# Investigating the effects of organic pollutants on amphibian populations in the UK

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## Abstract

Amphibians are undergoing dramatic population declines, with environmental pollution reported as a significant factor in such declines. Technologies are required that are able to monitor populations at risk of deteriorating environmental quality in a rapid, high-throughput and low-cost manner. The application of biospectroscopy in environmental monitoring represents such a scenario. Biospectroscopy is based on the vibrations of functional groups within biological samples and may be used to signature effects induced by chemicals in cells and tissues. Here, attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy in conjunction with multivariate analysis was implemented in order to distinguish between embryos, whole tadpoles at an early stage of development and individual tissues of late-stage tadpoles of the common frog collected from ponds in the UK with varying levels of water quality, due to contamination from both urban and agricultural sources. In addition, a *Xenopus laevis* cell line was exposed to low-levels of fungicides used in agriculture and assessed with ATR-FTIR spectroscopy. Embryos, in general did not represent a sensitive life stage for discriminating between ponds based on their infrared spectra. In contrast, tadpoles exposed to agricultural and urban pollutants, both at early and late stages of development were readily distinguished on the basis of their infrared spectra. ATR-FTIR spectroscopy also readily detected fungicide-induced changes in *X.laevis* cells, both as single-agent and binary mixture effects. Data reported in this study confirm the use of ATR-FTIR spectroscopy as a sensitive technique capable of detecting small changes in cellular groups, and as such represents a valuable starting point for its use in the monitoring of amphibian populations. However further research is needed in order to overcome confounding factors existent in natural populations of complex organisms.

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## 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

Al: Aluminium

AMPA: Aminomethylphosphonic acid

ANN: Artificial neural networks

ANOVA: Analysis of variance

ATR: Attenuated total reflectance

BCI: Body condition index

Ca: Calcium

Cl: Chloride

CYP: Cytochrome P-450

CYP51: Sterol-14α-demethylase

DCM: Dichloromethane

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

ESI+: Electrospray positive

FBS: Fetal bovine serum

Fe: Iron

FFS: Forward feature selection

FPA: Focal plane array

FSH: Feature selection histogram

FTIR: Fourier-transform infrared

GC-MS: Gas chromatography–mass spectrometry

$HNO_3$: Nitric acid

HSI: Hepatosomatic index

HW: Head width

ICP-OES: Inductively coupled plasma optical emission spectrometry

IR: Infrared

IRE: Internal reflection element

K: Potassium

*k*NN: *k*-nearest neighbours

LC-MS: Liquid chromatography–mass spectrometry

LDA: Linear discriminant analysis

LDC: Linear discriminant classifier

LoQ: Limits of quantification

Low-E: Low-emissivity

MeOH: Methanol

Mg: Magnesium

MRM: Multiple reaction monitoring

MS-222: Tricaine methanesulfonate

MSMA: Monosodium methyl arsenate

Na: Sodium

$NO_3$-N: Nitrate

OP: Organophosphorus

OWC: Organic wastewater contaminant

PAH: Polycyclic aromatic hydrocarbon

PBDE: Polybrominated diphenyl ether

PC: Principal component

PCA: Principal component analysis

PCB: Polychlorinated biphenyl

PLS: Partial least squares

PLS-DA: Partial least squares-discriminant analysis

QCL: Quantum cascade lasers

QDA: Quadratic discriminant analysis

QoI: Quinone outside inhibitor

RNA: Ribonucleic acid

SG: Savitzky-Golay

SHE: Syrian Hamster Embryo

SIMCA: Soft independent modelling of class analogies

$SO_4$-S: Sulphate

SNR: Signal to noise ratio

SVL: Snout to vent length

SVM: Support vector machines

TBP: Tributylphosphate

TCEP: Tris(2-chloroethyl)phosphate

TCPP: Tris(1-chloro-2-propyl)phosphate

TEP: Triethylphosphate

TON: Total organic nitrogen

UPLC: Ultra performance liquid chromatograph

UV: Ultraviolet

# Chapter 1. General Introduction

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## 1. Introduction

Amphibian populations are declining on a global scale (Stuart et al., 2004), which is thought to be due to several factors including habitat destruction (Cooke and Ferguson, 1976), climate change (Beebee, 1995; Todd et al., 2010), diseases (Daszak et al., 1999; Daszak et al., 2003) and pollution (Blaustein et al., 2003; Sparling et al., 2001). There is likely to be significant overlap amongst these factors, as well as variation among species, populations and different life stages within populations (Blaustein et al., 2011; Brühl et al., 2011; Greulich and Pflugmacher, 2003; Hof et al., 2011), rendering the situation highly complex with no single factor wholly responsible for the worldwide declines recorded.

While habitat loss is regarded as the greatest threat to amphibian biodiversity (GAA, 2004), environmental pollution has also been linked to population declines (Davidson et al., 2002; Rohr et al., 2008), particularly on a local scale (Cooke, 1972a, 1981). With habitat loss due to agricultural intensification, some amphibian species are also increasingly utilising urban habitats, exposing them to pollutants associated with these environments (Hamer and McDonnell, 2008). Small water bodies typical of amphibian habitats, such as ditches and ponds, may be the first to receive run-off containing contaminants from sources such as sewage effluent, industrial waste, accidental spills and agriculture, indicating that amphibians may be considered sentinel species for assessing ecosystem health as whole (Ralph & Petras, 1997). Amphibians are considered particularly vulnerable to contaminant exposure due to their highly permeable skin and life cycle, which comprises both aquatic and terrestrial phases, thus offering multiple routes of exposure (Brühl, Pieper, & Weber, 2011; Cooke, 1981; Kloas & Lutz, 2006; Ralph & Petras, 1998). This together with the observation that application of contaminants such as pesticides and fertilisers coincides with breeding and larval development in shallow water bodies, suggests that amphibians may be at particular risk of deleterious effects due to exposure from agricultural sources (Hayes et al., 2006; Mann et al., 2009).

A method which may be applicable to determining the effects of environmental contamination on amphibians is infrared (IR) spectroscopy. IR spectroscopy is a technique used to signature effects induced by chemicals in biological cells and tissues based on the vibrations of functional groups within the sample (Ellis and Goodacre,

2006). This technology has previously been employed in environmental toxicology in the laboratory in both cells (Holman et al., 2000b; Johnson et al., 2014; Llabjani et al., 2010), and tissues (Cakmak et al., 2006; Palaniappan et al., 2011) and also in the field (Malins et al., 2006; Malins et al., 1997; Malins et al., 2004).

This chapter will examine threats to amphibian health from urban and agricultural pollution, and the current status of amphibians in the UK, focusing on the most widespread species, the Common frog, *Rana temporaria*. The use of infrared spectroscopy coupled with computational analysis as a novel environmental monitoring technique will be explored, and its application to the monitoring of the health of amphibian populations exposed to urban and agricultural contaminants in the field will be elucidated.

## 2. Threats to amphibian health from pollution

Amphibians are regarded as a group sensitive to environmental pollution due to their biphasic life style, comprising aquatic and terrestrial phases and therefore multiple exposure routes (Hayes et al., 2006), and the permeability of their skin, which allows the diffusion of contaminants across it (Quaranta et al., 2009). There have been many studies exploring the link between environmental pollution and amphibian population declines, with correlations found between proximity to agricultural and urbanised land and declining populations (Bishop et al., 1999; Davidson, 2004; Davidson et al., 2002; Hamer and Parris, 2011; Houlahan and Findlay, 2003; Sparling et al., 2001).

Current methods used to assess the effects of environmental contamination on amphibian populations include both controlled exposure in the laboratory, often employing a surrogate test species, such as the African-clawed frog, *Xenopus laevis* (Coady et al., 2005; Gillardin et al., 2009) and sampling in the field at sites where contamination is a concern such as constructed wetlands and agricultural land (Christin et al., 2013; Ouellet et al., 1997; Ruiz et al., 2010). Endpoints commonly measured include mortality, growth, time to metamorphosis, deformities, effects on sexual differentiation, thyroid and immune function, hormone systems, energy/stress response and genotoxicity, examples of which are presented in Table 1.

**Table1.** Examples of endpoints in amphibians commonly measured following exposure to environmental contaminants in the field and in laboratory and mesocosm studies.

| Endpoint | Study type | Pollutant | Life stage | Species | Example reference |
|---|---|---|---|---|---|
| Survival | Laboratory microcosm | Stormwater pond sediment | Embryo and tadpole | *Bufo americanus* and *Rana sylvatica* | (Snodgrass et al., 2008) |
| | In situ field study | Agricultural gradient | Tadpole | *Pseudacris regilla* | (Sparling et al., 2014) |
| | Outdoor mesocosm | Glyphosate | Tadpole | *R.pipiens, B.americanus, Hyla versicolor* | (Relyea, 2005) |
| Growth | Laboratory | Naphthenic acids | Tadpole | *Lithobates (Rana) pipiens* | (Melvin et al., 2013) |
| | In situ field study | Agricultural intensity | Embryo, tadpole and metamorphs | *Bufo bufo* | (Orton and Routledge, 2011) |
| | Outdoor mesocosm | Carbaryl, atrazine | Tadpole | *R.sphenocephala, B.americanus Ambystoma maculatum, A. Texanum* | (Boone and James, 2003) |
| Deformities | Laboratory | Triadimefon and triadimenol | Embryo and tadpole | *X. laevis* | (Groppelli et al., 2005) |
| | Field | Wastewater run-off | Tadpole | *R. catesbeiana* | (Ruiz et al., 2010) |
| | Outdoor mesocosm | Atrazine, chlorpyrifos, MSMA and methyl mercury | Tadpole | *H.chrysoscelis* | (Britson and Threlkeld, 1998) |
| Gonadal abnormalities | Laboratory | Atrazine | Tadpole | *X.laevis* | (Hayes et al., 2002) |
| | Field | Agricultural intensity gradient | Adult | *B. marinus* | (McCoy et al., |

| | | | | | |
|---|---|---|---|---|---|
| | | | | | 2008) |
| | Outdoor mesocosm | Atrazine | Tadpole | *R.pipiens* | (Langlois et al., 2010) |
| Time to metamorphosis | Laboratory | Chlorpyrifos and endosulfan | Tadpole | *P.regilla, R.boylii* | (Sparling and Fellers, 2009) |
| | Field | Oil sands processed material | Tadpole | *Lithobates (Rana) sylvaticus* | (Hersikorn and Smits, 2011) |
| | Outdoor mesocosm | Malathion and nitrate | Tadpole | *R.sylvatica, B.americanus* | (Smith et al., 2010) |
| Thyroid function | Laboratory | Triclosan | Tadpole | *R.catesbeiana* | (Veldhoen et al., 2006) |
| | Field | Oil sands processed material | Tadpole | *L.sylvaticus* | (Hersikorn and Smits, 2011) |
| Immune function | Laboratory | Pesticide mixture (atrazine, metribuzine, endosulfan, lindane, aldicarb and dieldrin) | Adult | *X.laevis, R.pipiens* | (Christin et al., 2004) |
| | Field | Agricultural pesticides | Adult | *R.pipiens* | (Christin et al., 2013) |
| | In situ field study | Lambda-cyhalothrin | Tadpole | *Scinax squalirostris* and *Leptodactylus mystacinus* | (Attademo et al., 2013) |
| Cholinesterase activity | Laboratory | Chlorpyrifos and endosulfan | Tadpole | *P.regilla, R.boylii* | (Sparling and Fellers, 2009) |
| | Field | Pesticide mixtures associated with rice fields | Adult | *Leptodactylus chaquensis* | (Attademo et al., 2011) |
| | Outdoor mesocosm | Chlorpyrifos | Tadpole | *R.sphenocephala* | (Widder and Bidwell, 2006) |

| | | | | | |
|---|---|---|---|---|---|
| Glutathione-S-transferase activity | Laboratory | Diazinon and endosulfan | Adult | *B.regularis* | (Ezemonye and Tongo, 2010) |
| | Field | Pesticide mixtures associated with rice fields | Adult | *Chaunus schneideri* | (Attademo et al., 2007) |
| | Outdoor microcosm | Contaminated sediment from agricultural/industrial pollution | Tadpole | *Trachycephalus typhonius* | (Peltzer et al., 2013) |
| Corticosterone level | Laboratory | Chlorothalonil | Tadpole | *Osteopilus septentrionalis* | (McMahon et al., 2011) |
| | Field | Chlorinated hydrocarbons | Adult | *Necturus maculosus* | (Gendron et al., 1997) |
| Energy storage | Laboratory | Pharmaceutical mixture | Tadpole | *L.pipiens* | (Melvin, 2015) |
| | Field | Chlorinated hydrocarbons | Adult | *Necturus maculosus* | (Gendron et al., 1997) |
| | Laboratory | Atrazine, glyphosate and quinclorac | Tadpole | *Lithobates (Rana) catesbeiana* | (Dornelles and Oliveira, 2014) |
| Genotoxicity | Laboratory | Endosulfan | Tadpole | *H.pulchella* | (Lajmanovich et al., 2005) |
| | Field | Agricultural and industrial run-off | Tadpole | *R.clamitans, R.pipiens* | (Ralph and Petras, 1997) |
| | Outdoor microcosm | Contaminated sediment from agricultural/industrial pollution | Tadpole | *T.typhonius* | (Peltzer et al., 2013) |

## 2.1 Pollution from urban environments

Increased urbanisation has been cited as a possible reason for population declines in certain areas and amongst particular species of amphibian (Hamer and McDonnell, 2008); however constructed wetlands, which are utilised in urban areas to capture storm-water and treated wastewater run-off also have the added benefit of providing potential habitats for wildlife, with several amphibian species found to be abundant in such locations (Brand and Snodgrass, 2010; O'Brien, 2014; Ruiz et al., 2010; Snodgrass et al., 2008). Amphibian species which are habitat generalists, such as *R.temporaria* are more likely to be able to utilise such habitats. Indeed some studies have demonstrated the importance of urban ponds as breeding sites for *R.temporaria*, where this species shows increased persistence in urban and suburban areas in comparison to rural areas where other species are unable to tolerate due to their life history (Beebee, 1979; Carrier and Beebee, 2003; Cooke, 1975). As a consequence, species breeding and developing in urban environments may be exposed to environmental pollutants associated with storm run-off, wastewater and leachates from landfill from both residential and industrial sources (Brand and Snodgrass, 2010; Pablos et al., 2011; Ruiz et al., 2010; Snodgrass et al., 2008).

Pollutants associated with water bodies located in urban areas include those referred to as organic wastewater contaminants (OWCs) such as antibiotics, analgesic and anti-inflammatory drugs, beta-blockers, antiepileptic and antidepressant drugs and antineoplastics used in cancer treatment (Ashton et al., 2004; Fent et al., 2006; Kolpin et al., 2002). Flame retardants, both brominated (PBDEs) and organophosphorus (OP) compounds are also frequently found in surface waters (Hale et al., 2006; Kolpin et al., 2002; Oros et al., 2005; Reemtsma et al., 2008; Regnery and Püttmann, 2010; van der Veen and de Boer, 2012). Other pollutants associated with urban environments include heavy metals, pesticides, road de-icing salts and polycyclic aromatic hydrocarbons (PAHs) from surface run-off, and increased nutrient levels (nitrate and phosphate) from detergents in treated wastewater (Efroymson et al., 2007; Harris et al., 2001; Pablos et al., 2011; Sanzo and Hecnar, 2006; Snodgrass et al., 2008). However, there is currently a lack of knowledge regarding the ecotoxicological effects of OWCs on aquatic and terrestrial organisms, with chronic effects being particularly under researched (Fent et al., 2006).

Studies examining exposure of amphibians directly to wastewater in the field have found high incidences of skin lesions, edema and calcinosis of soft tissue, as well as deleterious effects on growth and development rates (Keel et al., 2010; Ruiz et al., 2010). Laboratory studies have revealed effects toxic effects of wastewater, including deleterious effects on thyroid morphology, metamorphosis and sexual development in males following exposure to different concentrations of estrogenic municipal wastewater (Sowers et al., 2009). Specific contaminants commonly associated with wastewater, such as caffeine and acetaminophen have also been shown to have detrimental impacts on amphibian behaviour and physiology at environmentally relevant concentrations (Fraker and Smith, 2004).

## 2.2. Pollution from agriculture

Agricultural pollutants primarily cover pesticides (herbicides, insecticides and fungicides) and fertilisers, which are associated with increased nutrient levels in water bodies (Smith et al., 1999). Agricultural pollutants may be from point sources, where a single source of pollution is identified, or from a non-point source such as land run-off, atmospheric deposition, precipitation or drainage (Humenik et al., 1987; Sparling and Fellers, 2009; Tilman et al., 2001). Environmental contamination from agricultural sources is widely considered to have a detrimental impact on amphibian health and survival (Bishop et al., 1999; Mann et al., 2009; Smalling et al., 2015; Sparling et al., 2001). Despite this, many amphibian species are able to survive and persist in modified habitats, indeed; where natural wetlands are scarce, constructed agricultural ponds may provide important breeding habitats if properly managed (Da Silva et al., 2012; da Silva et al., 2011; Knutson et al., 2004; Smalling et al., 2015). There is also emerging evidence that some amphibian species located in close proximity to agricultural areas may evolve tolerance to pesticides over time (Cothran et al., 2013; Hua et al., 2013a; Hua et al., 2013b). However, assessments of long-term health and survival are necessary as the populations may still be under chronic stress following sub-lethal exposures to agricultural contaminants, leading to increased risk of disease, reduced survivorship and negative effects on reproduction (Smalling et al., 2015).

There is a large amount of research on the effect of pesticides and fertilisers on amphibian health both in laboratory and field studies, with detrimental effects recorded on growth, survival, reproduction, development, and immune function, examples of which are presented in Table 1 (reviewed in Mann *et al.* 2009, Orton and Tyler 2014, Egea-Serrano *et al.* 2012 and Sparling *et al*. 2010). Naturally, there exists considerable variation between different species, life stages and the agricultural chemicals studied (Mann et al., 2009).

### 2.2.1. Fungicides

In the UK, the largest quantities of plant-protection pesticides are used in arable agriculture; with crops treated including wheat, barley, oats, rye, triticale, oilseed rape, linseed, ware and seed potatoes, dry harvest pea, field beans, and sugar beet, with wheat covering the largest area (45%). Of the products used, fungicides account for the largest area treated (40%), with chlorothalonil, an organochlorine fungicide, and tebuconazole, a triazole fungicide most widely applied. Herbicides account for the next largest area (31%), with glyphosate and iodosulfuron-methylsodium/mesosulfuron-methyl used the most frequently (Garthwaite et al., 2015).

Although fungicides are the most widely used plant-protection product in temperate regions, this is not reflected in the number of research studies conducted on their effects in non-target species such as amphibians (Ghose et al., 2014; Reilly et al., 2012; Smalling et al., 2013; Sparling et al., 2010), with fewer still examining the chronic sub-lethal effects. Of the studies available, there is an indication that fungicides may have deleterious effects in amphibians such as decreased growth, increased mortality, morphological abnormalities, delayed metamorphosis, reduced swimming abilities and an altered immune response (Belden et al., 2010; Bernabò et al., 2016; Di Renzo et al., 2011; Ghose et al., 2014; Groppelli et al., 2005; Hartman et al., 2014; Hooser et al., 2012; Junges et al., 2012; McMahon et al., 2011; Menegola et al., 2006; Papis et al., 2006; Teplitsky et al., 2005; Yoon et al., 2008).

Types of fungicides include strobilurins, azoles and imidazoles, benzimidazoles, chloronitriles, carbamates, carboxamides and morpholines; Table 2 shows a list of commonly used fungicides in the UK and their mechanism of action.

**Table 2.** List of commonly used fungicides in the UK and their mechanism of action (Garthwaite et al., 2015)

| Chemical Group | Examples | Mode of Action |
| --- | --- | --- |
| Strobilurin | Azoxystrobin<br>Fluoxastrobin<br>Picoxystrobin<br>Trifloxystrobin<br>Pyraclostrobin | Quinone Outside Inhibitors (QoI), which inhibit respiration. |
| Triazole | Cyproconazole<br>Difenoconazole<br>Epoxiconazole<br>Fluquincazole<br>Flusilazole<br>Metconazole<br>Tebuconazole | Inhibit sterol biosynthesis |
| Imidazole | Prochloraz | Inhibit sterol biosynthesis |
| Triazolinthione | Prothioconazole | Inhibits sterol biosynthesis |
| Benzimidazole | Carbendazim | Inhibits microtubule assembly and therefore cell division |
| Cyanoimidazole | Cyazofamid | Inhibits respiration |
| Pyrazolium | Bixafen<br>Fluxapyroxad<br>Isopyrazam | Succinate dehydrogenase inhibitor |
| Carbamate | Mancozeb | Disrupts lipid metabolism |
| Carboxamide | Boscalid<br>Penthiopyrad | Succinate dehydrogenase inhibitor, inhibits spore germination. |
| Benzamide | Fluopicolide | Affects spectrin-like proteins in the cytoskeleton of oomycetes |
| Mandelamide | Mandipropamid | Inhibits spore germination with preventative action. Inhibits cellulose synthesis. |
| Phthalimide | Folpet | Inhibits cell division |

| | | |
|---|---|---|
| Morpholine | Dimethomorph<br>Fenpropimorph<br>Spiroxamine | Inhibits ergosterol biosynthesis |
| Chloronitrile | Chlorothalonil | Binds to glutathione, disrupting cellular respiration |
| Phenylpyridinamine | Fluazinam | Uncoupler of oxidative phosphorylation in mitochondria |
| Anilinopyrimidine | Cyprodinil | Inhibits protein synthesis |
| Quinazolinone | Proquinazid | Translocates and inhibits appressoria (specialised fungal cell used to infect host plant) development, stopping infections. |

Among the fungicides, triazole compounds are perhaps the most studied due to their usage in both agriculture and medicine, where they are used to treat mycoses and as non-steroidal anti-estrogens in the treatment of some breast cancers (Menegola et al., 2006). Triazole fungicides share the structural element of a five-membered azole ring containing two carbon and three nitrogen atoms. Triazole fungicides act by blocking the synthesis of the essential cell membrane component ergosterol in yeast and fungi, which in turn affects the structure of the plasma membrane making it susceptible to further attack (Chambers et al., 2014; Georgopapadakou, 1998). This is achieved through inhibition of the Cytochrome-P450-mediated (CYP51, lanosterol-14-demethylase) conversion of lanosterol to ergosterol, leading to an accumulation of lanosterol and other 14-methylated sterols and a depletion in ergosterol, which changes the shape and properties of the plasma membrane (Menegola et al., 2006).

