K.M., Fielden, P.R., Fogarty, S.W., Fullwood, N.J., **Heys, K.A.**, Hughes, C., Lasch, P., Martin-Hirsch, P.L., Obinaju, B., Sockalingum, G.D., Sulé-Suso, J., Strong, R.J., Walsh, M.J., Wood, B.R., Gardner, P., Martin, F.L., 2014. Using Fourier transform IR spectroscopy to analyze biological materials. Nature Protocols 9, 1771-1791.

2. Halliwell, D., Morais, C.L.M., Gomes De Lima, K.M., Trevisan, J., Siggel-King, M.R.F., Craig, T., Ingham, J., Martin, D.S., **Heys, K.**, Kyrgiou, M., Mitra, A., Paraskevaidis, E., Theophilou, G., Martin-Hirsch, P.L., Cricenti, A., Luce, M., Weightman, P., Martin, F.L., 2016. Imaging cervical cytology with scanning near-field optical microscopy (SNOM) coupled with an IR-FEL. Scientific Reports 6, 11p.

3. Theophilou, G., Fogarty, S.W., Trevisan, J., Strong, R.J., **Heys, K.A.**, Patel, I.I., Stringfellow, H.F., Martin-Hirsch, P.L. and Martin, F.L., 2016. Spatial and temporal age-related spectral alterations in benign human breast tissue. Journal of Molecular Structure 1106, 390-398.

Appendix 3. Conference Abstracts

1. Heys K.A., Shore R.F., Pereira M.G.d.S., Jones K.C., Martin F.L. 2013. Mixture effects of genotoxic and endocrine disrupting agents in MCF-7 cells as assessed by biospectroscopy. Mutagenesis 29 (1), 81-81.

Although many studies test contaminants in isolation, one must also consider interactions between chemicals to gain a more accurate understanding of how they generate their effects within an environmental context. The study of mixture effects is particularly thought-provoking, especially if the agents in questions have different mechanisms of action. Here, we look at the effects of mixtures of a known genotoxin, benzo[a]pyrene (B[*a*]P), and endocrine disruptors, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) on MCF-7 cells. B[*a*]P, found in car exhaust fumes and cigarette smoke, is not carcinogenic in its parent form, but when metabolically activated by cytochrome P450 enzymes, it forms DNA adducts. Thus, the metabolised form of B[*a*]P has genotoxic effects. In contrast, brominated flame retardants such as PCBs and PBDEs are endocrine disruptors. PCBs can bind to aryl hydrocarbon receptors which, via interaction with the gene-specific dioxin response elements, can alter synthesis of cytochrome P450 enzymes. PBDEs interfere with thyroid hormone function by altering thyroxine levels. Infrared (IR) spectroscopy is a non-destructive, high-throughput technique which has previously been used to look at the effects of a range of individual environmental contaminants. Using this method, MCF-7 cells were treated, for 24 hours, with individual agents and combinations of B[*a*]P (10^{-6} or 10^{-10} M) and PCBs or PBDEs at environmentally-relevant concentrations (10^{-8}-10^{-12} M). Cells were then fixed in ethanol before attenuated total reflection Fourier-transform IR (ATR-FTIR) spectroscopy and multivariate analysis. Initial results suggest that the combined effects of B[*a*]P and some PBDE congeners mean that category clusters for such treatments segregate further away from the corresponding control than treatment with individual agents. This would indicate that the effects on cells of combination treatments are significantly different

from those seen with single treatments. Further work will focus on investigating the mechanisms by which mixtures of PCB congeners and B[*a*]P generate their effects on cells.

2. Heys K.A., Riding M.J., Strong B.J., Shore R.F., Pereira M.G.d.S., Jones K.C., Semple K.T., Martin F.L. 2014. Mid-infrared spectroscopic assessment of nanotoxicity in Gram-negative vs. Gram-positive bacteria. Mutagenesis 29 (6), 531-531.

Nanoparticles appear to induce toxic effects through a variety of mechanisms including generation of reactive oxygen species (ROS), physical contact with the cell membrane and indirect catalysis due to remnants from manufacture. The development and subsequent increasing usage of nanomaterials has highlighted a growing need to characterize and assess the toxicity of nanoparticles, particularly those that may have detrimental health effects such as carbon-based nanomaterials (CBNs). Due to interactions of nanoparticles with some reagents, many traditional toxicity tests are unsuitable for use with CBNs. Infrared (IR) spectroscopy is a non-destructive, high throughput technique, which is unhindered by such problems. We explored the application of IR spectroscopy to investigate the effects of CBNs on Gram-negative (Pseudomonas fluorescens) and Gram-positive (Mycobacterium vanbaalenii PYR-1) bacteria. Two types of IR spectroscopy were compared: attenuated total reflection Fourier transform infrared (ATR-FTIR) and synchrotron radiation-based FTIR (SR-FTIR) spectroscopy. This showed that Gram-positive and Gram-negative bacteria exhibit differing alterations when exposed to CBNs. Gram-positive bacteria appear more resistant to these agents and this may be due to the protection afforded by their sturdier cell wall. Markers of exposure also vary according to Gram status; Amide II was consistently altered in Gram-negative bacteria and carbohydrate altered in Gram-positive bacteria. ATR-FTIR and SR-FTIR spectroscopy could both be applied to extract biochemical alterations induced by each CBN that were consistent across the two bacterial species; these may represent potential biomarkers of nanoparticle-induced alterations. Vibrational spectroscopy approaches may provide a novel means of fingerprinting the effects of CBNs in target cells.

3. Heys K.A., Shore R.F., Pereira M.G.d.S., Jones K.C., Martin F.L. 2015. Infrared spectroscopy as a tool to assess the effects of multi-component mixtures in an avian cell line. Mutagenesis 30 (6), 872-872.

Many studies focus on the effects of chemical pollutants in isolation, but in the environment organisms are exposed to many different contaminants at the same time. In order to understand the ecotoxic risk that chemicals will pose, we must study their individual toxicities and their toxicity within mixtures containing other contaminants. Due to interactions between mixture components, the overall toxicity of a mixture may be greater than or less than the sum of the components. Here, we have investigated the toxicity of benzo[*a*]pyrene (B[*a*]P) in binary mixtures with two types of brominated flame retardants, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs). Mallard fibroblast cells were treated for 24 hours with binary mixtures of B[a]P at 10^{-6} or 10^{-10} M and PCBs or PBDEs at environmentally relevant concentrations of 10^{-8} and 10^{-12} M. Then cells were fixed in 70% ethanol and transferred onto Low-E glass slides before interrogation with attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy. Traditional cytotoxicity methods are often time consuming and expensive so we used vibrational spectroscopy to analyse the effects of the binary mixtures. Vibrational spectroscopy techniques are reagent-free, non-destructive and high-throughput and have previously been used to study at the effects of environmental contaminants in different species. Coupled with multivariate analysis, this method has the benefit of identifying biochemical alterations that have been induced by pollutants and gives an insight into how they exert their toxicity which may not be possible using traditional methods. Initial results suggest that the toxicity of the binary mixtures and individual agents is significantly different and the effects of the mixture cannot always be accurately modelled using data from individual treatments.