There have been notable developmental effects of azole fungicides in non-target organisms including amphibians, where they have been associated with developmental problems such as skeletal defects, craniofacial abnormalities and hydrocephaly (Bernabò et al., 2016; de Jong et al., 2011; Di Renzo et al., 2011; Groppelli et al., 2005; Menegola et al., 2006; Papis et al., 2006). Recent studies have demonstrated the accumulation of triazole fungicides in the tissues of amphibians and evidence of endocrine disruption at low concentrations (Hansen et al., 2014; Poulsen et al., 2015; Smalling et al., 2013).

Other fungicides associated with negative effects in amphibians include chlorothalonil, which led to high mortality rates and elevated corticosterone levels in *R.sphenocephala, O.septentrionalis* and *H.squirella* tadpoles following exposure at the expected environmental concentration (McMahon et al., 2011). Strobilurin fungicides such as pyraclostrobin at environmentally relevant concentrations have demonstrated acute mortality rates of up to 100% in *Bufo cognatus* tadpoles (Belden et al., 2010; Hooser et al., 2012), with chronic effects on development, growth and mortality also noted at sub-lethal concentrations (Hartman et al., 2014). However, sensitivity to strobilurins has also been shown to vary between amphibian species (Junges et al., 2012).

## 3. Life stages of anuran amphibians and sensitivity to environmental pollutants

Exposure to pollutants across amphibian life stages potentially varies significantly due to the different interactions with the environment associated with each stage of metamorphosis (egg to larvae, larvae to metamorph etc) (Sparling et al., 2010). Both laboratory and field studies have indicated varying sensitivities to pollutant exposure across different stages of development in amphibians.

### 3.1 Life cycle of anuran amphibians

According to Gosner (1960), there are 46 stages in the metamorphosis of anuran amphibians (frogs and toads), from fertilisation of the embryo through to emergence as a frog. A simplified life cycle of *R.temporaria*, showing embryos after fertilisation is shown in Figure 1. After fertilisation, the eggs take 10-14 days to develop depending on temperature. Stages 1-25 comprise the embryonic pre-feeding stages, with stages 21-23 the period at which full development of the external gills occurs. Tadpoles hatch and remain on the surface of the spawn clump or attached to plants until the yolk is fully absorbed (Beebee and Richard, 2000). Stages 23-25 represent when the external gills disappear and the operculum develops, covering the internal gills. At this stage the tadpoles become free-swimming and free-feeding and disperse

from the spawn site. After this stage the hind limb buds begin to develop (stages 26-30), followed by the development of the toes of the hind limbs from stages 31-38 and subarticular tubercles at stages 39-40. Metamorphosis rapidly occurs following the emergence of the front limbs and reabsorbtion of the tail from stages 41-46 (Gosner, 1960). Froglets appear usually in the middle of summer and grow in size before entering hibernation. Frogs reach sexual maturity and 2-3 years of age and at a minimum size of 50 mm (Beebee and Richard, 2000).

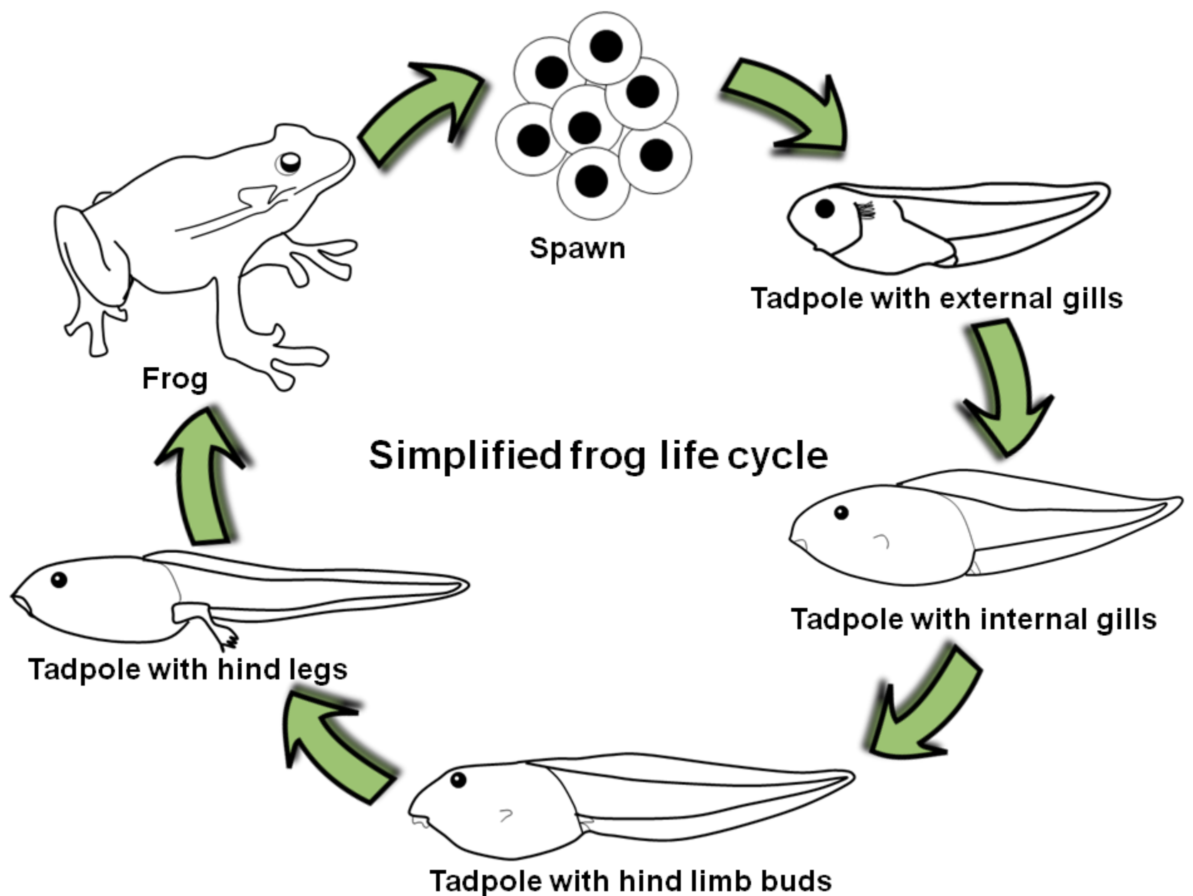


**Figure 1.** Simplified life cycle of the common frog, *Rana temporaria*.

## 3.2. Embryos

Eggs of amphibians can be used to perform biological and chemical assays; this practice is usually regarded as non-destructive and is therefore favoured to reduce the number of animals used in experiments (Scholz et al., 2013; Walker, 1998). However, embryos are generally considered a less sensitive stage of development due to the jelly coat and perivitelline membrane that surrounds the embryo. The jelly coat is

composed of mucin-type glycoproteins, which are highly glycosylated molecules synthesised by oviduct cells and which play an important part in the fertilisation process, species recognition and as protection to the developing embryo (Maes et al., 1997). The jelly layers vary in thickness and viscosity depending on species (Hatch and Burton, 1998) and act like a barrier to a number of chemicals (Berrill et al., 1994; Berrill et al., 1998; Brodeur et al., 2009; Edginton et al., 2007; Hall and Swineford, 1980; Wagner et al., 2015). However, in some this cases, this gelatinous matrix can react with chemicals and become more toxic, for example solvent toxicity in *R.temporaria* embryos was enhanced by the presence of the jelly coat (Marquis et al., 2006). The jelly coat may also not offer sufficient protection against contaminants such as α-cypermethrin, where significant abnormalities occurred following exposure (Greulich and Pflugmacher, 2003).

Embryos may also show less sensitivity to pollutants which affect the nervous system, as the nervous system at this early ontogenic stage is not yet fully developed (Ortiz-Santaliestra et al., 2006). This is also true of nitrates, which are transformed into the more toxic nitrites and nitrosamines by gut bacteria, thus incomplete differentiation of the gut in early life stages may have a protective effect (Egea-Serrano et al., 2012)

## 3.3. Tadpoles

Earlier larval stages of amphibians are generally regarded at the most sensitive life stage when assessing the effects of environmental pollution (Anguiano et al., 1994; Berrill et al., 1994; Berrill et al., 1998; Cooke, 1972b; Greulich and Pflugmacher, 2003; Ortiz-Santaliestra et al., 2006). Tissue and organ development begins at fertilisation, continuing through to metamorphosis; this incomplete organ and tissue differentiation could make earlier larval stages more sensitive to chemical contamination (Egea-Serrano et al., 2012). Differences in the sensitivity of larval stages may also be due to changes in surface area: volume ratios, permeability of the skin and metabolism (Ralph and Petras, 1998b). However, a recent meta-analysis found no difference in sensitivity to pollutants between embryos and larvae (Egea-Serrano et al., 2012). The authors stated this may be due to a lack of carry-over effects, as many studies only lasted until the embryos hatched. Indeed, there is

evidence that an earlier exposure to pollutants at the embryonic stage of development results in detrimental effects, such as sexual differentiation and deformities that may only be apparent at metamorphosis (Bridges, 2000; Orton and Routledge, 2011; Orton and Tyler, 2014).

Later larval stages of tadpoles, i.e. pro-metamorphic tadpoles with emerged hind legs may be more tolerant to pollutants than earlier stages. For example, *Rhinella arenarum* tadpoles were more sensitive to atrazine exposure at Gosner stage 25 in comparison to those exposed at stage 38-39 (Brodeur et al., 2009). This differential sensitivity may be due to a higher metabolic detoxification ability in tadpoles at a later stage of development (Bucciarelli et al., 1999). However, there are studies which demonstrate increased sensitivity of later larval stages in comparison to earlier larval stages after exposure to certain contaminants. For example, Howe *et al.* (1998) exposed larval *Rana pipiens* and *Bufo americanus* at an early (Gosner stage 29) and late (Gosner stage 40) of development to the herbicides alachlor and atrazine, with the finding that both species were more sensitive to both chemicals when exposed at the later stage of development. This was suggested to be due to the complex changes that are taking place during this stage as the tadpole enters metamorphosis, adding extra physiological and developmental stress (Howe et al., 1998). A recent study has demonstrated differential sensitivity in larval *Hyla intermedia* exposed to the fungicides tebuconazole and pyrimethanil throughout the development period, with low concentrations (5 µg/L) associated with greater effects on survival and deformity incidences than high concentrations (50 µg/L) if exposure occurred prior to metamorphic climax. During metamorphic climax, there was a clear dose-response (Bernabò et al., 2016). Therefore, there is likely to be differential sensitivity between life stages as well as between species, depending on the chemical to which larval amphibians are exposed, thus it is critical to consider different life stages in any monitoring study.

## 3.4. Amphibian cell lines

The use of cell cultures in environmental risk assessment is a growing area, which allows the number of vertebrates used in experimental procedures to be reduced in line with the 3R principles – replacement, reduction and refinement, of animal tests (Scholz et al., 2013). In addition, the key interaction between chemical contaminants and organisms initially occurs within cells, therefore determining the effects of environmental pollutants at the cellular level is of great importance, in order to elucidate mechanistic information (Fent, 2001). Using cell lines is cost-effective, reduces waste and allows multiple experiments to be conducted, including those using chemical mixtures, and is thus less time-consuming than using whole organisms (Schirmer, 2006). However, there may be issues with extrapolation from cell lines to whole organisms, with toxicity sometimes underestimated (Schirmer, 2006; Walker, 1998).

There are already a large number of studies confirming the use of several fish cell lines in the *in vitro* cytotoxicity testing of chemicals (Fent, 2001; Schirmer, 2006; Walker, 1998). Although fewer in number, there are also studies using amphibian cell lines to determine the effects of environmental contaminants at the cellular level. Available amphibian cell lines are those derived from *Xenopus laevis*: A6 and XLK-WG, both epithelial cell lines derived from the kidney of *X.laevis* (Martin et al., 1998; Rafferty Jr, 1969), the fibroblast FT cell line, derived from *R.catesbeiana* tongue (Wolf and Quimby, 1964), and the ICR-2A/-134 fibroblast cell lines derived from *R.pipiens* embryos (Freed and Mezger-Freed, 1970). Of the small number of continuous cell lines available, A6 cells are most widely used, as they are well-characterised, simple to culture, growing at 22°C without carbon dioxide, and have a population doubling time of 24-36 hours (Ikuzawa et al., 2007; Kitamoto et al., 2005; Perkins and Handler, 1981; Rafferty Jr, 1969). A6 cells have previously been used in toxicity studies, measuring responses such as levels of heat shock proteins (HSPs), intracellular calcium and cell cycle progression after exposure to a variety of environmental contaminants, including toxic metals, detergents, and nanoparticles (Bjerregaard, 2007; Bjerregaard et al., 2001; Brunt et al., 2012; Darasch et al., 1988; Faurskov and Bjerregaard, 1997; Faurskov and Bjerregaard, 2000; Faurskov and Bjerregaard, 2002; Heikkila et al., 1987; Khamis and Heikkila, 2013; Khan et al., 2015; Music et al., 2014; Thit et al., 2013, 2015; Woolfson and Heikkila, 2009; Young

et al., 2009; Yu et al., 2007). The use of cell lines alongside methods involving whole organisms thus provides further mechanistic information, as well as reducing the number of animals used in research.

## 4. Amphibians as biological indicators

Amphibians are frequently regarded as sentinels or biological indicators of water quality due to their high sensitivity to environmental pollution (Blaustein et al., 1994; Burkhart et al., 2000). Small bodies of water like those frequented by amphibians are often the first to be impacted by contaminants, and also tend to be closed systems, thus an accumulation of contaminants may occur (Ralph and Petras, 1997).Tadpoles in particular go through many physiological and anatomical changes during development, therefore the risk of deleterious changes occurring during larval development is increased (Cooke, 1981). The use of an indicator species in the field is of benefit in situations where a broad spectrum of contaminants may be detected, such as that from agricultural or wastewater run-off. Analysis of water and sediment samples is expensive and knowing the concentrations of contaminants in such samples does not necessarily give information concerning biological relevance (Cooke, 1981). Additionally, whilst exposure to single agents at concentrations found in the environment may not elicit a harmful response, repeated exposures and the exposure to multiple agents at environmental concentrations, as found in the field may have deleterious consequences (Hayes et al., 2006; Hua and Relyea, 2014; Relyea, 2009; Relyea and Diecks, 2008). Laboratory studies also tend not to replicate the complexity of natural habitats (Ralph and Petras, 1998a; Relyea and Mills, 2001; Thompson et al., 2004). Using an indicator species in the field therefore represents a more environmentally-realistic scenario, although there are limitations to this approach due to the inability to control the heterogeneous environment of natural systems (Rowe and Dunson, 1994).

## 4.1. Suitability of the common frog as a biological indicator

The common frog has numerous characteristics that make it a suitable indicator species, among them a widespread distribution, high reproductive output and ability to exploit multiple habitats and it has been previously used in several studies to assess environmental impacts (Beebee, 1979; Cooke, 1981; Piha et al., 2009).

### 4.1.1. Identification

There are few native species of amphibian in the UK; in addition to *R.temporaria*, there is the common Toad*, Bufo bufo*, the pool frog *Pelophylax lessonae*, and the Natterjack Toad, *Epidalea calamita,* which comprise the anurans. There are also three species of newt (urodeles): the Great-crested Newt, *Triturus cristatus,* the smooth newt, *Lissotriton vulgaris* and the palmate newt, *Lissotriton helveticus*. This makes identification relatively simple; frog and toad spawn is distinguished by appearance, with frogs producing clumps of spawn in shallow water, as shown in Figure 2A, whereas toads produce long thin strings of spawn wrapped around vegetation in relatively deep water (Beebee and Richard, 2000). Pool frogs were reintroduced to two sites in Norfolk in 2005 (Beebee, 2014) and breed later in the year than the common frog, making the distinction between these two species straightforward. Newts lay single eggs on the underside of leaves and their larvae look identical to the adult newts making them quite distinct from anuran species. Tadpoles of frogs and toads are distinguishable by appearance and behaviour, with tadpoles of *R.temporaria* developing flecks of brown/gold as they grow (Fig. 2B), whereas toad larvae remain uniformly black. Common frog tadpoles do not shoal, in contrast to tadpoles of the common toad, which often shoal in open water (Beebee and Richard, 2000). This makes using *R.temporaria* spawn or tadpoles as a monitoring species a relatively simple process.

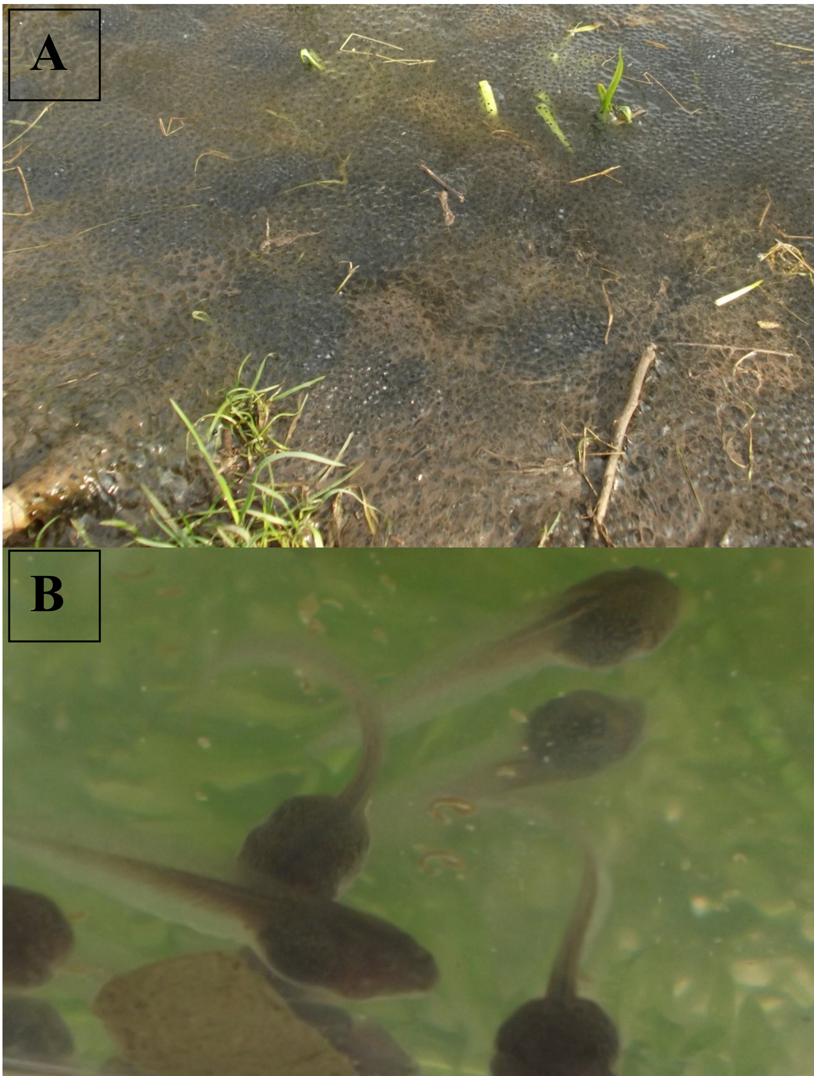


**Figure 2.** *Rana temporaria* spawn (A) and tadpoles (B).

## 4.1.2. Reproduction

Breeding of *R.temporaria* in the UK generally occurs in early Spring, however there is considerable variation in spawning dates with frogs in Southern Britain spawning as early as November, whereas those in Northern Britain may spawn as late as April, with rising temperature used as a cue to begin spawning (Scott et al., 2008). Spawning occurs in shallow, small water bodies, including temporary ponds (Cooke, 1975). *R.temporaria* has a high reproductive output and is regarded as an 'explosive breeder', completing spawning within 1-2 weeks unless interrupted by cold weather (Beebee and Richard, 2000). Each clutch of spawn comprises around 700-2000 eggs and averages 1300 eggs per clump (Cummins, 1986; Ryser, 1996). Figure 3 shows *R.temporaria* spawning in a shallow pond in March.

Metamorphosis takes 40-80 days from fertilisation, with water temperature, population origin and population density causing variation in the length of time development takes (Loman, 2002; Scott et al., 2008). *R.temporaria* shows high site fidelity, and has a summer home range close to the breeding pond, allowing the same populations to be studied each year, making it an ideal species for longer term monitoring studies (Cummins, 1986; Haapanen, 1970).

**Figure 3.** *Rana temporaria* photographed breeding in March in the UK.

### 4.1.3. Routes of exposure to environmental pollutants

The common frog may be useful as an indicator species due to its widespread distribution in the UK (and much of Europe), where it forms an important part of ecological communities (Johansson et al., 2006). This species is regarded as a habitat generalist (Van Buskirk, 2005), capable of exploiting both urban and agricultural environments and thus has the potential to be exposed to a variety of environmental contaminants . Like most species of amphibian, it has a biphasic life cycle, with spawning and larval development occurring in the aquatic environment in Spring and Summer, whilst metamorphosed frogs lead a predominantly terrestrial lifestyle, returning to ponds for breeding; thus there is the possibility of both aquatic and

terrestrial exposure routes. The period of larval development coincides with the application of pesticides to adjacent land, with tadpoles potentially exposed following pesticide spray drift or run-off following heavy rain (Johansson et al., 2006). Larval *R.temporaria* are filter feeders, which allows them to exploit a wide range of food sources in detritus, algae and sediment and thus play an important role in aquatic ecosystems (Viertel, 1990). The combination of a restriction to an aquatic environment and their feeding behaviour mean that tadpoles stand to have a greater exposure to pollutants than their terrestrial counterparts (Roe et al., 2005; Semlitsch, 2000).

### 4.1.4. UK research on the effect of pollutants on native amphibians

Overall numbers of *R.temporaria* are not thought to be declining; however some populations may be susceptible to local extinctions, with pollution examined as a possible reason. As well as the loss in habitat driven by agricultural intensification, it was postulated that certain areas in Britain may have had declines in numbers of *R.temporaria* and *Bufo bufo* due to usage of pesticides in the 1950s and 1960s in highly agricultural areas (Beebee, 2014; Cooke and Ferguson, 1976). Several studies were conducted around this time using native amphibian species in the laboratory and *in situ* to investigate the effects of pesticides and fertilizers in common usage with the finding that acute and chronic levels of these contaminants can have deleterious effects on larval amphibians, including altered development and abnormal feeding behaviour, as well as direct mortality at high concentrations (Cooke, 1970, 1972b, 1973a, b, 1977, 1981; Oldham et al., 1997; Osborn et al., 1981). Since these studies, there has been relatively little research conducted on the effect of contaminants on native amphibian species in the UK despite the continued production of new pesticides (Beebee, 2014), and the projected increase in environmental pollution due to a growing human population and therefore increased agricultural and industrial activities (Tilman et al., 2001). The small numbers of studies conducted in the UK recently have noted significant effects on development, growth, sexual differentiation and reproductive physiology in a native species (the common toad, *Bufo bufo)* in areas of high agricultural intensity and  high human impact (Orton et al., 2014; Orton and Routledge, 2011).

## 5. Biospectroscopy

Infrared spectroscopy is a powerful and sensitive technique which has been previously utilised in chemistry and physics to identify the molecular structure of unknown chemical entities, as well as providing quantitative information such as the concentration of a molecule in a sample (Smith, 2011). With advances in instrumentation and concurrent computational analysis, this technique is now widely applied in the characterisation of biochemical components in complex biological samples (Baker et al., 2014). Following exposure to chemical agents, commonly used toxicological measurements in cells and tissues include cell viability and proliferation assays (Fotakis and Timbrell, 2006; Soto et al., 1995), genotoxicity and carcinogenicity assays such as the Syrian Hamster Embryo (SHE) and micronucleus assays (Ahmadzai et al., 2012; Fenech, 2000), induction of CYP enzymes (Malins et al., 2006; Malins et al., 2004), changes in histological structures (Malins et al., 2006; Malins et al., 2004), and growth inhibition in plants (Wang, 1986). Whilst these assays are extremely useful, they may be labour-intensive, subjective, expensive and require the use of reagents which are potentially polluting to the environment. In contrast, spectroscopy approaches are rapid, often requiring minimal sample preparation and non-destructive, thus allowing samples to be used for subsequent applications (Ellis and Goodacre, 2006; Martin et al., 2010; Naumann, 2000). For example, the SHE assay is laborious and the visual scoring of transformed colonies may be prone to subjectivity, whereas use of FTIR spectroscopy in assessing cellular transformation is objective (Ahmadzai et al., 2012; Trevisan et al., 2010). Cytotoxicity assays such as the MTT assay use colorimetric methods to assess cell viability; in contrast IR spectroscopy does not require the use of reagents, allowing cells to be used for subsequent applications and produces comparable results (Fale et al., 2015). Results obtained using the E-screen assay to identify estrogenic compounds, whilst rapid in comparison to other methods (Soto et al., 1995), is still relatively time consuming, whereas FTIR spectroscopy may offer comparable sensitivity in a shorter time-frame (Johnson et al., 2014). Infrared spectroscopy also has the advantage of providing a metabolic fingerprint as it is able to analyse carbohydrates, lipids, proteins and amino acids simultaneously, thus providing integrated information in a short time frame (Ellis and Goodacre, 2006; Hu et al., 2016).

## 5.1. Background to Fourier-transform infrared spectroscopy

Infrared spectroscopy is the study of the interaction of infrared light with matter (Smith, 2011). An interferometer takes a beam of light, splits it into two beams and makes one beam travel a different distance to the other, known as the optical path difference. A Michelson interferometer is the most commonly used interferometer, and comprises four arms: one a source of IR light, the next a fixed mirror, the third a moving mirror and the fourth arm is open. Intersecting the four arms is a beamsplitter, usually comprised of potassium bromide, which transmits half of the IR radiation, striking the fixed mirror and reflecting the other half, which then strikes the moving mirror. Following this, the two light beams recombine at the beamsplitter and then leave the interferometer to interact with the sample and strike the detector, as shown in Figure 4. There are several choices available for the light source, including globar used in benchtop instruments (Miller and Smith, 2005), synchrotron, which offers a much brighter light source and improved resolution, thus allowing subcellular resolution (Diem et al., 2004), and quantum cascade lasers (QCLs), which preclude the necessity for an interferometer (Yeh et al., 2014). There are also a variety of choices for the detector, including 2D focal plane array (FPA), linear array and single element (Carter et al., 2009). FPA and linear array are used to generate image maps, whereas single element detectors are used to generate point spectra across a whole sample, therefore choice of detector is based on whether imaging or point spectra with a high signal to noise ratio is required (Miller and Smith, 2005).

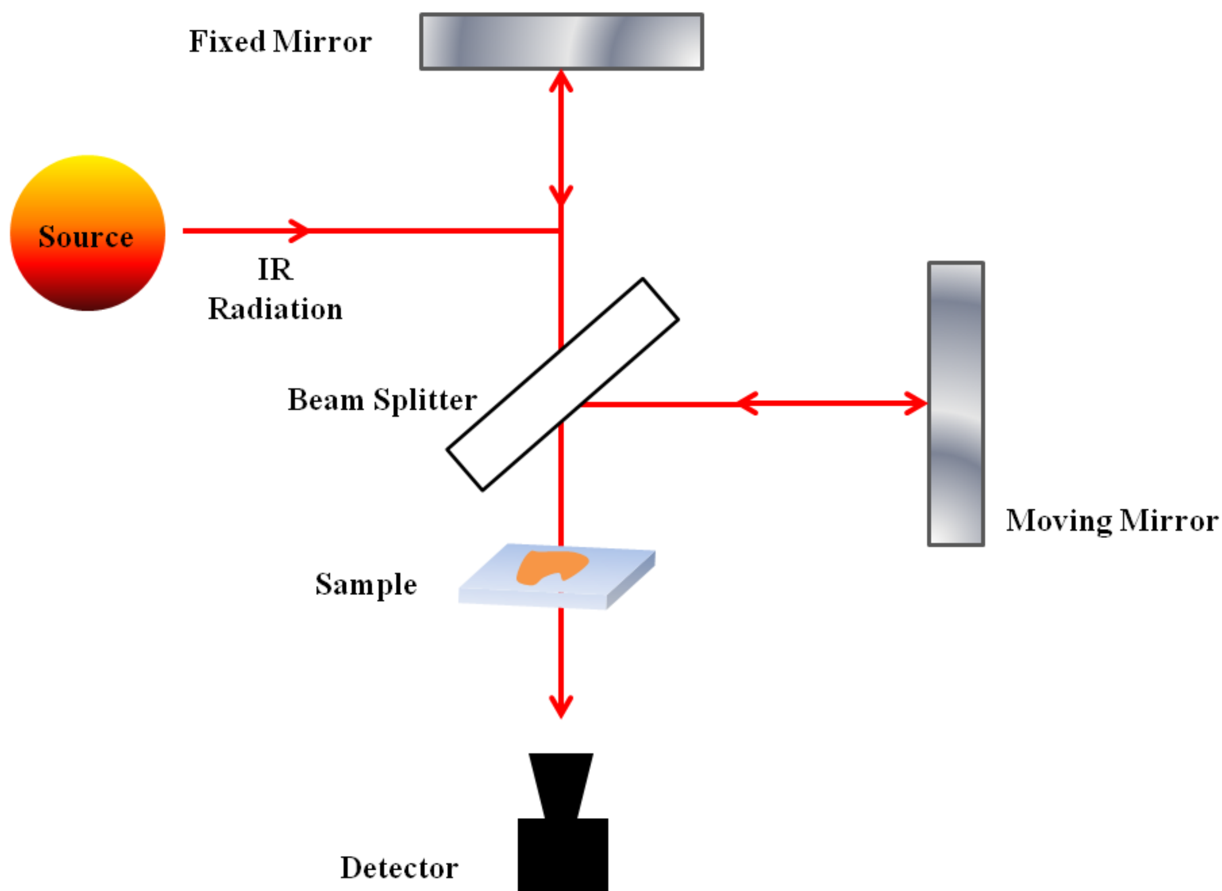


**Figure 4.** A schematic of a Michelson interferometer, the most commonly used in FTIR spectroscopy.

The measurement obtained from the detector is known as an interferogram and comprises a plot of light intensity versus optical path difference and is essentially a large number of sinusoidal waves added together. This interferogram undergoes a Fourier-transformation to give a spectrum, plotted as absorbance against wavenumber, as shown in Figure 5, hence Fourier-transform infrared (FTIR) spectroscopy (Smith, 2011). Here, the wavenumber of a wave of light is simply defined as the reciprocal of the wavelength; the wavelength is the distance between adjacent crests or troughs, hence the unit of wavenumber is $cm^{-1}$, and is a measure of the number of waves (crests or troughs) per centimetre. Spectra obtained from FTIR measurements are plotted from high wavenumber to low wavenumber, in order of decreasing energy intensity. The wavenumber positions of the peaks in the spectrum correspond to

molecular structure, and quantitative information may also be obtained, making an infrared spectrum highly information-rich (Smith, 2011).

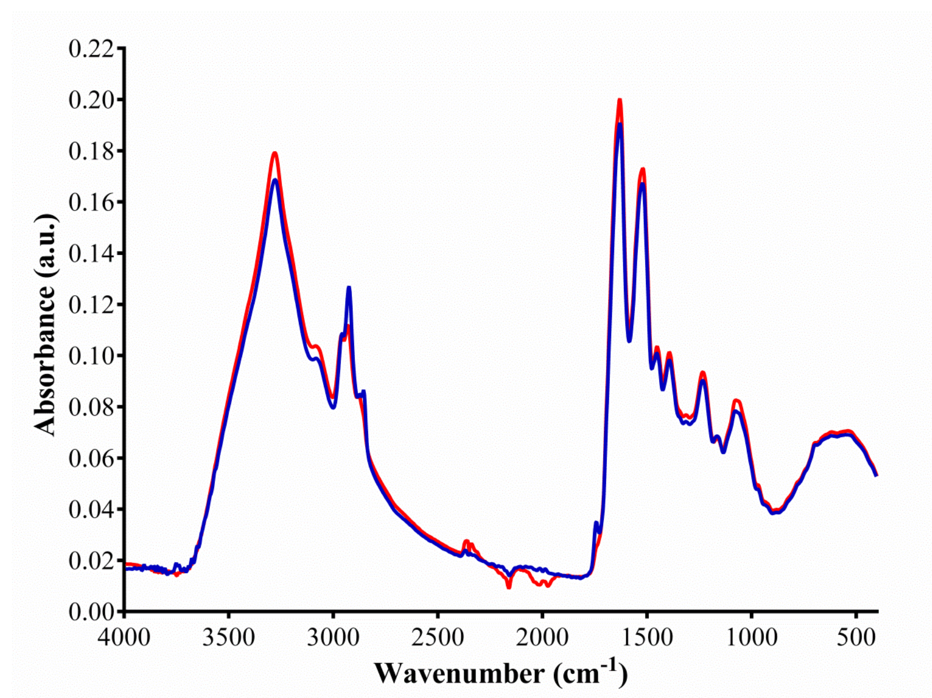


**Figure 5.** Representative raw FTIR spectra following Fourier -transformation of the interferogram. The sample analysed was *Rana* t*emporaria* kidney tissue.

## 5.2. Principles of FTIR spectroscopy

FTIR spectroscopy is based on the vibrations of the atoms of a molecule. Exposure of a sample to IR radiation will cause the functional groups within the sample to absorb the radiation and vibrate in a number of ways; stretching, bending, deformation or a combination of these vibrations (Ellis and Goodacre, 2006; Stuart, 2005). Examples of these vibrational modes are shown in Figure 6. IR spectroscopy is a measure of this absorption, with peaks in the spectrum corresponding to the chemical structure of a particular molecule (Kelly et al., 2011; Stuart, 2005).

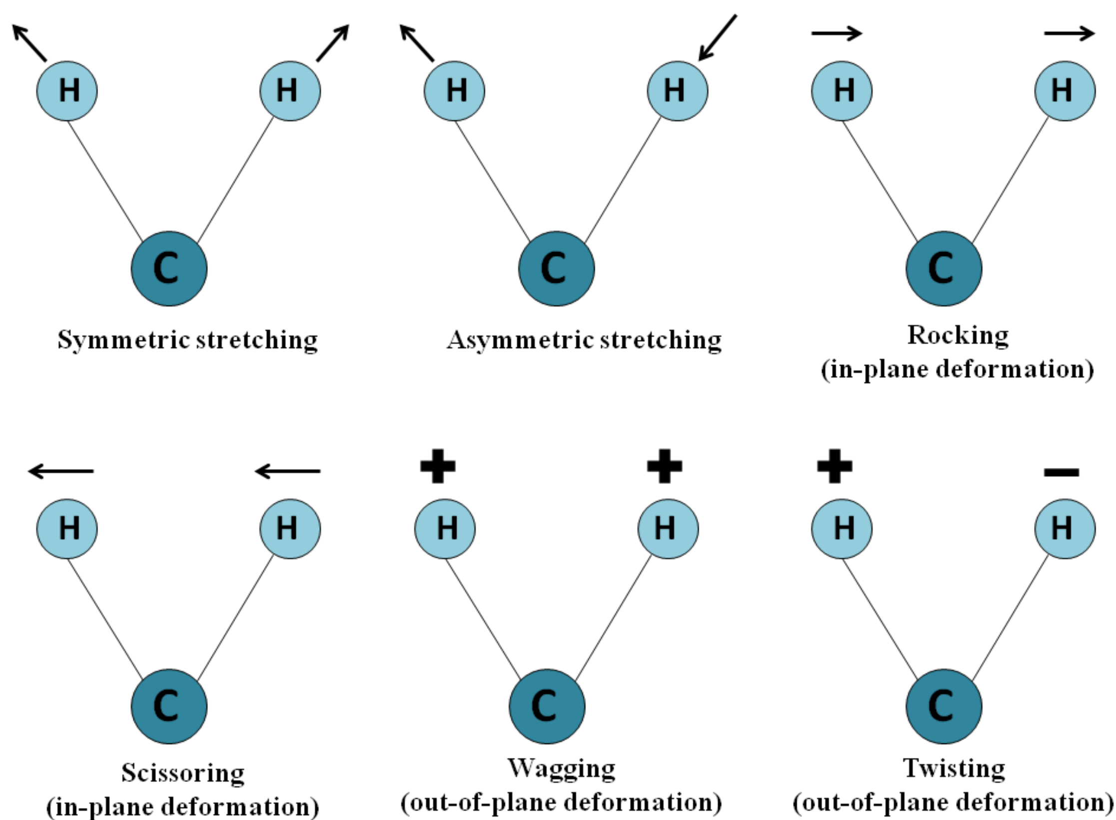


**Figure 6.** Vibrational modes in infrared spectroscopy.

The mid IR region (400-4000 cm$^{-1}$) of the electromagnetic spectrum is absorbed by biomolecules based on the chemical bonds present, with the range 1800-900 cm$^{-1}$ regarded as the biological fingerprint region and commonly used when analysing biological samples (Griffiths and De Haseth, 2007; Stuart, 2005). FTIR spectroscopy provides information down to the molecular-level, which allows the investigation of functional groups, types of vibrational mode and molecular conformations within biological samples (Movasaghi et al., 2008). The spectral bands obtained from the resulting spectra are molecule-specific, as shown in Figure 7, where the fingerprint region with corresponding assignment of major peaks is denoted; thus providing direct information about the biochemical composition of the sample (Movasaghi et al., 2008). The assignment of peaks can be difficult in complex biological systems where absorbance bands overlap, and thus can be considered tentative (Naumann, 2000); however, many studies have been conducted which have recorded marked similarities in the spectral interpretation of equivalent areas in the derived spectra, creating detailed tables of wavenumber assignments for diverse biological samples (Cakmak et

al., 2006; Movasaghi et al., 2008). Changes in the absorbance intensity or area of particular peaks can thus be correlated to histological structures and are directly related to the concentration of molecules in the sample (Yang et al., 2011). For example, decreases in the intensity of the glycogen band at 1042 $cm^{-1}$ and the asymmetric CO-O-C band at 1152 $cm^{-1}$, also due to glycogen in tissues, were observed in fish liver tissue exposed to 17β-estradiol in comparison to control fish. These decreases are due to the liver being the main site for gluconeogenesis in fish and may be related to the energy requirement of estradiol-induced vitellogenin synthesis (Cakmak et al., 2006).

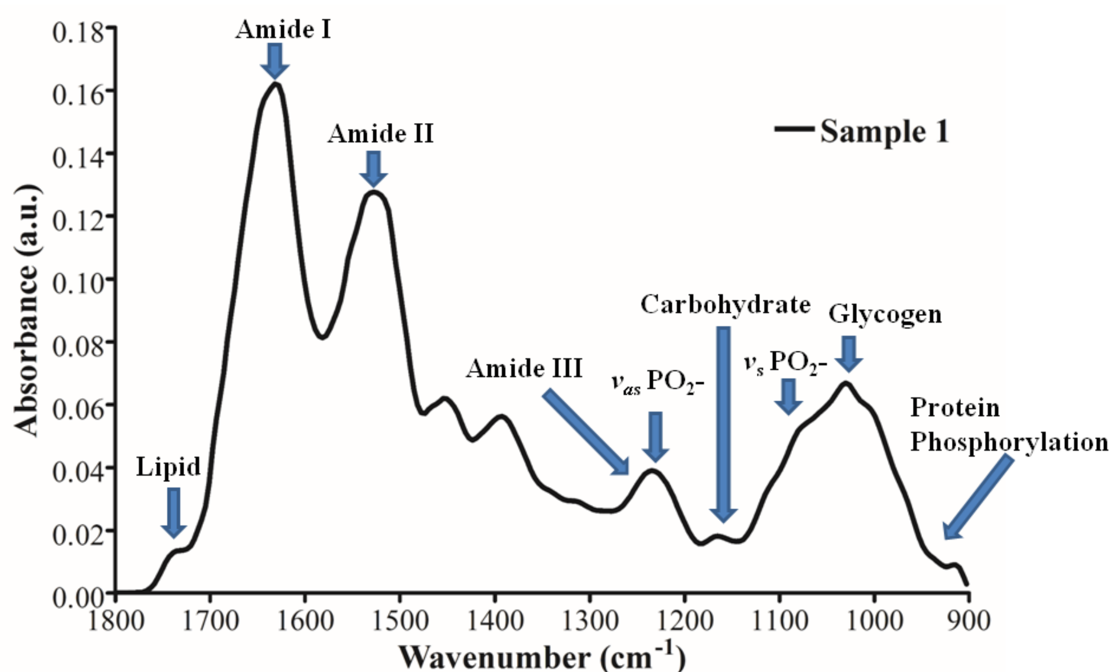


**Figure 7.** A representative spectrum obtained of the fingerprint region with chemical peaks tentatively assigned. The sample analysed was *Rana temporaria* kidney tissue using ATR-FTIR spectroscopy.

## 5.3. Sampling modes in FTIR spectroscopy

The three main sampling modes in FTIR spectroscopy are transmission, transflection and attenuated total reflection (ATR) as shown in Figure 8. In transmission (Figure 8A), the infrared beam is passed directly through the sample before it is detected; therefore the substrate must be of an IR transparent material such as barium fluoride or zinc selenide. This type of sampling mode has a high signal-to-noise ratio (SNR), but it is limited by the thickness of the sample, with samples greater than 20 μm thick absorbing too much radiation, making it impossible to obtain a spectrum. Therefore more time may be spent appropriately preparing samples for analysis (Baker et al., 2014; Smith, 2011). In transflection sampling modes (Figure 8B), the infrared beam is bounced off the sample, rather than passing straight through it. This sampling mode has the advantage of requiring less sample preparation than for samples prepared for transmission as the sample can be thicker than 20 μm. This technique is also non-destructive, allowing samples to be used for other applications. However, the depth to which the infrared beam penetrates into the sample is not accurately known, plus the surface of the sample contributes more than the bulk of the sample, as the depth of penetration varies between 1-10 μm, thus giving variable results (Smith, 2011). Attenuated total reflection Fourier-transform infra-red spectroscopy (ATR-FTIR) (Figure 8C) is based on the same principles as transmission and transflection, however, it uses a different technique to transmit the IR and obtain the absorbance values (Kazarian and Chan, 2006). ATR-FTIR spectroscopy is carried out using an accessory that fits into the sample compartment of an FTIR. Within the accessory is a crystal of IR transparent material with a high refractive index, usually composed of zinc selenide, germanium or diamond, known as the internal reflection element (IRE) (Smith, 2011). The IR radiation is focussed onto the face of the crystal using mirrors on the accessory. Under the right conditions, that is, the crystal has the correct refractive index and the light has the correct angle of incidence, the IR radiation, instead of leaving the crystal undergoes total internal reflection. As the IR radiation is inside the crystal, a standing wave of radiation known as an evanescent wave is created. The evanescent wave is slightly larger than the crystal and so penetrates a small distance beyond the crystal surface into the sample. The sample in close contact with the crystal, interacts with the evanescent wave, absorbs the IR, and an IR spectrum is detected. The evanescent wave is attenuated by the absorbance of the

sample, hence attenuated total reflectance (Kazarian and Chan, 2006; Smith, 2011). ATR-FTIR, in contrast to FTIR, is unable to distinguish cell type as it is unable to differentiate cellular characteristics at a microscopic level (Martin et al., 2010). However, the main advantage of ATR-FTIR spectroscopy is the minimal sample preparation that is required, as the penetration depth of IR light in the sample is independent of sample thickness (Kazarian and Chan, 2006). While FTIR spectroscopy is subject to interference from water in the mid-IR region, this is not the case with ATR-FTIR and as such it is more suitable for a wider variety of biological specimens (Winder and Goodacre, 2004).

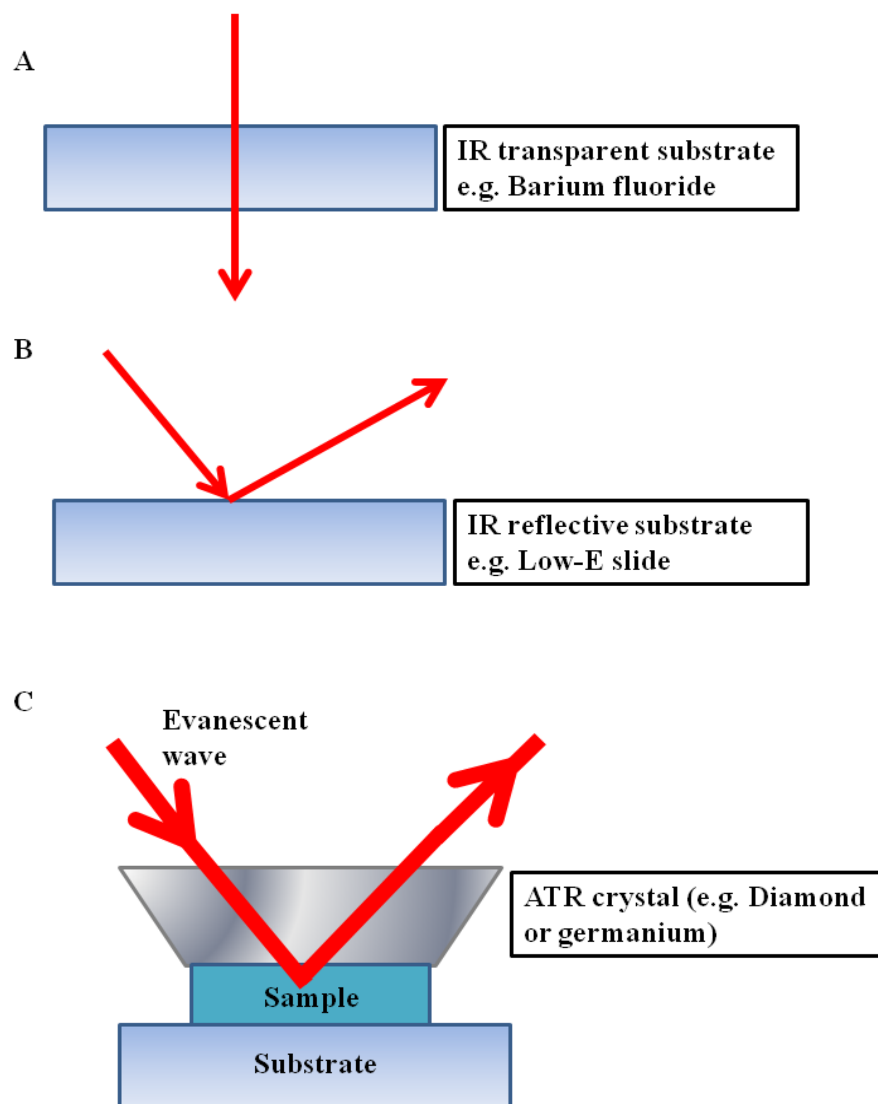


**Figure 8.** Sampling modes in FTIR spectroscopy. **A.** Transmission. **B.** Transflection. **C.** Attenuated total reflection (ATR).

## 6. Data analysis

Large amounts of complex data are generated following spectral acquisition, which require robust methods to extract key information from the resulting spectra. This requires pre-processing which removes unwanted spectral artefacts from the data sets, and data analysis methods which reduce the inherent complexity of the IR spectrum, whilst retaining its most useful features.

### 6.1. Spectral pre-processing

Following spectral acquisition, pre-processing of the raw data is an important step for subsequent data analysis, particularly when using classification models. An overview of commonly used spectral pre-processing steps is shown in Figure 9. Pre-processing raw data aims to improve the robustness and classification accuracy of the acquired spectra and to ease the interpretability of complex data sets.

Prior to any corrections to the baseline or normalisation, data are often cut to particular areas of interest, most commonly the region 1800-900 $cm^{-1}$, which is known as the 'fingerprint region', and is an information-rich area in biological samples. Other areas of interest may include the regions 3600-3030 $cm^{-1}$ and 3030-2800 $cm^{-1}$ (Cakmak et al., 2006). Unwanted spectral contributions may arise from carbon dioxide, paraffin (in paraffin-embedded samples) and changes in atmospheric conditions. Carbon dioxide and paraffin contributions (~2,954 $cm^{-1}$, 2,920 $cm^{-1}$, 2,846 $cm^{-1}$, 1,462 $cm^{-1}$ and 1,373 $cm^{-1}$) can be dealt with by cutting these regions out of the spectrum, (Baker et al., 2014). Changes in atmospheric conditions can be accounted for by taking a background reading before the acquisition of spectra from the sample commences; this is then automatically subtracted from the resulting spectrum.

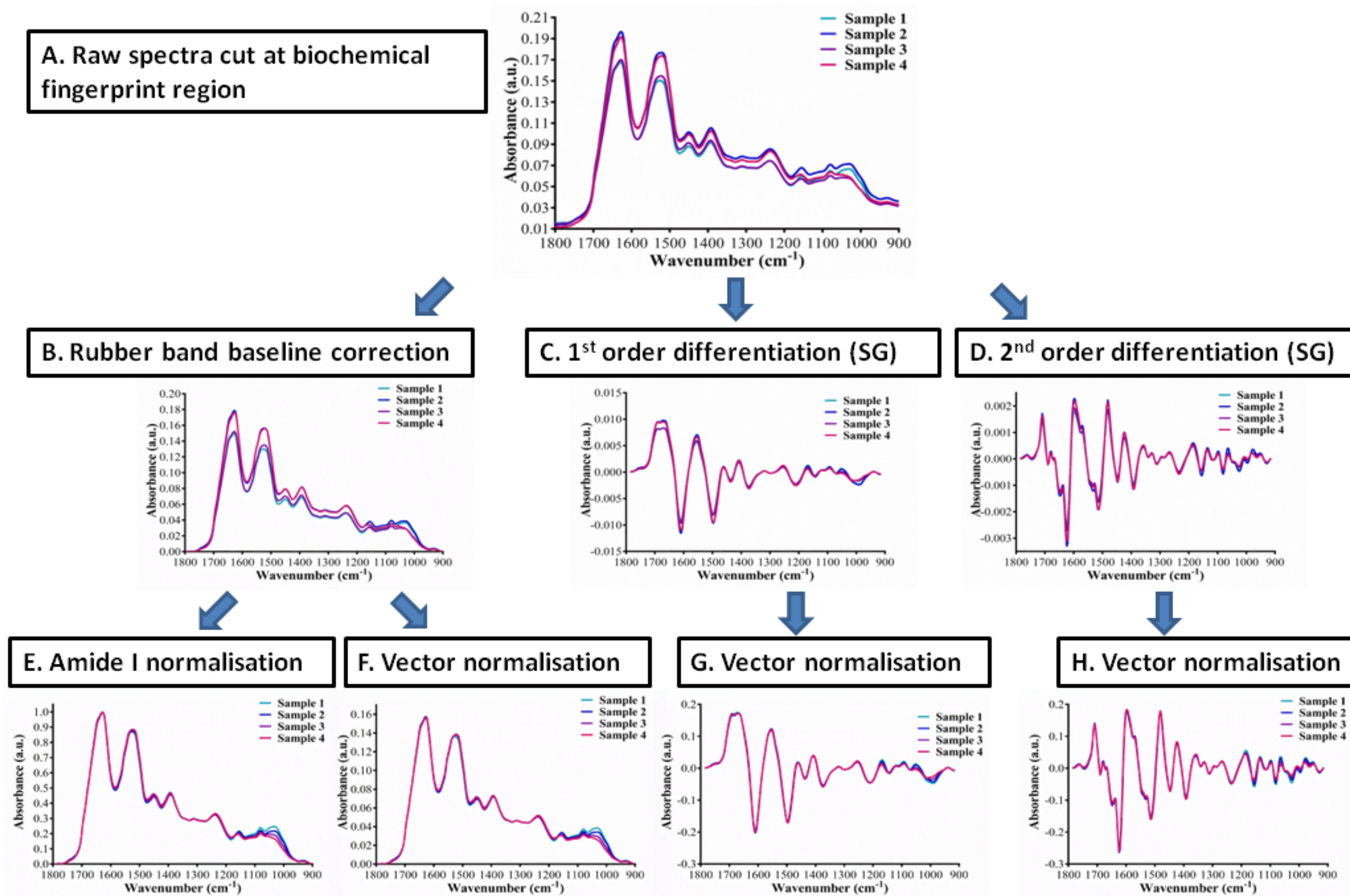


**Figure 9.** Overview of commonly used pre-processing steps in infrared spectroscopy. The samples analysed are *Rana temporaria* muscle

### 6.1.1 Baseline correction

A step necessary to many datasets is baseline correction, due to a sloped or oscillatory baseline. Typical mean raw ATR-FTIR spectra cut to the fingerprint region prior to any baseline correction is shown in figure 9A. Spectral baselines may be distorted as a result of scattering, substrate absorption, alterations in experimental conditions during data acquisition and variability in instrumental parameters (Kelly et al., 2011; Lasch, 2012). All methods for baseline correction aim to reduce baseline distortions, unwanted spectral offsets and positive and negative sloping (Lasch, 2012). Frequently used methods to correct sloping baselines include rubber band baseline correction and differentiation (either 1$^{st}$ or 2$^{nd}$ order).

Rubber band baseline correction operates by dividing the spectrum into ranges of equal size, the minimum $y$-value is then found in each range. The baseline is then created by connecting these minima with a straight line. Starting from below, the rubber band (a convex polygonal line) is stretched over this curve, which becomes the baseline. Any points which do not lie on this baseline are discarded (Baek et al., 2015). An advantage of this method is the ease of interpretability, as the spectra still retain the appearance of the original spectrum (Kelly et al., 2011). Mean spectra following rubber band baseline corrections are shown in figure 9B.

Differentiation (usually 1$^{st}$ or 2$^{nd}$ order) is a technique also employed as a baseline correction, as constant and linear components of baseline errors are removed in the differentiation, thus allowing quantitative information to be obtained from the spectrum (Rieppo et al., 2012; Trevisan et al., 2012). Differentiation also has the advantage of resolving overlapping peaks in the spectrum so that more detailed analysis of individual peaks is possible (Rieppo et al., 2012). For broad spectra the derivative intensity decreases with increasing derivative order, whereas for sharp spectra, the reverse is true. Therefore the underlying shape of the spectrum determines the intensity of the derivative spectrum, with flat peaks decreasing in intensity with each derivative order, and sharp peaks increasing in intensity, thus allowing small sharp peaks overlapped by broad flat peaks to be exposed (Kus et al.). However, the magnitude of spectral noise is increased with each differentiation order (Rieppo et al., 2012); therefore a smoothing technique, most frequently the Savitzky-Golay (SG) algorithm is applied at the same time as the application of differentiation (Savitzky

and Golay, 1964; Trevisan et al., 2012). The original shape of the spectrum is also changed when using differentiation (Kelly et al., 2011). First derivatives cross the *X*-axis at the wavelength where the absorbance peak is at its maximum value and has maximum values either side of this in the positive and negative directions. The second derivative has its maximum value at the same wavelength as the underlying peak, in the negative direction, and is flanked by two positive artefact peaks (Mark and Workman Jr, 2010). First and second derivatives of the raw mean spectra shown in figure 9A are shown in figures 9C and 9D respectively.

### 6.1.2. Normalisation

Following baseline correction, spectra must be normalised to account for differences in sample thickness in heterogeneous samples, or to account for variations in the intensity of the source (Lasch, 2012; Randolph, 2006). Common methods include Min-Max normalisation and vector normalisation. Min-Max normalisation, most frequently to the Amide I peak can be applied when this peak is consistently present in all spectra (Kelly et al., 2011; Trevisan et al., 2012) and is commonly applied after rubber band baseline correction. Spectra are offset-corrected by setting the minimum intensity of the spectrum to zero; spectra are then scaled, with the maximum intensity value set to one (Lasch, 2012). Amide I normalisation, where the maximum intensity is set to the Amide I peak is shown in figure 9E. Vector normalisation is used as an alternative to normalisation to a particular peak after rubberband baseline correction as shown in figure 9F. Vector normalisation is also used when there is no consistent peak in the spectrum where each spectrum is divided by its Euclidean norm (Kelly et al., 2011). Therefore this normalisation technique is used after differentiation as shown in figures 9G and 9H.

## 6.2 Feature extraction

The resulting output from IR spectroscopy is in the form of an absorbance spectrum. Within the cut and processed spectrum, there are over two hundred wavenumbers some correlated and overlapping, which can make data analysis challenging. Therefore using multivariate analysis can be an efficient tool in extracting patterns

from the dataset in a more readily interpretable fashion by reducing the dimensionality of the data (Ellis and Goodacre, 2006). Multivariate pattern recognition techniques can be divided into unsupervised and supervised methods: unsupervised methods use no prior knowledge of the classes the data are divided into, whereas in supervised pattern recognition the information about which classes the data are divided into is incorporated into the learning algorithm (Wang and Mizaikoff, 2008).

### 6.2.1 Principal component analysis

Principal component analysis (PCA) is an unsupervised method of data analysis, which looks for inherent similarities in the data and then groups them in the way the data 'naturally' cluster (Ellis and Goodacre, 2006; Wang and Mizaikoff, 2008). This method is used as a variable reduction technique to compress the variance in large complex datasets, like the ones generated from spectroscopic analysis, into a smaller number of principal components (PCs), simplifying interpretation and retaining the most relevant analytical information. PCA operates by resolving datasets into orthogonal components, the linear combinations of which approximate the original dataset (Jolliffe, 2002). The PCs are eigenvectors of the correlation coefficient matrix of squared deviations, with the first PC presenting the most variance, the second PC presenting the maximum amount of the remaining variance and so on (Davies and Fearn, 2004). PCA is commonly applied and is useful for small datasets, where the number of observations may be small in comparison to the number of variables (i.e. wavenumbers), like in many spectroscopic data sets (Ellis and Goodacre, 2006; Martínez and Kak, 2001). The disadvantage of PCA is that it does not distinguish between within group and between group variances (Wang and Mizaikoff, 2008).

### 6.2.2. Linear discriminant analysis

Supervised methods of data analysis include linear discriminant analysis (LDA), a technique used to find a linear combination of features that correctly discriminate between two or more data classes, rather than simply those that best describe the data. LDA maximises the differences between classes and minimises the heterogeneity within classes (Martínez and Kak, 2001).  In order for the output from LDA to be

unbiased, the number of features (samples) must be much larger than the number of variables (wavenumbers). Therefore a variable reduction technique, such as PCA or partial least squares (PLS) is often employed before input into LDA.

### 6.2.3 PCA-LDA

PCA is frequently employed to reduce the dimensionality of the data, as typically the data sets generated from IR spectroscopy have many more features (wavenumbers) than observations and without feature reduction, the model would overfit the data, thus resulting in good separation in the data by chance. Use of PCA also overcomes the issue of colinearity in the data matrix, as the principal components generated are orthogonal to each other (Gromski et al., 2015). Implementation of PCA prior to LDA reduces the number of features to a much smaller number of variables (principal components), whilst still preserving the majority of the variance in the data; this is achieved without prior knowledge of the classes within samples in the original dataset (Gromski et al., 2015; Trevisan et al., 2012) The number of PCA factors retained prior to input into LDA must be carefully selected, usually through percentage of variance captured, or by cross-validation based upon the mean-square error in the spectrum reconstruction (Baker et al., 2008). All supervised methods must be cross-validated, commonly with leave-one-out or *k*-fold cross validation (where $k = 5$ or 10), which uses a small portion of the data set to train the model, so as to prevent bias in the output (Trevisan et al., 2012).

## 6.3. Data visualisation

The resulting outputs from both PCA and PCA-LDA/LDA are scores and loadings plots. Each score corresponds to an individual observation (in this case, spectrum), which can be viewed in several possible dimensions and are thus commonly viewed as one, two or three-dimensional scatter plots. For PCA, the number of dimensions is the number of principal components retained (usually 5-20), and in PCA-LDA/LDA it is the number of classes minus one. Score plots give information regarding how the classes cluster i.e. do different classes cluster together or away from one another. Loadings plots give information about the contribution individual wavenumbers contribute in forming the new features (scores) (Trevisan et al., 2012). An alternative to viewing loadings to determine the wavenumbers to attributable to the data

separation seen in the scores plots is to use cluster vectors. Cluster vectors generate 'pseudo-spectra'; which have a direct relation to the original absorbance spectra and are used to reveal biochemical alterations specific to each data class relative to the control, which is set at the origin (Trevisan et al., 2012). Examples of the outputs from PCA/PCA-LDA are shown in Figure 10.

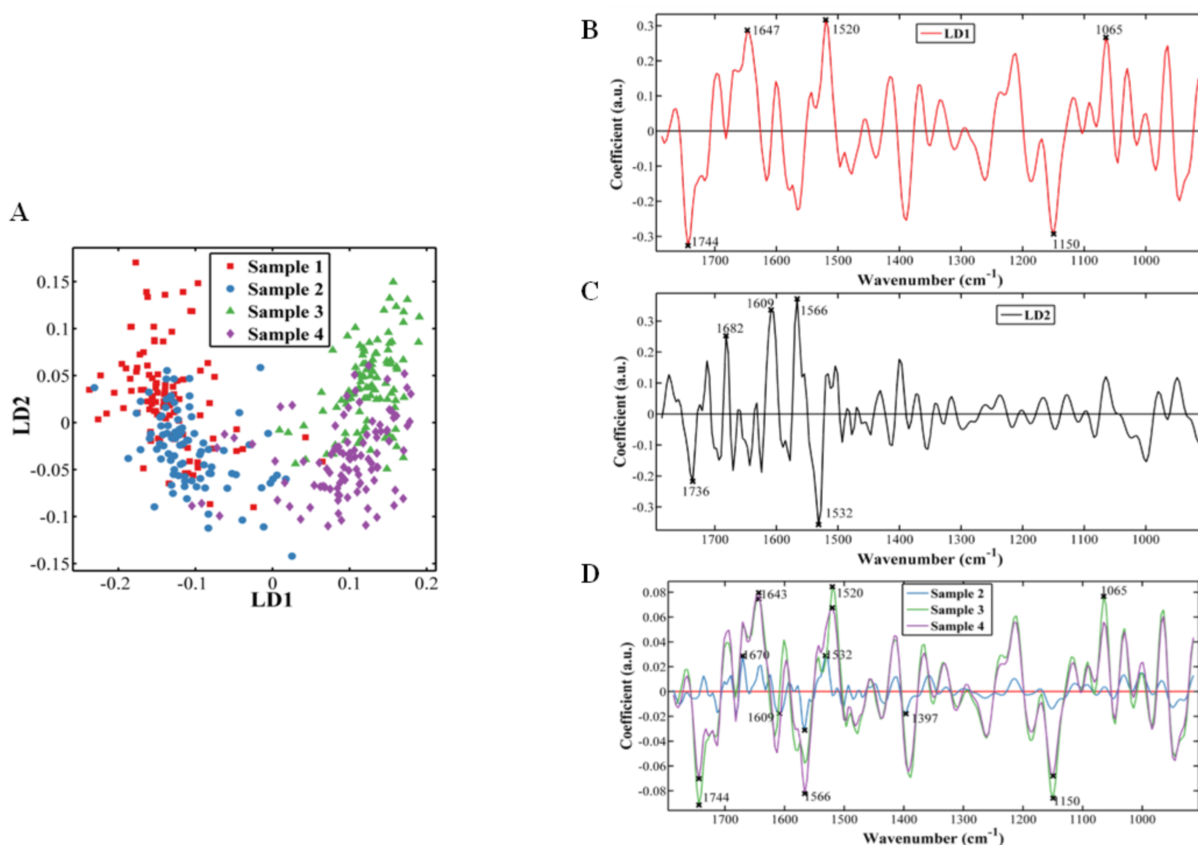


**Figure 10.** Example outputs following PCA-LDA analysis of *Rana temporaria* tissue. **A:** Two-dimensional scores plot; **B:** Loadings plot corresponding to LD1 of scores plot, representing wavenumbers distinguishing between sample 1/2 and sample 3/4; **C:** Loadings plot corresponding to LD2 of scores plot, representing wavenumbers distinguishing between sample 1 and sample 2, and sample 3 and sample 4; **D:** Cluster vector plot, where sample 1 is set at the origin and the wavenumbers represent alterations relative to this.

Data generated from IR spectroscopy may also be inputted into a classification model and the classification accuracy rate, defined as the average between sensitivity and specificity determined where appropriate (Owens et al., 2014). There are many classifiers in use in FTIR analysis for classification of data based upon particular

parameters e.g. cancer identification (Baker et al., 2008; Gajjar et al., 2013), taxonomical classification of microorganisms (Maquelin et al., 2002; Mariey et al., 2001; Winder and Goodacre, 2004), in food analysis to identify adulteration of consumer products (Rohman and Man, 2010) or chemical modification (Fernández Pierna et al., 2005) and to some extent in environmental pollution research (Gómez-Carracedo et al., 2012; Llabjani et al., 2012). For supervised classification, mathematical models are generated which estimate the correct classification of an unknown dataset based upon knowledge gained from a training dataset. Classifiers are therefore divided into 'training' and 'testphases (Trevisan et al., 2012). Feature extraction, such as PCA or PLS may first be implemented as a data reduction technique as datasets are typically large and complex (Fernández Pierna et al., 2005). A list of some of the methods available is presented in Table 3 and a more detailed analysis of each classification method is presented in a number of reviews (Krafft et al., 2009; Mariey et al., 2001; Trevisan et al., 2012).

**Table 3.** A list of commonly used methods for classification of datasets generated from IR spectroscopy.

| Method | Example references |
|---|---|
| Linear Discriminant Classifier (LDC)/PCA-LDC | (Gómez-Carracedo et al., 2012; Liu et al., 2006; Pereira et al., 2006; Wu et al., 1996) |
| Partial Least Squares-Discriminant Analysis (PLS-DA) | (Gómez-Carracedo et al., 2012; Liu et al., 2006) |
| Support Vector Machines (SVM) | (Fernández Pierna et al., 2005; Gómez-Carracedo et al., 2012; Huang et al., 2008) |
| Artificial Neural Networks (ANN) | (Goodacre et al., 1996; Gómez-Carracedo et al., 2012) |
| Quadratic Discriminant Analysis (QDA) | (Khanmohammadi et al., 2013; Wu et al., 1996) |
| *k*-Nearest Neighbours (kNN) | (Gómez-Carracedo et al., 2012; Kansiz et al., 1999) |
| Soft Independent Modelling of Class Analogies (SIMCA) | (Gómez-Carracedo et al., 2012; Kansiz et al., 1999) |

## 6.4. Applications of biospectroscopy in environmental toxicology

Biospectroscopy has been employed in several studies in order to identify changes in cells and tissues associated with environmental contamination; this has been explored *in vitro* and also *in vivo*. For example, *in vitro*, ATR-FTIR has been implemented in order to signature the effect of sub-lethal concentrations of several different environmental contaminants in algal, bacterial and human cell types (Barber et al., 2006; Corte et al., 2010; Heys et al., 2014; Holman et al., 2000a; Holman et al., 2000b; Johnson et al., 2014; Kardas et al., 2014; Li et al., 2016; Llabjani et al., 2010, 2011; Mecozzi et al., 2007; Riding et al., 2012; Ukpebor et al., 2011). This technique therefore has the potential to be utilised in other cell-lines not yet explored, in particular cell-lines relevant to environmental toxicology such as those derived from amphibians. Results obtained using IR spectroscopy show concordance with those obtained from traditional cell-based assays, For example MCF-7 cells exposed to 17*β*-estradiol showed comparable $EC_{50}$ values when assessed with either the E-screen assay or FTIR spectroscopy, with the added benefit that the results obtained using FTIR spectroscopy were obtained in a much shorter time frame (Johnson et al., 2014). A positive correlation was found between CYP1A1 expression and IR absorption of the phosphate band in HEPG2 cells exposed to TCDD (Holman et al., 2000b). Results obtained using ATR-FTIR spectroscopy were similar to those obtained from the SHE assay, when assessing transformation of cells exposed to potential carcinogens (Trevisan et al., 2010).

FTIR spectroscopy has been implemented in order to develop biomarkers for assessing contamination in the aquatic and terrestrial environment, both in the laboratory and *in situ*. Laboratory studies using the fish species *Labeo rohita* demonstrated that exposure to arsenic instigated significant structural changes in gill and kidney tissue, with major alterations in lipids, proteins and nucleic acids (Palaniappan and Vijayasundaram, 2009; Palaniappan et al., 2011). Changes in liver structure, including decreases in glycogen and protein concentration and increases in lipids and nucleic acids were found in Rainbow trout (*Onchorhynchus* mykiss) exposed to both estradiol and the estrogenic compound nonylphenol (Cakmak et al., 2006; Cakmak et al., 2003). FTIR spectroscopy has recently been utilised in order to enhance the duckweed (*Lemna minor* L.) toxicity test, where growth inhibition is used as an endpoint. Following exposure of duckweed to metals, industrial wastewater and

herbicides, similar $EC_x$ values were obtained using FTIR spectroscopy in comparison to those obtained from standard toxicity endpoints (frond number, chlorophyll content), with FTIR spectroscopy also able to determine biochemical alterations and thus explain potential toxic mechanisms (Hu et al., 2016). In field studies using the fish species *Parophrys vetulus,* differences have been observed in the deoxyribose nucleic acid (DNA) of liver and gill samples of fish from sites contaminated with PAHs and polychlorinated biphenyls (PCBs) compared to those from 'clean' sites, using FTIR (Malins et al., 2006; Malins and Gunselman, 1994; Malins et al., 1997; Malins et al., 2004). This allowed the establishment of a DNA damage index, which provided information about the extent of damage to liver and gill DNA due to contaminant exposure under environmental conditions, with the results obtained using FTIR spectroscopy showing consistency with those obtained from assessment of histology and CYP1A expression (Malins et al., 2006; Malins et al., 2004). Recent studies have demonstrated the use of IR spectroscopy in assessing fish tissues and bird feathers in order to identify areas at risk of environmental pollution, as well as to ascertain the effects such contamination have on tissues at a biochemical level (Abdel-Gawad et al., 2012; Li et al., 2015; Llabjani et al., 2012; Obinaju et al., 2014; Obinaju et al., 2015).

## 7. Aims and objectives

This thesis consists of four main research projects, which aim to explore the use of biospectroscopy as a novel method in assessing the health of amphibian populations, both using an amphibian cell line and in tissues of the widespread anuran species *R.temporaria* collected from ponds with differing water quality at several life stages. In addition, included in the appendix are several co-author projects, which apply IR spectroscopy in other areas of biological research, as well as a paper which explores the potential to standardise methods and protocols in order to optimally apply IR spectroscopy in biological applications. An overview to the aims and objectives of the thesis is provided in schematic form in Figure 11. The main objectives of the four projects are as follows:

- To determine the use of ATR-FTIR spectroscopy in identifying incipient water quality problems in ponds at risk of environmental contamination by assessing differences in spawn and early stage tadpoles of *R.temporaria* from ponds with presumed differing water quality, and confirm the differences in water quality parameters in a pilot study (Chapter 2).

- Once the use of ATR-FTIR spectroscopy in distinguishing spawn and early stage tadpoles from different ponds is established, implement this technique over a longer-term period in order to explore its use in a multi-generational study, and to identify temporal differences in populations (Chapter 3).

- To provide a spectroscopic assessment of individual tissues (liver, muscle, kidney and skin) of pro-metamorphic late-stage Common frog tadpoles collected from ponds with differing water quality in order to establish the tissues most sensitive to environmental pollutants (Chapter 4).

- To assess the effects of low-dose fungicide (carbendazim and flusilazole) exposure to the A6 kidney epithelial cell line derived from *X.laevis* in both single agent and binary mixture experiments, as determined by analysis with ATR-FTIR spectroscopy (Chapter 5).

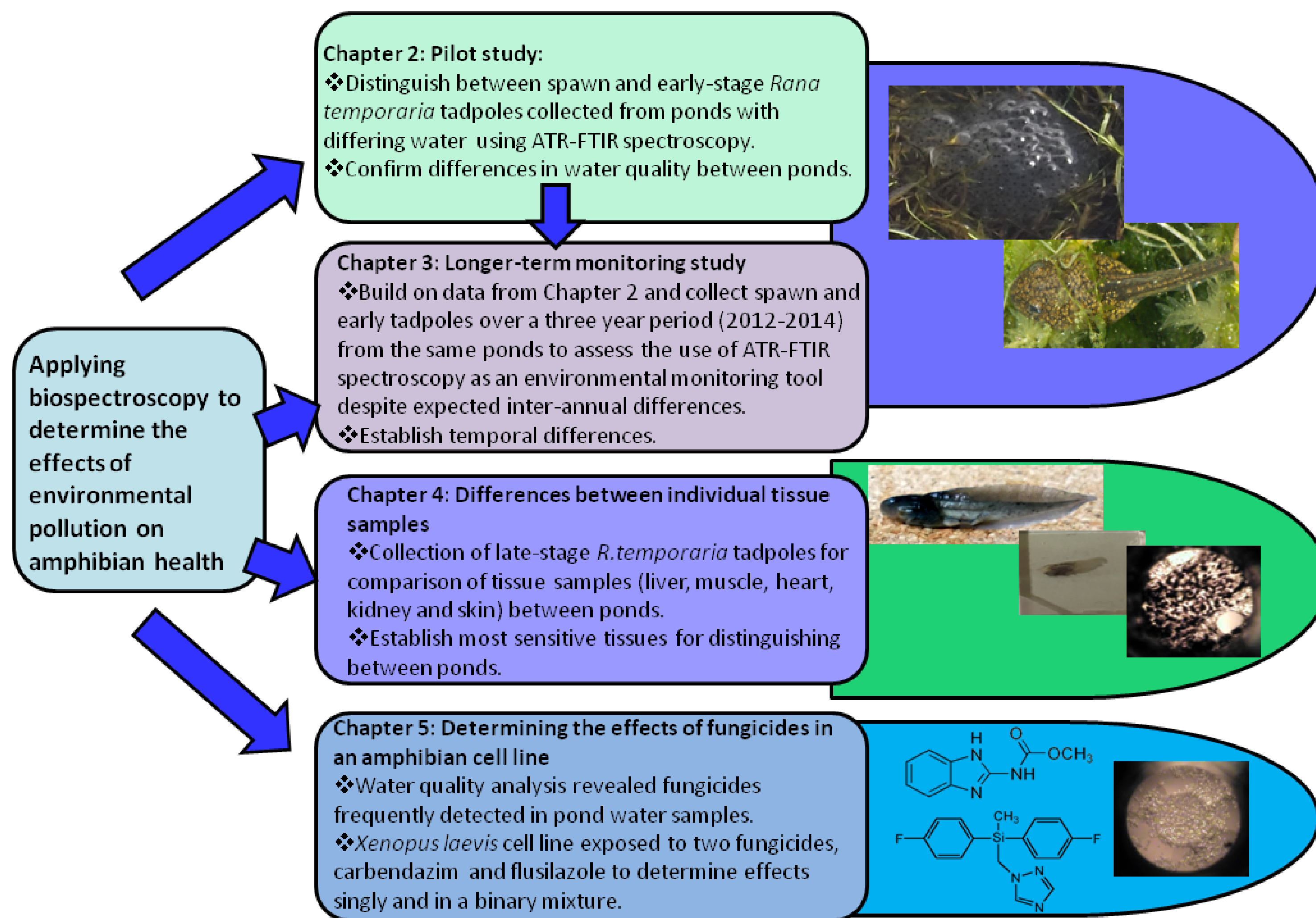


**Figure 11.** Schematic diagram giving an overview of the aims and objectives of the thesis.

# 8. Overview to methods

## 8.1. Field sites

One of the aims of this thesis was to utilise biospectroscopy in identifying incipient water quality problems in ponds at risk of environmental contamination by using *R.temporaria* as a sentinel organism. With that in mind, ponds were selected to give a comparison between a relatively clean site with minimal pesticide input, a site impacted by commonly used agricultural pesticides and an urban location with run-off from wastewater and storm water based on land-use data and information from the land owners/managers. Approximate locations of the study sites on a map of the UK together with photographs of each site is provided in Figure 12.

The ponds were as follows:

1. **Minimally impacted pond:** Crake Trees (CT), Crosby Ravensworth is a farm used as beef grazing land and marginal arable land, which has been accepted onto Natural England's Higher Level Environmental Stewardship Scheme and uses minimal quantities of pesticides, with buffer zones to prevent pesticide run-off into water courses (see Fig.12A).

2. **Pesticide impacted pond:** Whinton Hill (WH), Plumpton, Cumbria is a farm consisting of arable land used for winter wheat, maize and potatoes, and grazing land for beef and sheep, which is routinely sprayed with herbicides and fungicides (Fig. 12B). The pond sampled receives run-off from the surrounding farmland.

   The ponds surveyed at WH and CT are constructed wetlands, created as part of the MOPS2 (Mitigation Options for Phosphorus and Sediment) project monitored by Lancaster University http://mops2.diffusepollution.info/

3. **Urban pond:** Pennington Flash Country Park (PF) is a nature reserve and recreational park located in Leigh, Lancashire. The site consists of a lake formed through the subsidence of mine workings, situated in a country park, and there are numerous small ponds located around the site which receive run-off from landfill and wastewater from a nearby wastewater treatment works.

The site is also liable to flooding; therefore water quality is likely to vary (Fig.12C).

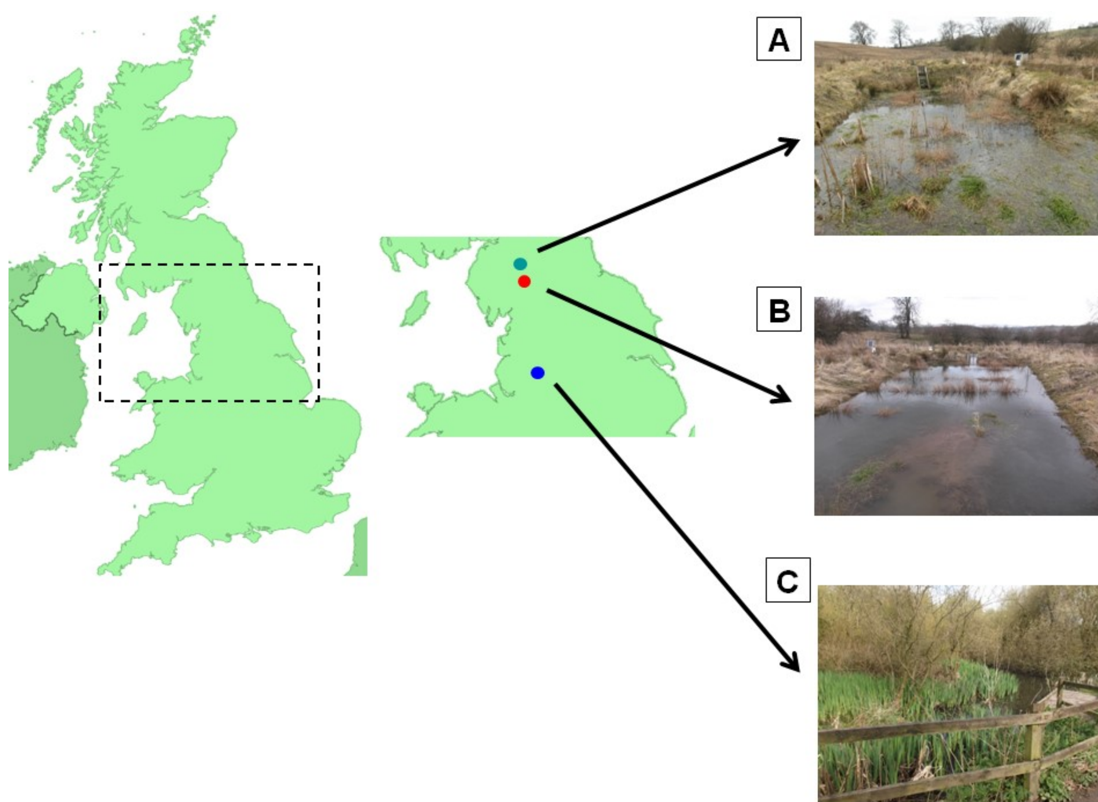


**Figure 12.** Approximate locations of the study sites on a map of the UK, together with photographs of each site. Sites are as follows: **A:** Crake Trees, a pond located on a farm which does not receive pesticides; **B:** Whinton Hill, a pond impacted by agricultural pesticides; **C:** Pennington Flash, a pond located in an urban park which receives run-off from landfill and wastewater.

## 8.2. Sample preparation

Samples of *R.temporaria* spawn, early-stage but free-swimming tadpoles (Gosner stage 25-28) and late-stage tadpoles (Gosner stage 38-40) were collected from the three ponds annually. Samples were prepared using a Stadie-Riggs tissue slicer to obtain slices ~ 0.5 mm thick, mounted onto Low-E slides and spectra obtained using an ATR-FTIR spectrometer. An outline of this procedure using a late-stage tadpole as an example is shown in Figure 13.

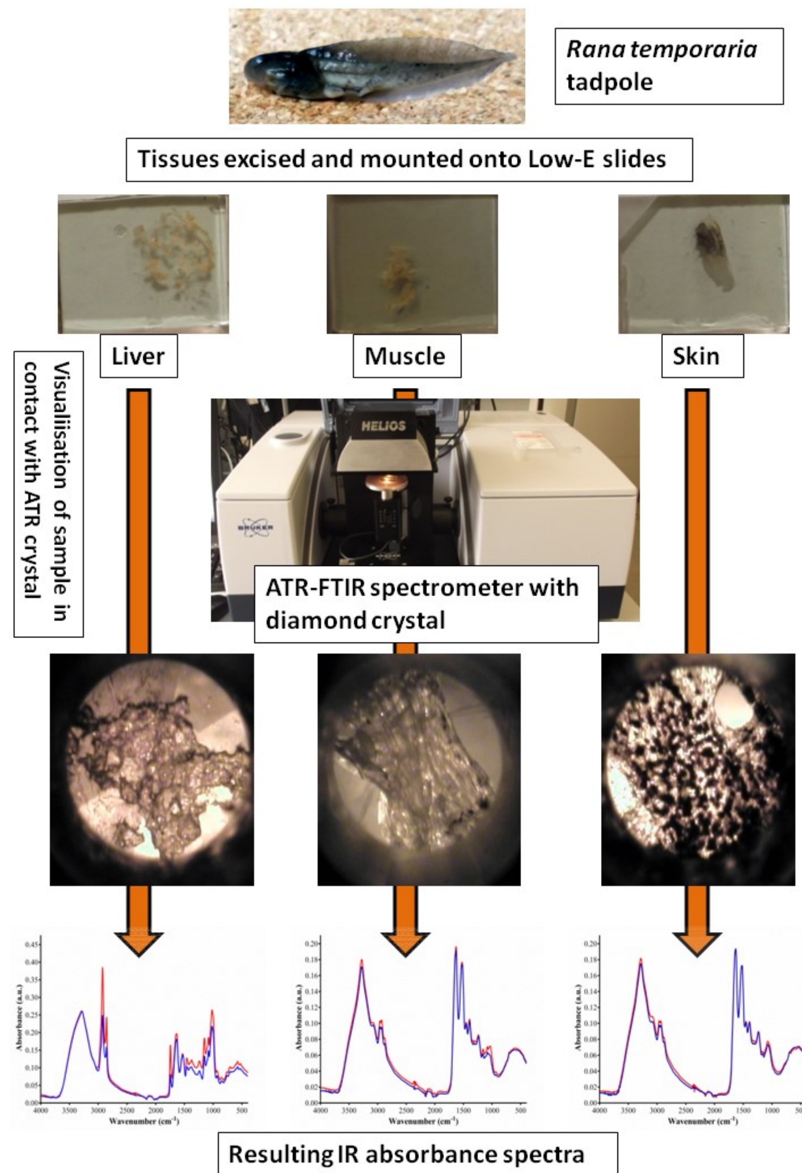


**Figure 13.** A workflow of the procedure used to obtain ATR-FTIR spectra of tissue samples of *Rana temporaria* tadpoles.

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# Chapter 2.

**Biospectroscopy as a tool to monitor subtle effects of environmental stress on the early life stages of the Common frog: a pilot study.**

**Rebecca J. Strong**, Crispin J. Halsall, Martin Ferenčík, Kevin C. Jones, Richard F. Shore and Francis L. Martin.

**Manuscript for submission**

Contribution:

- I acquired the samples required for the project.
- I prepared, processed and acquired data for spawn and tadpole samples including conducting computational analysis.
- Water analysis for organic contaminants was acquired by Martin Ferenčík at Povodí Labe, Czech Republic.
- Nutrient analysis was carried out by the Centre for Ecology and Hydrology, Lancaster.
- I prepared the first draft of the manuscript.

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Rebecca J. Strong Prof. Francis L. Martin

**Biospectroscopy as a tool to monitor subtle effects of environmental stress on the early life stages of the Common frog: a pilot study.**

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## Abstract

Worldwide amphibian populations are declining due to habitat loss, disease and pollution. Vulnerability to environmental contaminants such as pesticides will be dependent on the species, the sensitivity of the ontogenic life stage and hence the timing of exposure and the exposure pathway. Here we focus on the Common frog, *Rana temporaria,* and use biospectroscopy to investigate the biochemical tissue 'fingerprint' in spawn and early-stage tadpoles from urban and agricultural ponds with contrasting water quality. Tissue analysis using attenuated total reflection-Fourier-Transform infrared (ATR-FTIR) spectroscopy revealed only subtle differences in biochemistry between spawn from the different ponds. For tadpoles of the same Gosner life stage, gross morphological differences (e.g. head width and snout-to-vent length) were also not apparent between the ponds. However, marked differences ($p < 0.05$) were observed in the ATR-FTIR spectra between tadpoles from a rural agricultural pond with no pesticide input and those from both an agricultural pond impacted by pesticides and an urban pond affected by wastewater and landfill run-off. These differences related principally to carbohydrates, particularly the glycogen region of the spectra (1030-1150 $cm^{-1}$) and to a lesser extent the phosphate chain vibrations in nucleic acids and phospholipids (e.g. 1003 $cm^{-1}$) Tadpoles from the intensive agricultural and urban ponds have altered levels of glycogen in comparison to those from the rural agricultural pond. In the absence of population surveys our results demonstrate that levels of stress (marked by biochemical constituents involved in compensatory metabolic mechanisms) can be observed in tadpoles in freshwater systems with low water quality.

## Introduction

Globally, amphibians are facing precipitous declines (Alford and Richards, 1999; Wake and Vredenburg, 2008), with environmental pollution cited as a major threat to amphibian health and survival (Carey and Bryant, 1995; Mann et al., 2009). The vulnerability of amphibians to contaminant exposure is due to their highly permeable skin and complex lifecycle comprising both aquatic and terrestrial phases (Brühl et al., 2011; Cooke, 1981; Ralph and Petras, 1998). In addition, certain environmental contaminants, such as pesticides used in agriculture, are applied to adjacent land at the same time breeding and larval development occurs; a period thought to be particularly susceptible to the effects of chemical exposure (Hayes et al., 2006a; Mann et al., 2009). Environmental contamination, in particular contamination from agricultural sources, was implicated in local reductions in abundance of the Common frog, *Rana temporaria* and Common toad, *Bufo bufo* in the UK during the 1960s and a number of subsequent studies demonstrated deleterious effects of pesticides in use at that time on UK amphibian species (Cooke, 1970, 1972, 1973a, b, 1981; Osborn et al., 1981). More recent studies have examined the effects of agricultural intensification on native species of amphibian (Oldham et al., 1997; Orton and Routledge, 2011; Watt and Jarvis, 1997). Although populations of *R.temporaria* have since stabilised, there remains a paucity of data regarding the effect of agricultural perturbations and newer pesticides on UK amphibian populations (Beebee, 2014).

Amphibians are thought to vary in their vulnerability to environmental contamination depending on ontogenic stage; therefore it is important to take this into account in any monitoring study. Several studies have shown that the jelly coat surrounding the embryo may act as a physical barrier to several contaminants, offering some protection against mortality and developmental abnormalities (Berrill et al., 1994; Berrill et al., 1998; Cooke, 1972; Edginton et al., 2007; Marquis et al., 2006; Meredith and Whiteman, 2008; Ortiz-Santaliestra et al., 2006). However, the jelly coat offers little in the way of protection against certain toxic chemicals such as the pyrethroid insecticide α-cypermethrin (Greulich and Pflugmacher, 2003). Additionally, some chemicals, particularly those that are lipophilic may be maternally transferred to spawn (Bergeron et al., 2010; Hopkins et al., 2006; Kadokami et al., 2004; Orton and Routledge, 2011; Wu et al., 2009; Wu et al., 2012). Therefore this exposure route

must be considered in addition to that from the surrounding environment. Exposure to environmental pollutants at the embryonic stage may result in developmental abnormalities, which are only apparent at metamorphosis following this earlier exposure (Bridges, 2000; Orton and Routledge, 2011; Orton and Tyler, 2014) . Within larval stages, there may also be variation in susceptibility to particular contaminants, with the earlier larval stages generally regarded as most vulnerable (Cooke, 1972; Greulich and Pflugmacher, 2003), although this can vary between species and contaminant type (Richards and Kendall, 2002). This variation in susceptibility may be important if the more vulnerable stages coincide with pesticide application.

Due to the widely accepted sensitivity of amphibians to environmental contamination, several studies have attempted to establish possible biomarkers of effect (Mann et al., 2009; Venturino et al., 2003). Endpoints commonly measured include growth (Relyea and Diecks, 2008; Widder et al., 2008), behavioural abnormalities (Cooke, 1972), time to metamorphosis (Greulich and Pflugmacher, 2003; Hersikorn and Smits, 2011; Relyea and Diecks, 2008), deformities (Ruiz et al., 2010), endocrine disruption (Harris et al., 2001; Hayes et al., 2006a; Hayes et al., 2006b), induction or suppression of enzymes and endogenous compounds related to oxidative metabolism such as glutathione s-transferases and $\beta$-esterases (Buryskova et al., 2006; Ferrari et al., 2009; Hersikorn and Smits, 2011; Lajmanovich et al., 2010), suppression of immune function (Carey et al., 1999; Christin et al., 2004), and genotoxicity (Clements et al., 1997; Ralph and Petras, 1997).

A technique also employed to develop biomarkers for use in environmental monitoring is infrared spectroscopy (IR); a tool which can potentially be used to monitor the effects of a suite of contaminants in a non-destructive and high throughput manner (Holman et al., 2000; Johnson et al., 2014; Llabjani et al., 2011). Spectra derived using this approach represent a “biochemical cell fingerprint”, with wavenumbers corresponding to particular biochemical entities; such constituents include those related to the secondary structure of proteins (Amide I, II and III at ~1650 $cm^{-1}$, ~1550 $cm^{-1}$, ~1250 $cm^{-1}$ respectively), lipids (~1750 $cm^{-1}$), carbohydrates (~1150 $cm^{-1}$ and ~1030 $cm^{-1}$) and DNA/RNA (~1225 $cm^{-1}$ and ~1080 $cm^{-1}$) (Bellisola and Sorio, 2012; Movasaghi et al., 2008). The IR spectrum produced is highly information-rich and therefore key features may be extracted by determining changes

in absorbance values at key peaks in the spectrum, or by using multivariate data analysis techniques such as principal component analysis in order to reduce the complexity of the data sets (Cakmak et al., 2006; Ellis and Goodacre, 2006). The assignment of peaks can be difficult in complex biological systems like whole organism and tissue samples where absorbance bands overlap, and thus can be considered tentative (Naumann, 2000). Nevertheless, many studies have been conducted which have recorded marked similarities in the spectral interpretation of equivalent areas in the derived spectra, creating detailed tables of wavenumber assignments, thus assisting greatly in determining the chemical structure of diverse biological samples using IR spectroscopy (Cakmak et al., 2006; Movasaghi et al., 2008). In addition, the use of spectral derivatives allows the more detailed resolution of underlying peaks in the broad bands of the spectrum (Rieppo et al., 2012). Fourier-transform IR (FTIR) and attenuated total reflection-FTIR (ATR-FTIR) spectroscopy has previously been employed in order to identify potential biomarkers in fish and bird species exposed to contaminants both in the laboratory and field; such contaminants include heavy metals (Henczova et al., 2008; Henczova et al., 2006; Llabjani et al., 2012; Palaniappan and Vijayasundaram, 2009; Palaniappan et al., 2011), nanoparticles (Palaniappan and Pramod, 2010), polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Malins et al., 2006; Malins et al., 2004; Obinaju et al., 2014; Obinaju et al., 2015) and endocrine disruptors (Cakmak et al., 2006; Cakmak et al., 2003) . However, to our knowledge this technique has yet to be employed in examining any contaminant-induced stresses in amphibians exposed to environmental pollution.

The aim of this study was to establish the viability of using ATR-FTIR spectroscopy to interrogate embryos and early-stage tadpoles of *R.temporaria* collected from ponds with differing water quality due to pollution from agricultural and urban sources. Subsequent data analysis of the derived spectra was implemented in order to determine molecular modifications suggestive of exposure to chemical stressors. The detection of differences in the IR spectra of embryos and tadpoles could suggest the use of IR spectroscopy as an environmental monitoring technique, capable of identifying incipient water quality problems in ponds at risk of environmental contamination.

## Materials and methods

### Ponds

Sites were selected in order to give a comparison between agricultural and urban ponds and were based on site characteristics and information from landowners/land managers.

1. Whinton Hill (WH), Plumpton, Cumbria is a farm consisting of arable land used for winter wheat, maize and potatoes, and grazing land for beef and sheep, which is routinely sprayed with herbicides and fungicides (see supplementary information table S1 for agronomist's report). The pond surveyed was the second pond of a pair of deep and shallow ponds (8 m long x 8 m wide and 32 m long x 8 m wide), located in a boggy field, and fed by a field drain from approximately 30 ha of farmland.

2. Crake Trees (CT), Crosby Ravensworth is a farm used as beef grazing land and marginal arable land, which has been accepted onto Natural England's Higher Level Environmental Stewardship Scheme and uses minimal quantities of pesticides, with buffer zones to prevent pesticide run-off into water courses. The pond surveyed was the second pond of a pair of shallow ponds (each 17 m long x 6 m wide), located in a field corner, and fed by surface runoff from approximately 20 ha of farmland.

   The ponds surveyed at WH and CT constructed wetlands, created as part of the MOPS2 (Mitigation Options for Phosphorus and Sediment) project monitored by Lancaster University http://mops2.diffusepollution.info/

3. Pennington Flash Country Park (PF) located in Leigh, Lancashire is a site managed by Wigan and Leigh Culture Trust. The 'Flash' is a large lake formed over time by mining subsidence. The southern part of the Flash was filled with domestic waste during the 1950s to prevent the regular flooding of nearby St Helens Road. The pond sampled (PF) is adjacent to Westleigh Brook, which receives treated wastewater from Leigh sewage works. Several areas of this site are liable to flooding, so the quality of the water received is likely to vary.

### Collection and processing of spawn and tadpoles

Samples of *R.temporaria* spawn were taken from all of the sites at different times (WH and CT 07/03/2012, PF 16/03/2012) due to the variation in spawning date. Spawn was collected in solvent-rinsed glass jars and transported back to the laboratory before the jelly coat was removed with forceps and the embryo fixed in 70% ethanol overnight at 4°C. The Gosner stage (Gosner, 1960) of spawn samples was noted prior to fixation. Spawn from all sites was classified as Gosner stage 10-11 (dorsal lip). Whole fixed embryos were mounted directly onto Low-E reflective glass slides (Kevley Technologies, Chesterland, OH, USA), dried overnight and stored in a desiccator before subsequent interrogation with ATR-FTIR spectroscopy.

Common frog tadpoles were caught using dip nets (ten from each site) and euthanised using a solution of tricaine methanesulfonate (MS-222) (200mg/L) buffered with sodium bicarbonate (both from Sigma Aldrich, Poole, Dorset UK), in accordance with Schedule 1 of the British Home Office Animals (Scientific Procedures) Act 1986. Tadpoles were rinsed in distilled water and then fixed immediately in the field in 70% ethanol (Fisher Scientific, UK). Ethanol was replaced after 24 hours with fresh. Tadpoles were weighed and measurements were taken of snout-vent length (SVL) and head width (HW) using digital callipers to the nearest 0.01mm after fixation and tadpoles were staged according to Gosner (1960)(Gosner, 1960). All tadpoles were between stages 25-28 (full details of stages and tadpole mass, SVL and HW measurements are in Table S2 of supplementary information), with variation both within and between sites. In order to process samples for ATR-FTIR spectroscopy, a longitudinal slice (~ 0.5 mm thick) was taken from the ventral side of the tadpole using a Stadie-Riggs tissue slicer; a technique previously employed for preparing tissue samples for analysis with IR spectroscopy (Maher et al., 2014; Obinaju et al., 2014; Taylor et al., 2011). The second slice was used for spectroscopy. Slices were mounted skin side down onto Low-E slides, dried overnight and stored in a desiccator before interrogation with ATR-FTIR spectroscopy.

**Water quality analysis**

Samples of surface water (15-20 cm depth) were collected concurrently with spawn and tadpole samples (March and April 2012 respectively). Water samples were collected in methanol-rinsed amber bottles for organics analysis and acid-washed bottles for nutrient analysis and then stored at 4°C until analysis. The concentrations of trace metals (Al, Fe, Mg, Ca, K and Na) were determined in filtered acidified samples ($HNO_3$) using inductively coupled plasma optical emission spectrometry (ICP-OES) using a Perkin Elmer DV 7300, while concentrations of major anions (Cl, $NO_3$-N, $SO_4$-S) as well as phosphate, ammonium and total organic N (TON) were determined using colorimetric methods performed by the Centre for Ecology and Hydrology (Lancaster) in a quality-assured, previously published method (Neal et al., 2000). For organic chemical analysis, 800 mL of sample water (adjusted to pH 9.5 with borate buffer) underwent liquid-liquid (1:1) extraction using dichloromethane (DCM) on a laboratory shaker (Gerhardt Shaker LS-500) followed by separation and evaporation of the DCM on a rotary evaporator (rotavapor Büchi R-210). The concentrated DCM extracts (700 µL) underwent initial qualitative screening using Gas chromatography–mass spectrometry (GC-MS) (Agilent 6890N GC and Agilent 5973 single quad MS) operated by ChemStation software (D.02.00.275) with subsequent mass spectral identification using Mass Hunter software and comparison to the NIST spectral library. The following chemicals were detected: aniline, metazachlor, acetochlor, dimetachlor, triethylphosphate (TEP), tributylphosphate (TBP), tris(2-chloroethyl)phosphate (TCEP), tris(1-chloro-2-propyl)phosphate (TCPP) and flusilazole. These compounds were quantitatively analysed using authentic standards using a 7-point calibration, with standards ranging from 0-2000 ng/L for each analyte. Internal standards comprising $^{13}C$-labelled aniline, acetochlor and metalochlor were added to sample extracts and calibration standards prior to analysis. Limits of quantification (LoQ) ranged from 5-10 ng/L (aniline 200 ng/L) with recoveries based on spiked water samples ranging from 80-120%.  Water samples were also analysed for more polar, water–soluble compounds. For this analysis, 10 mL of a water sample was filtered (using a 0.2 µm RC syringe filter), spiked with internal standards and analysis performed on a Waters Acquity Binary Ultra Performance Liquid Chromatograph (UPLC) (Waters Corporation, Milford, USA) coupled to a Waters Premier XE triple quadrupole mass spectrometer (LC-MS/MS) operated by MassLynx

software V 4.1.The MS was operated in electrospray positive (ESI+) ionisation mode with multiple reaction monitoring (MRM). A 250 µL aliquot was injected via an autosampler, with analyte separation performed under a methanol (MeOH)/$H_2O$ (with 5 mmol/L ammonium acetate added to both phases) mobile gradient eluted through an Acquity BEH $C_{18}$ column (1.7 µm, 2.1 mm x 50 mm) fitted with a VanGuard Acquity pre-column. The following compounds, including pesticides and pharmaceuticals, were qualified/quantified: chlorotoluron, isproturon, caffeine, tebuconazole, prochloraz, carbendazim, gabapentin, acetaminophen, benzotriazole, benzotriazole-methyl, ketoprofen, dimethyl-chlorotoluron, metconazole, spiroxamine, boscalid, erythromycin,   Samples were analysed separately for glyphosate and its degradation by-product, aminomethylphosphonic acid (AMPA), using LC-MS/MS. For the analysis of glyphosate and AMPA, 8 mL of a water sample was acidified to pH 1 (addition of 160 µL of 6 M HCl) and subject to derivatisation using 9-fluorenylmethyl chloroformate in a method detailed by Ibáñez *et al.* (Ibáñez et al., 2006). Analytes were separated using the same LC-MS/MS instrument and method above. Internal standards comprised of 1,2-$^{13}C_2$ $^{15}N$ Glyphosate and $^{13}C$ $^{15}N$ AMPA with a 7-point calibration with standards ranging from 0 to 2000 ng/L. Ionisation was through ESI+ (precursor ions) and MRM (product ions). LoQs were 10 ng/L for both glyphosate and AMPA and recoveries ranged from 70-130% (water spiked with internal standards). A list of the chemicals screened for in the analysis of water samples together with detection limits is provided in Table S3 in the SI.

**ATR-FTIR spectroscopy**

Spectra of both spawn and tadpoles were obtained using a Tensor 27 FTIR spectrometer with Helios ATR attachment (Bruker Optics Ltd, Coventry, UK) containing a diamond crystal (≈250 µm×250 µm sampling area). Spectra were acquired at 8 $cm^{-1}$ resolution with 2x zero-filling, giving a data-spacing of 4 $cm^{-1}$ over the range 400-4000 $cm^{-1}$. Ten embryos and ten tadpoles were analysed from each site, with twenty five spectra per slide acquired each time in order to account for the variability inherent in whole organisms. Distilled water was used to clean the crystal in between analysis of each sample. A new background reading was taken prior to the analysis of each sample in order to account for changes in atmospheric conditions.

## Data processing

A representative ATR-FTIR spectrum was obtained by taking the mean of the spectral measurements for each sample (*n* = 10 embryos and tadpoles for each site sampled). Spectra were then cut at the biochemical cell fingerprint region (1800-900 $cm^{-1}$), baseline corrected using Savitzky-Golay $2^{nd}$ order differentiation ($2^{nd}$ order polynomial and 9 filter coefficients), and vector normalised. Use of second derivative spectroscopy allows the resolution of overlapping peaks in the original spectrum, thereby allowing more detailed analysis of particular absorption peaks. Processing the data using differentiation also removes constant and linear components of baseline errors (Rieppo et al., 2012).

## Principal component analysis

Multivariate analysis using principal component analysis (PCA) allows rapid reduction of the large datasets generated from spectral measurements into a smaller number of principal components, whilst retaining the majority of the variance in the data (Jolliffe, 2002). PCA is an unsupervised and therefore unbiased analysis which reveals the underlying patterns in the data. Data points from PCA can then be viewed as 'scores' in several dimensions, with scores clustering together indicating similarities, whereas those clustering away from one another suggest differences (Kelly et al., 2011). This type of analysis also generates loadings vectors, which demonstrate which wavenumbers and corresponding biochemical entities are responsible for the separation between classes when viewed alongside the scores plots (Trevisan et al., 2012).

After the data were mean-centred, PCA was employed to reduce the 227 absorbance values into 10 principal components, which represented > 96 % of the variance in the datasets (see figures S1A and S1B in supplementary information). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests, or one-way ANOVA with Welch's correction followed by Games-Howell multiple comparison tests if there were inequality of variances was used to calculate the statistical significance of the PC scores in SPSS 22 (SPSS Inc., Chicago, IL, USA), the results of which are shown in Table S4 in the SI. The most statistically significant PCs were retained, a technique previously employed by Malins *et al.* (Malins et al., 2006;

Malins et al., 2004). Corresponding loadings from the most significant PCs were used to identify wavenumbers accounting for the separation between sites. A peak detecting algorithm was employed to determine the seven largest loadings values (constrained by a minimum of 20 $cm^{-1}$ spacing between values).

**Second derivative absorbance values**

Analysis of peak heights in the fingerprint region of the spectrum allows more detailed quantification of any differences between samples at specific wavenumbers. Second derivative spectroscopy has previously been used in order to quantify components measured in the infrared spectrum (Rieppo et al., 2012). The second derivative has its maximum value at the same wavelength as the underlying absorbance peak, but in the negative direction (Mark and Workman Jr, 2010).

The values of absorbance peak-heights were determined from the second derivative spectrum for samples of both spawn and tadpoles. Statistical significance at each absorbance peak was tested using one-way ANOVA followed by Tukey's post-hoc tests to determine differences between ponds. Data with unequal variances were tested using one-way ANOVA with Welch's correction followed by Games-Howell post hoc tests (SPSS 22 software, SPSS Inc., Chicago, IL, USA).

All spectral pre-processing and data analysis was implemented using the IRootLab toolbox https://code.google.com/p/irootlab/ (Martin et al., 2010; Trevisan et al., 2013) in Matlab (r2012a) (The MathWorks, Inc., USA), unless otherwise stated.

**Morphometric data**

Body condition indices (BCI) were calculated for each tadpole as follows: (body mass/$SVL^3$) X 100 (Melvin et al., 2013). One-way ANOVA was used to compare mass, BCI, HW and SVL between sites followed by post-hoc comparisons with Tukey multiple comparison tests where appropriate (SPSS 22 software, SPSS Inc., Chicago, IL, USA).

## Results

### Water quality analysis

Water samples collected at the same time as spawn and tadpole samples were screened for major anions and cations, the results of which are presented in Table 1. Nitrate concentrations were highest at PF in March in comparison to the other two sites, whereas in April concentrations were highest at WH, although concentrations still remained low (< 3 mg/L). Phosphate concentrations were low at all three sites during the March sampling period < 0.08 mg/L. However during April, concentrations at PF and WH increased to high levels (0.3 and 0.6 mg/L respectively). At CT concentrations of phosphate remained low as before.

**Table 1.** Analysis of water samples for inorganic anions and cations collected from CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides and PF: an urban pond impacted by wastewater and landfill run-off. Water samples were collected to coincide with the collection of spawn (March) and tadpoles (April) of *Rana temporaria.*

| **Anion/cations (mg/L)** | **CT March** | **PF March** | **WH March** | **CT April** | **PF April** | **WH April** |
|---|---|---|---|---|---|---|
| Ca | 84.40 | 46.30 | 53.20 | 77.60 | 36.80 | 56.60 |
| Cl | 9.06 | 21.60 | 64.10 | 10.40 | 11.60 | 47.80 |
| Fe | 0.47 | 0.01 | 0.03 | 0.01 | 0.76 | 0.03 |
| K | 1.97 | 3.88 | 11.30 | 1.57 | 4.01 | 18.10 |
| Mg | 2.95 | 10.00 | 9.35 | 4.37 | 7.99 | 10.70 |
| Na | 4.88 | 15.00 | 38.80 | 4.97 | 9.82 | 37.20 |
| NH4-N | 0.03 | 0.06 | 0.30 | 0.41 | 0.13 | 0.28 |
| NO3-N | < 0.01 | 0.43 | 0.01 | 0.22 | 1.18 | 2.49 |
| PO4-P | 0.03 | 0.07 | 0.01 | 0.03 | 0.30 | 0.64 |
| SO4-S | 0.71 | 6.59 | 9.92 | 0.20 | 2.61 | 12.70 |

Results from the analysis of water samples for micro-organics are shown in Table 2. The agronomist's report from WH (SI Table 1) determined which pesticides were likely to be detected in the water samples. Screening of the water samples collected from CT, PF and WH revealed large differences in the organic contaminants detected. CT appeared to be the least contaminated site, particularly when spawn was collected (~March 2012), with only TCPP, an organophosphorus (OP) flame retardant detected at this point. PF, in contrast showed detectable levels of naphthalene, glyphosate and its degradation product (AMPA), two OP flame retardants (TCEP and TCPP) and the

pharmaceutical drugs gabapentin and acetaminophen at this time point. Water samples collected from WH around the same time showed low levels of TCPP, like CT, and similar levels of gabapentin and acetaminophen to that found at PF. Additionally, the triazole fungicide tebuconazole and relatively high levels of aniline, a compound generated during the degradation of several herbicides and pesticides (Xiao et al., 2007) were detected at WH.

**Table 2.** Organic contaminant analysis of water samples collected from CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides and PF: an urban pond impacted by wastewater and landfill run-off. Water samples were collected to coincide with the collection of spawn (March) and tadpoles (April) of *Rana temporaria.* Chemicals recorded as < LD are below the limits of quantification.

| **Chemical (ng/L)** | **CT March** | **PF March** | **WH March** | **CT April** | **PF April** | **WH April** |
|---|---|---|---|---|---|---|
| Naphthalene | < LD | 10 | < LD | < LD | < LD | < LD |
| Aniline | < LD | < LD | 1100 | < LD | < LD | < LD |
| Dimethachlor | < LD | < LD | < LD | < LD | < LD | 26 |
| Chlorotoluron | < LD | < LD | < LD | < LD | < LD | 23 |
| Caffeine | < LD | < LD | < LD | 441 | 107 | < LD |
| Glyphosate | < LD | 40 | < LD | < LD | < LD | 50 |
| AMPA | < LD | 130 | < LD | 150 | 658 | 1470 |
| Tebuconazole | < LD | < LD | 76 | < LD | < LD | < LD |
| Carbendazim | < LD | < LD | < LD | < LD | < LD | 866 |
| Triethylphosphate, TEP | < LD | 11.00 | < LD | < LD | 11.00 | < LD |
| Tributhylphosphate, TBP | < LD | < LD | < LD | 13 | < LD | < LD |
| Tris(2-chloroethyl)phosphate, TCEP | < LD | 190 | < LD | 26 | 12 | 7.2 |
| Tris(1-chloro-2-propyl)phosphate, TCPP | 15 | 142 | 25 | 125 | 314 | 1600 |
| Flusilazole | < LD | < LD | < LD | < LD | < LD | 552 |
| Gabapentin | < LD | 75 | 21 | < LD | < LD | < LD |
| Acetaminophen | < LD | 20 | 50 | < LD | < LD | 33 |
| Benzotriazol | < LD | < LD | < LD | < LD | < LD | 85 |
| Benzotriazol-methyl | < LD | < LD | < LD | < LD | < LD | 268 |
| Spiroxamin | < LD | < LD | 30 | < LD | < LD | < LD |
| Erytromycin | < LD | < LD | < LD | < LD | < LD | 181 |

Water samples collected at the same time as tadpole samples (~April 2012) again showed CT to have relatively low levels of contamination, although with notable detection of caffeine, AMPA and OP flame retardants (TCPP and TCEP). PF also showed detectable levels of caffeine, AMPA, and OP flame retardants. In contrast,

water collected from WH demonstrated detectable levels of several pesticides, particularly the fungicides carbendazim and flusilazole, the antibiotic erythromycin and the corrosion inhibitor benzotriazole. OP flame retardants were also detected at relatively high levels.

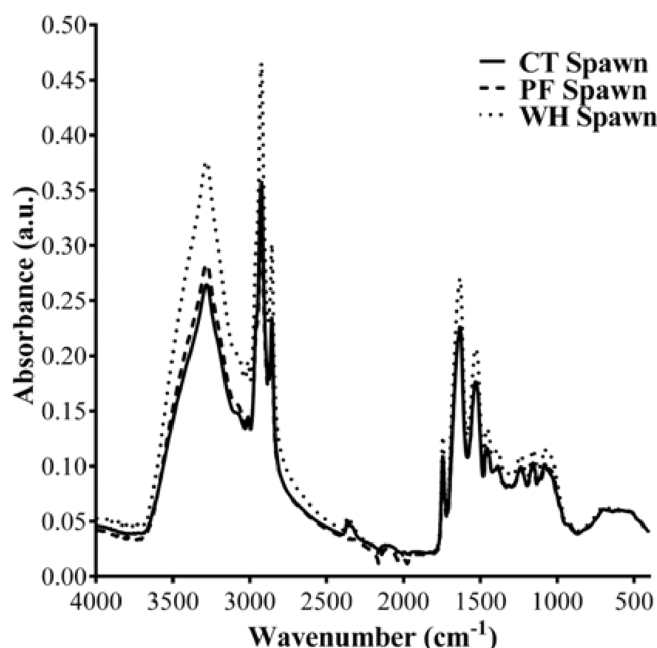
**Spawn**

Mean absorbance spectra of spawn samples prior to pre-processing are shown in Figure 1A, with figure 1B showing the mean spectra labelled with wavenumbers following pre-processing with second-order differentiation and vector normalisation . The assignment of the second derivative peaks are shown in Table 3.

Analysis of the peak heights of the second derivative spectra with one-way ANOVA and subsequent post-hoc tests demonstrated significant differences in regions assigned as $OCH_3$ and polysaccharides, where spawn collected from CT had a larger peak height than that from WH. Spawn collected from PF also had a larger peak height in comparison to spawn from WH in the region assigned as the stretching mode of phosphate groups in RNA. No other comparisons were significant (see Table 3).

Analysis of the data generated from the IR spectra of *R.temporaria* spawn with PCA revealed significant differences between CT and WH along PC4 (Fig. 2A), in regions associated with lipids and fatty acids (~1755-1730 $cm^{-1}$), with some contribution from proteins (~1600-1630 $cm^{-1}$), as shown in the loadings plot in Fig. 2B. Significant differences were also detected between spawn from CT and PF along PC7 (Fig. 2C) in regions associated with DNA (1096 $cm^{-1}$) and protein phosphorylation (1069 $cm^{-1}$) with additional contributions from the amide I absorbance of proteins (~1670-1610 $cm^{-1}$) , as denoted in the loadings plot in Fig. 2D. All other comparisons were not significant ($P > 0.05$). The largest seven discriminating loadings values for each significant PC and corresponding wavenumber assignments are shown in Table 4.

**1A**




**1B**

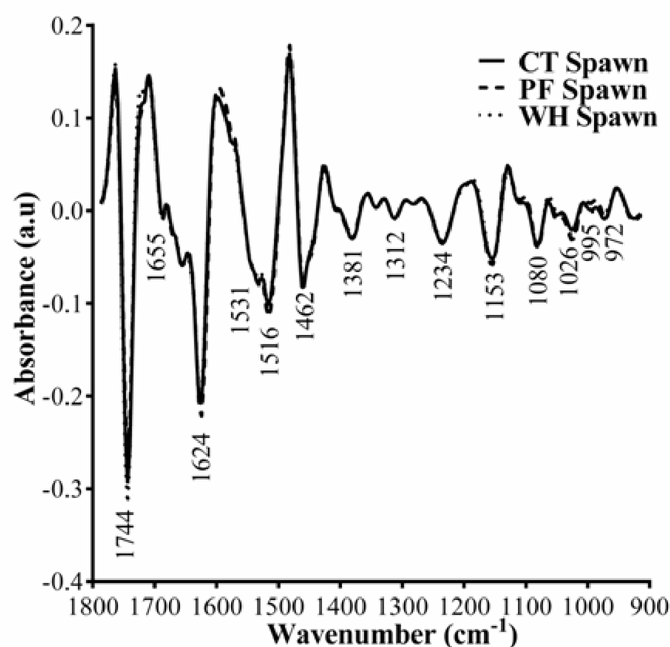



**Figure 1.** Mean ATR-FTIR spectra of *Rana temporaria* spawn collected from ponds with different contamination profiles: CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides; PF: an urban pond impacted by wastewater and landfill run-off. Spectra are shown prior to pre-processing **(A)** and following pre-processing **(B)**. Pre-processed spectra were cut to the 1800-900 cm$^{-1}$ region, the second derivative calculated using the Savitzky Golay algorithm (9 smoothing points) and vector normalised.

**Table 3.** Wavenumbers and assigned bands of infrared peaks following ATR-FTIR analysis of spawn of *Rana temporaria*. Absorbance values of second derivatives were compared between CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides and PF: an urban pond impacted by wastewater and landfill run-off.

| Wavenumber ($cm^{-1}$) | Proposed assignment [a] | Site comparison |
|---|---|---|
| 972 | $OCH_3$ polysaccharides | [b]CT = PF<br>CT > WH **<br>PF = WH |
| 995 | Stretching mode of phosphate groups in RNA | [b]CT = PF<br>CT = WH<br>PF > WH * |
| 1026 | Glycogen absorption (C-O stretching) | CT = PF<br>CT = WH<br>PF = WH |
| 1080 | $PO_2^-$ symmetric stretching: nucleic acids and phospholipids | CT = PF<br>CT = WH<br>PF = WH |
| 1153 | Stretching vibrations of hydrogen-bonded C-OH groups | CT = PF<br>CT = WH<br>PF = WH |
| 1234 | $PO_2^-$ asymmetric stretching, with overlap from Amide III | CT = PF<br>CT = WH<br>PF = WH |
| 1312 | Amide III of proteins | CT = PF<br>CT = WH<br>PF = WH |
| 1381 | Bending $CH_3$ | CT = PF<br>CT = WH<br>PF = WH |
| 1462 | $CH_2$ stretching of lipids | CT = PF<br>CT = WH<br>PF = WH |
| 1516 | Amide II of proteins | CT = PF<br>CT = WH<br>PF = WH |
| 1531 | Amide II, C≡N stretching | CT = PF<br>CT = WH<br>PF = WH |
| 1624 | Amide I, β-sheet | CT = PF<br>CT = WH<br>PF = WH |

| | | |
|---|---|---|
| 1655 | Amide I of proteins (α helix) | CT = PF<br>CT = WH<br>PF = WH |
| 1744 | C=O stretching of lipids | CT = PF<br>CT = WH<br>PF = WH |

[a] Sources: (Bellisola and Sorio, 2012; Cakmak et al., 2006; Cakmak et al., 2003; Chu et al., 2001; Maziak et al., 2007; Movasaghi et al., 2008).
Asterisks denote significance at the $P < 0.05$ (*) or < 0.001 level (**) following one way ANOVA and subsequently by Tukey's post-hoc comparison tests.
[b] Assumption of equality of variances not met, therefore one-way ANOVA with Welch's correction applied followed by Games-Howell post-hoc comparison tests.

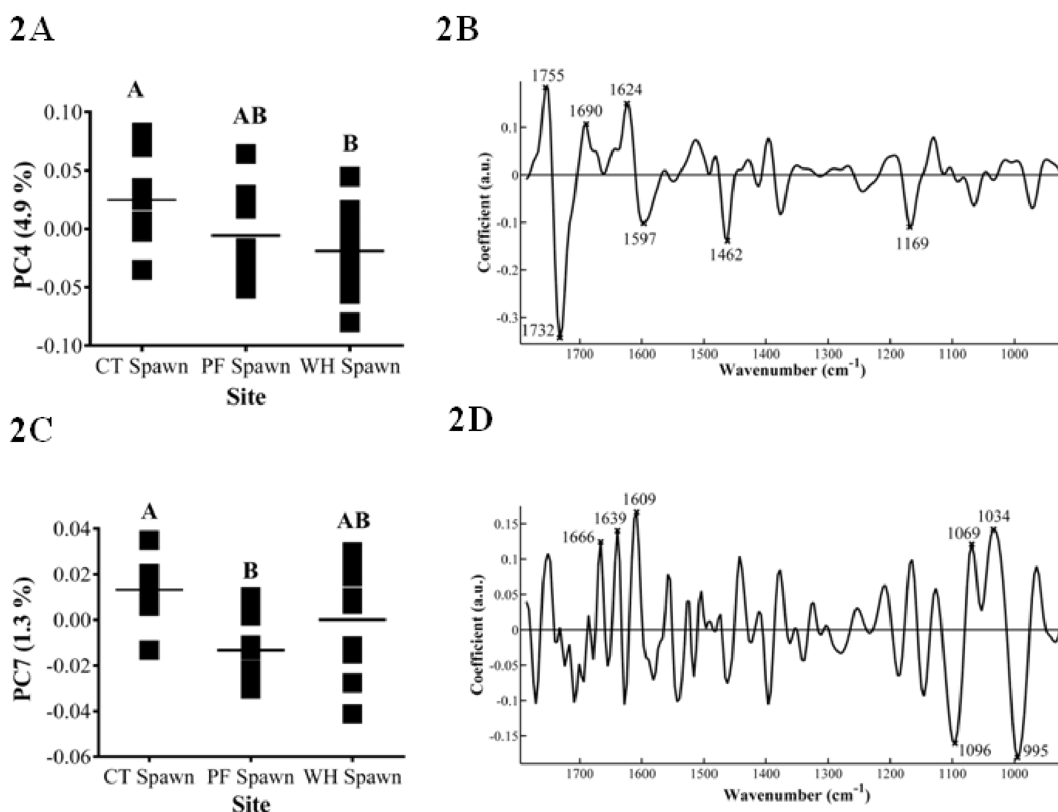



**Figure 2.** PCA scores and loadings generated from ATR-FTIR spectral data of *Rana temporaria* spawn collected from ponds with different contamination profiles: CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides; PF: an urban pond impacted by wastewater and landfill run-off ($n$ = 10 embryos for each pond sampled, total 30). The most discriminating PCs as determined by one-way ANOVA (where $P < 0.05$) were used to generate scores and corresponding loadings which best described the separation seen in the data: **A, B**: PC4 scores and loadings respectively; **C, D**: PC7 scores and loadings respectively. Tukey's or Games-Howell multiple comparison tests were used to determine where the significant differences were between sites. Different letters denote a significant difference between spawn ($P < 0.05$).

**Table 4.** Distinguishing wavenumbers and proposed assignments obtained from analysis of *Rana temporaria* spawn and tadpoles with ATR-FTIR spectroscopy following analysis with PCA. The seven largest loadings values for the most discriminating principal components are shown.

| Loading and Stage | Wavenumber ($cm^{-1}$) | Proposed Assignment [a] |
|---|---|---|
| Loading 4 Spawn | 1732 | C=O stretching of lipids |
| | 1755 | Lipids and fatty acids |
| | 1624 | Amide I |
| | 1462 | $CH_2$ bending vibration (lipids and proteins) |
| | 1169 | C-O bands from glycomaterials and proteins |
| | 1690 | Peak of nucleic acids from base carbonyl stretching and ring breathing mode |
| | 1597 | C=N, $NH_2$ adenine |
| Loading 7 Spawn | 995 | Stretching modes of phosphate groups in RNA |
| | 1609 | Adenine vibration in DNA |
| | 1096 | Symmetric stretching of phosphate $PO^-_2$ |
| | 1034 | Collagen |
| | 1639 | Amide I of $\beta$-pleated sheet structures |
| | 1666 | C=O stretching vibration of pyrimidine base |
| | 1069 | Stretching C-O DNA, RNA, phospholipid, phosphorylated protein |
| Loading 1 Tadpole | 1003 | Sugar phosphate chain vibrations in nucleic acids |
| | 1030 | Glycogen vibration |
| | 1076 | Symmetric phosphate stretching |
| | 957 | Stretching of phosphorylated protein |
| | 1049 | Glycogen, C-O stretching and bending of carbohydrates |
| | 1150 | C-O stretching of carbohydrates |
| | 1616 | Amide I (carbonyl stretching vibrations in side chains of amino acids) |
| Loading 3 Tadpole | 1624 | Amide I, $\beta$-sheet |
| | 1462 | $CH_2$ bending vibration (lipids and proteins) |
| | 1497 | C=C deformation, C-H |
| | 1693 | High frequency vibration of Amide I (β-sheet) |
| | 1034 | Collagen |
| | 1551 | Amide II base vibrations |
| | 1717 | C=O vibration of purine base |
| Loading 5 Tadpole | 1616 | Amide I (carbonyl stretching vibrations in side chains of amino acids) |
| | 1643 | Amide I (C=O stretching vibrations) |
| | 1501 | Amide II (N-H bending vibration coupled to C-N stretching) |
| | 1069 | Stretching C-O ribose |
| | 1042 | C-O stretching: polysaccharides |
| | 1555 | Ring base |
| | 1721 | C=O band |

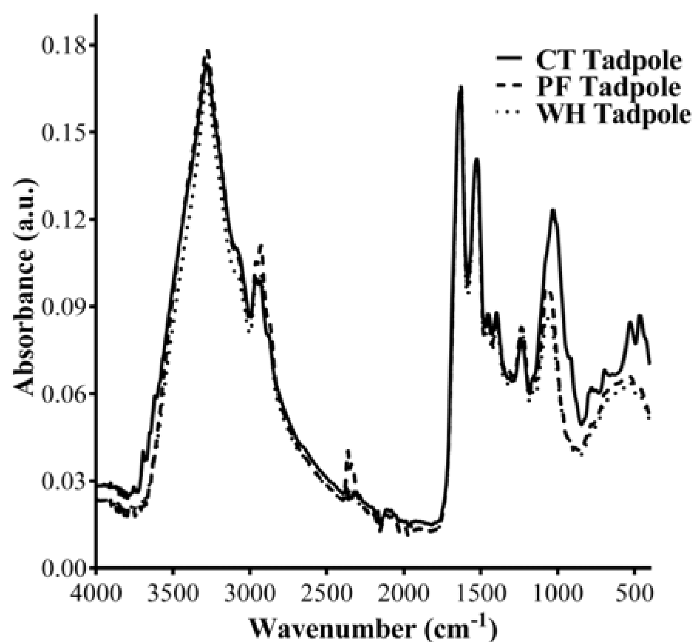
[a] Sources: (Bellisola and Sorio, 2012; Cakmak et al., 2006; Cakmak et al., 2003; Chu et al., 2001; Maziak et al., 2007; Movasaghi et al., 2008).

**Tadpoles**

Mean absorbance spectra of tadpole samples prior to pre-processing are shown in Figure 3A, with figure 3B showing the mean spectra labelled with wavenumbers following pre-processing with second-order differentiation and vector normalisation. The assignment of the second derivative peaks are shown in Table 3. Analysis of the peak heights from the second derivative spectra of *R.temporaria* tadpoles shown in figure 3B with one-way ANOVA revealed that there were significant differences between tadpoles from each pond for the majority of the wavenumbers tested, as shown in Table 5. Subsequent post-hoc tests demonstrated that in general the differences were between tadpoles from CT and those from PF and/or WH (in 12 out of 15 wavenumbers tested), with differences between PF and WH only apparent at 5 out of 15 wavenumbers tested.

In regions associated with DNA vibrations and phospholipids (964, 1080, 1234 $cm^{-1}$), peak heights generated from tadpoles from CT were generally significantly smaller than those from PF and WH. In contrast, in regions associated with glycogen (1030 $cm^{-1}$), peak heights measured in tadpoles from CT were significantly larger than those from PF and WH. The peak at ~1740 $cm^{-1}$ associated with lipids was absent from the second derivative spectrum of the tadpoles measured (see Fig. 3B); other peaks associated with lipids and fatty acids at 1447 and 1393 $cm^{-1}$ demonstrated no differences between ponds and a significantly larger peak measured in tadpoles from CT compared to PF respectively. Protein absorbance varied between ponds, with peak height in tadpoles collected from CT significantly smaller at some measures of protein absorbance in comparison to those from PF and in some cases WH (1312, 1157, 1531, and 1624 $cm^{-1}$), but larger at others (1643 $cm^{-1}$).

3A




3B

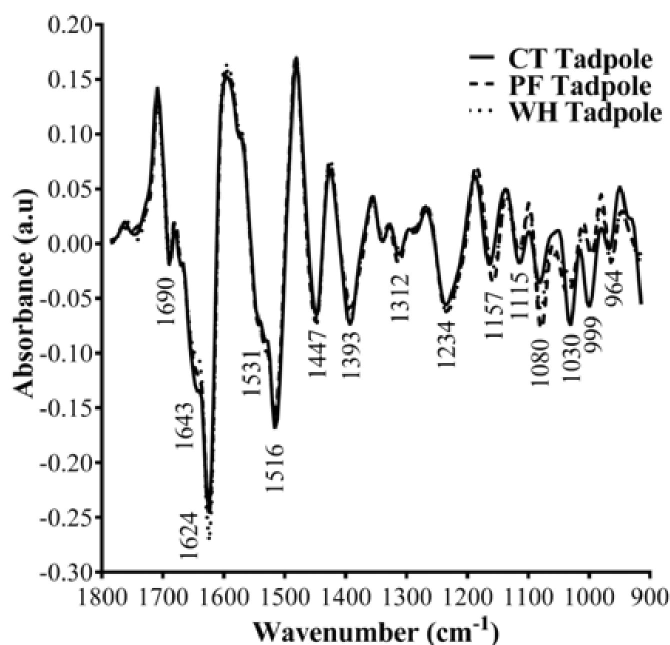



**Figure 3.** Mean ATR-FTIR spectra of *Rana temporaria* tadpoles collected from ponds with different contamination profiles: CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides; PF: an urban pond impacted by wastewater and landfill run-off. Spectra are shown prior to pre-processing **(A)** and following pre-processing **(B)**. Pre-processed spectra were cut to the 1800-900 $cm^{-1}$ region, the second derivative calculated using the Savitzky-Golay algorithm (9 smoothing points) and vector normalised.

**Table 5.** Wavenumbers and assigned bands of infrared peaks following ATR-FTIR analysis of whole tadpoles of *Rana temporaria*. Absorbance values of second derivatives were compared between CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides and PF: an urban pond impacted by wastewater and landfill run-off.

| Wavenumber ($cm^{-1}$) | Proposed assignment [a] | Site comparison |
|---|---|---|
| 964 | C-C stretch of nucleic acids | [b] CT < PF *<br>CT < WH *<br>PF = WH |
| 999 | C-C vibration of DNA | [b] CT > PF **<br>CT > WH **<br>PF = WH |
| 1030 | Glycogen vibration | [b] CT > PF **<br>CT > WH **<br>PF = WH |
| 1080 | $PO_2^-$ symmetric stretching: nucleic acids and phospholipids | CT < PF **<br>CT < WH **<br>PF > WH ** |
| 1115 | Symmetric stretching P-O-C | CT > PF **<br>CT > WH **<br>PF = WH |
| 1157 | C-O vibrations of proteins and carbohydrates | CT < PF **<br>CT = WH<br>PF > WH ** |
| 1234 | $PO_2^-$ asymmetric stretching | CT < PF **<br>CT < WH **<br>PF = WH |
| 1312 | Amide III of proteins | [b] CT < PF **<br>CT > WH *<br>PF > WH ** |
| 1393 | $CH_3$ bending of proteins and lipids | CT > PF **<br>CT = WH<br>PF = WH |
| 1447 | $CH_2$ bending of lipids and fatty acids | CT = PF<br>CT = WH<br>PF = WH |
| 1516 | Amide II of proteins | CT = PF<br>CT = WH |

| | | |
|---|---|---|
| | | PF = WH |
| 1531 | Amide II, C≡N stretching | CT < PF *<br>CT < WH **<br>PF = WH |
| 1624 | Amide I, β-sheet | CT =PF<br>CT < WH **<br>PF < WH ** |
| 1643 | Amide I (C=O vibrations) | CT > PF *<br>CT > WH **<br>PF = WH |
| 1690 | Peak of nucleic acids due to carbonyl stretching | CT = PF<br>CT = WH<br>PF < WH * |

[a] Sources: (Bellisola and Sorio, 2012; Cakmak et al., 2006; Cakmak et al., 2003; Chu et al., 2001; Movasaghi et al., 2008).
Asterisks denote significance at the $P < 0.05$ (*) or $< 0.001$ level (**) following one way ANOVA and subsequently by Tukey's post-hoc comparison tests.
[b] Assumption of equality of variances not met, therefore one-way ANOVA with Welch's correction applied followed by Games-Howell post-hoc comparison tests.

Analysis of the data generated from the IR spectra of *R.temporaria* tadpoles with PCA revealed significant segregation along PC1, with tadpoles from CT segregating very clearly away from those collected from PF and WH but no differences between tadpoles from PF and those from WH along this dimension, as shown in Figure 4A. The wavenumbers attributable to this separation were from regions associated with protein phosphorylation (~960-1000 $cm^{-1}$) and carbohydrates, particularly glycogen (~1030-1050 $cm^{-1}$, 1150 $cm^{-1}$), with smaller contributions from DNA (1076 $cm^{-1}$) and protein (1616 $cm^{-1}$) (see Fig. 4B). In contrast, along PC3 (Fig. 4C), tadpoles from WH segregate away from PF and CT tadpoles in regions mainly associated with protein (amide I and II) and lipid (see Fig. 4D).

PC5 accounts for separation between tadpoles from WH and PF only (Fig. 4E); here the wavenumbers responsible for the separation were in regions associated with protein (amide I and II), DNA, carbohydrate and lipids (Fig. 4F). No other PCs accounted for significant segregation between PC scores (Table S4 in SI).The largest seven discriminating loadings values for each significant PC and corresponding wavenumber assignments are shown in Table 4.

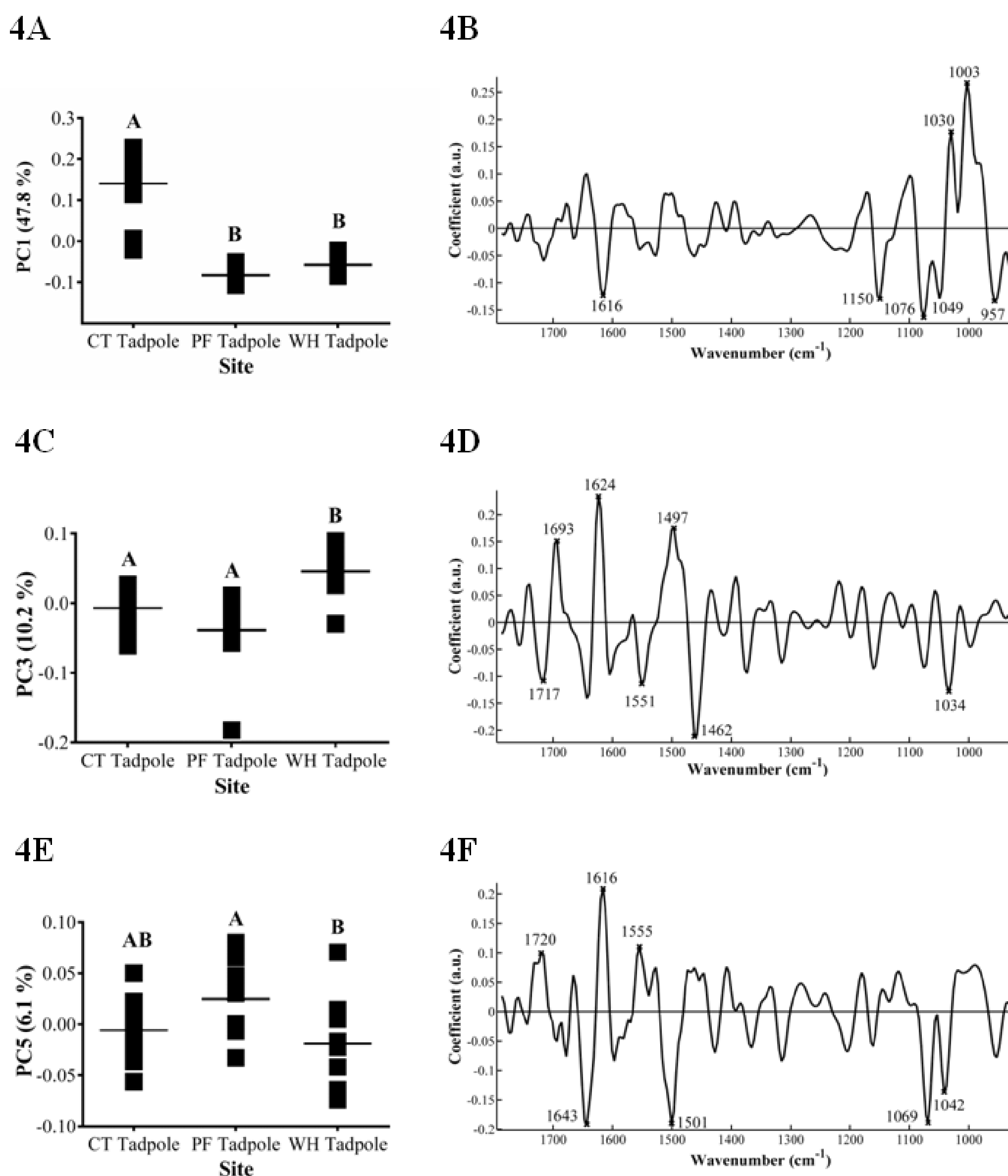



**Figure 4.** PCA scores and loadings generated from ATR-FTIR spectral data of *R.temporaria* tadpoles collected from ponds with different contamination profiles: CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides; PF: an urban pond impacted by wastewater and landfill run-off ($n$ = 10 tadpoles per pond, total 30). The most discriminating PCs as determined by one-way ANOVA (where $P < 0.05$) were used to generate scores and corresponding loadings which best described the separation seen in the data: **A, B**: PC1 scores and loadings respectively; **C, D**: PC3 scores and loadings respectively; **E, F**: PC5 scores and loadings respectively. Tukey's or Games-Howell multiple comparison tests were used to determine where the significant differences were between sites. Different letters denote a significant difference between tadpoles ($P < 0.05$).

### Morphometric measurements

Tadpoles did not differ significantly between sites in measurements of mass, BCI, HW or SVL (One-way ANOVA: Mass: $F_{2, 27}$ = 1.34, $P$ > 0.05; BCI: $F_{2, 27}$ = 3.17, $P$ > 0.05; HW: $F_{2, 27}$ = 0.67, $P$ > 0.05; SVL: $F_{2, 27}$ = 0.33, $P$ > 0.05).

## Discussion

Chemical analysis of the water (Tables 1 and 2) confirmed the classification of ponds based on their land-use data i.e. CT was defined as a rural agricultural site, with the input sources confirmed as nutrients and commonly found water contaminants such as caffeine, but no input from pesticides. Levels of nitrate and phosphate at this site remained low during the sampling period (< 0.22 and <0.03 mg/L respectively). PF was defined as an urban site with input from general-use herbicides (glyphosate), some pharmaceuticals such as acetaminophen, and the PAH naphthalene. Additionally there was input from phosphates at this site, reaching high levels (0.07-0.30 mg/L), most likely due to wastewater run-off containing detergents (Mainstone and Parr, 2002). WH was defined as an agricultural site impacted by pesticides, which was confirmed by detectable levels of several herbicides, particularly fungicides, in agreement with agronomist's report on pesticide use in the surrounding farmland (SI Table S1).

Fungicides, particularly azole fungicides as measured at WH, have been associated with negative effects on development and sexual differentiation in amphibians and other aquatic species even at low levels of exposure (Andrade et al., 2016; Menegola et al., 2001; Poulsen et al., 2015; Yoon et al., 2008). Glyphosate and its metabolite AMPA was detected at both WH and PF. Glyphosate has been associated with detrimental effects on survival in some species of amphibian (Relyea, 2005) and with decreases in biochemical parameters such as glycogen and triglycerides in bullfrog tadpoles at relatively low (18 µg/L) concentrations (Dornelles and Oliveira, 2014; Dornelles and Oliveira, 2016). Pharmaceutical drugs and corrosion inhibitors, were also detected in water samples at WH as well nutrient input from nitrate and phosphate, the latter reaching levels of 0.64 mg/L during April, which is regarded as

relatively high and associated with moderate/poor water quality in lowland non-alkaline water bodies in the UK and likely due to agricultural run-off (UKTAG, 2013; Williams et al., 2004). Exposure of tadpoles to acetaminophen has previously shown to have either no effect on activity levels, or significant effects depending on the species of tadpole exposed, although at higher concentrations than those found here (Fraker and Smith, 2004; Smith and Burgett, 2005). Caffeine was found in both CT and PF water samples; levels similar to those measured in this study have previously shown no effect on survivorship or activity levels of *Bufo* americanus tadpoles (Smith and Burgett, 2005), although activity levels were significantly reduced and startle response increased in *Rana pipiens* tadpoles (Fraker and Smith, 2004), suggesting the response is species-specific. Interestingly, all three sites had detectable levels of OP flame retardants (TCEP, TCPP TEP and TBP), the highest of which were found at PF and WH. These compounds are used as plasticizers and flame retardants and frequently detected in surface waters due to their lack of biodegradability in wastewater treatment (Fries and Puttmann, 2003; Regnery and Püttmann, 2010). As PF receives treated wastewater as well as run-off from landfill, this may explain the higher levels found here. WH also received water run-off from a septic tank system; this was upgraded in February 2012 (Ockenden et al., 2014). Research on the toxicity of OP flame retardants is limited, however in human cell lines and zebrafish, endocrine disrupting activity has been noted, with the ratio of estradiol to testosterone disrupted following exposure to these compounds (Liu et al., 2012).

Analysis of *R.temporaria* spawn with ATR-FTIR spectroscopy revealed small but significant differences between embryos from CT and those from PF and WH. These differences were small in comparison to those found between tadpoles, which is in agreement with other studies and likely to be due in part to the jelly capsule surrounding the embryo, affording protection against some environmental contaminants (Anguiano et al., 1994; Berrill et al., 1994; Berrill et al., 1998; Cooke, 1972; Edginton et al., 2007; Marquis et al., 2006; Meredith and Whiteman, 2008; Ortiz-Santaliestra et al., 2006), but not others (Greulich and Pflugmacher, 2003). This may explain why the differences detected between embryos in the current study were relatively smaller in comparison to that of the tadpoles despite water quality differences between the sites.

The regions of the IR spectrum associated predominantly with lipids and proteins (~1730-1755, 1462, 1624 $cm^{-1}$) were responsible for differences between spawn from CT and WH, whereas symmetric phosphate stretching and protein phosphorylation/phospholipids (1096, 1069 $cm^{-1}$) and the amide I protein region (1666, 1639 $cm^{-1}$) accounted for differences between spawn from CT and that from PF. Increases in phospholipids and decreases in protein content have previously been observed in *Bufo arenarum* embryos exposed to the OP insecticide malathion (Rosenbaum et al., 1988). Further analysis of the second derivative peak heights demonstrated that spawn from CT had higher levels of polysaccharides in comparison to WH, and PF had greater absorbance in the region associated with the stretching modes of phosphate groups in RNA in comparison to WH. The yolk platelets are the main energy store of vertebrate embryos, including amphibians, and contain small amounts of nucleic acids and polysaccharides, which are released into the cytoplasm of embryonic cells during degradation of the yolk in early embryogenesis (Komazaki and Hiruma, 1999). The differences in polysaccharides and nucleic acids detected between spawn from different ponds in this study may simply be reflective of slightly different stages in embryogenesis (Fagotto and Maxfield, 1994).

Clear differences were apparent in the mean spectra of *R.temporaria* tadpoles collected from CT, PF and WH, with the largest differences apparent between CT and the other two ponds. This was confirmed by analysis with PCA and analysis of the second derivative peak heights, with the greatest segregation between tadpoles from CT and the other two ponds. Regions of the IR spectrum associated with carbohydrates, particularly glycogen showed marked decreases in the peak heights in spectra of tadpoles from PF and WH in comparison to those from CT. Several studies have measured glycogen levels in tissues of both larval and adult amphibians following exposure to various environmental contaminants, including pesticides such as atrazine (Dornelles and Oliveira, 2014; Ezemonye and Tongo, 2009; Zaya et al., 2011), glyphosate (Dornelles and Oliveira, 2014; Dornelles and Oliveira, 2016), quinclorac (Dornelles and Oliveira, 2014), basudin (Ezemonye and Ilechie, 2007), naphthenic acids (Melvin et al., 2013) and PAHs (Gendron et al., 1997). In general, these studies found depleted levels of glycogen in response to pesticide exposure, although not in all cases (Zaya et al., 2011). Tadpoles exposed to sub-lethal wastewater mixtures also show decreased glycogen levels (Melvin et al., 2016).

Glycogen levels can be depleted in response to other environmental stressors, such as hypoxia/anoxia, as glycogen is the main energy substrate utilised during metabolic depression in ranid species (Emel'yanova et al., 2007; Loumbourdis and Kyriakopoulou-Sklavounou, 1991; Smith, 1950). Given that the levels of phosphate were relatively high during tadpole development at PF and WH (0.3 mg/L and 0.6 mg/L respectively); these ponds could potentially be at risk of eutrophication, resulting in hypoxia (Correll, 1998). Larval amphibians are susceptible to chemical insult during development, causing alterations in energy storage and metabolism as energy is allocated towards detoxification pathways and maintaining homeostasis, resulting in decreased levels of key energy stores such as glycogen. Thus the decreases seen in glycogen absorbance in the infrared spectra of tadpoles collected from ponds with urban and agricultural run-off may be due to increased glycogenolysis (Dornelles and Oliveira, 2014; Dornelles and Oliveira, 2016; Melvin et al., 2013; Melvin et al., 2016). This could have broader ecological consequences as organisms may divert energy away from other processes such as growth, development and reproduction, resulting in an overall negative effect on the health of the organism in response to environmental stress (Wingfield et al., 1998).

Loadings corresponding to PC1 also showed changes in regions associated with phosphate chain vibrations in nucleic acids (1003 $cm^{-1}$) and stretching of symmetric phosphate (1076 $cm^{-1}$); increases in both symmetric and asymmetric phosphate absorbance values were also observed in tadpoles from PF and WH in comparison to those from CT. Infrared spectroscopy has previously detected increases in asymmetric and symmetric phosphate absorbance values in fish following exposure to endocrine disruptors (Cakmak et al., 2006; Cakmak et al., 2003) and PAHs (Obinaju et al., 2014). Exposure to agricultural and urban run-off has been associated with genotoxicity in tadpoles (Ralph and Petras, 1997, 1998), with exposure to chemicals including those measured in this study such as glyphosate associated with DNA damage (Clements et al., 1997). Whilst only speculative, the changes in regions of the spectrum associated with DNA vibrations could therefore be due to exposure of tadpoles to potential genotoxic agents in the water, although caution must be exercised as tadpoles were exposed to a mixture of chemicals, as well as varying nutrient levels, so it is not possible to identify a single factor. PC3 showed separation between tadpoles from WH and those from both PF and CT, with the wavenumbers responsible

mainly associated with proteins and lipids. PC5, which accounted for the smallest amount of variance in the data (6.1%), revealed differences between WH and PF only); these differences were in regions associated with protein (amide I and II) and DNA mainly, with some contribution from carbohydrate and lipids.

Anuran amphibians also utilise lipids as a primary endogenous energy source, particularly during metamorphic climax, reproduction and hibernation (Gurushankara et al., 2007; Sheridan and Kao, 1998). The deposition and mobilisation of lipids in amphibians is controlled by hormone –sensitive enzyme systems, with whole-body levels of lipid low in early pre-metamorphic tadpoles (prior to significant hind-limb development, < Gosner stage 31 (Crespi and Denver, 2005)), followed by an increase in pro-metamorphic tadpoles ( during hind-limb development > Gosner stage 36 (Crespi and Denver, 2005)) (Sheridan and Kao, 1998). As the tadpoles in this study were Gosner stage 25-28, the absence of a distinct lipid peak ~1740 $cm^{-1}$ (Cakmak et al., 2006) in the spectrum is therefore not surprising. There were, however, other peaks associated with lipids and fatty acids detected in the infrared spectrum (1393, 1447 $cm^{-1}$); with either no differences between ponds or a greater peak height at CT in comparison to PF. Disturbances in lipid metabolism due to contaminant exposure have previously been associated with negative impacts on amphibian health (Gurushankara et al., 2007) , with some studies finding an increase in lipid levels (Melvin et al., 2013), while others found a decrease (Zaya et al., 2011). Changes in constituents associated with regulation of energy metabolism such as lipids and glycogen have been suggested as potential integrative markers of exposure to chemical stressors in larval amphibians due to the large physiological and metabolic changes associated with metamorphosis (Dornelles and Oliveira, 2016; Melvin, 2015; Melvin et al., 2013; Melvin et al., 2016).

Several studies have applied IR spectroscopy as an ecotoxicological tool in assessing the effects of environmental pollution in fish (Cakmak et al., 2006; Cakmak et al., 2003; Chu et al., 2001; Malins et al., 2006; Malins et al., 2004; Obinaju et al., 2014; Obinaju et al., 2015) The results here suggest that IR spectroscopy may also have promise as an environmental monitoring tool in amphibian populations and future work should aim to assess populations over longer periods of time in order to account for potential multi-generational effects (Sparling et al., 2010). None of these sites, to

the authors' knowledge have been subject to amphibian surveys to assess population numbers. In view of the fact that no major morphological differences were apparent between these sites, then biospectroscopy offers a unique tool to assess the stress status of wild populations living in contaminated sites. In addition, the use of hand-held IR devices could potentially allow the non-destructive monitoring of amphibians throughout their development, which could be of great benefit in the many species of amphibian vulnerable to extinction.

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## Supplementary information:

**Number of figures = 1**

**Number of tables = 4**

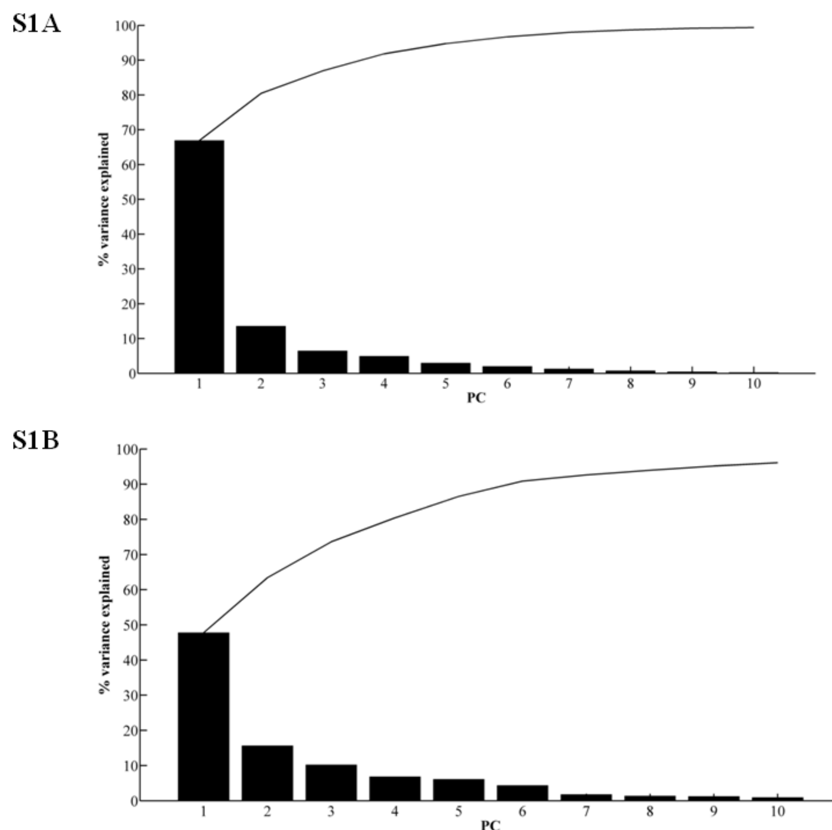


**Figure S1.** Percentage variance the first ten PCs contribute following PCA of ATR-FTIR spectral data generated from *Rana temporaria* spawn **(S1A)** and tadpoles **(S1B)**. The line represents cumulative variance. Data were cut at the 1800-900 $cm^{-1}$ region, the second derivative calculated using the Savitzky-Golay algorithm (9 smoothing points), vector normalised and mean-centred prior to PCA.

**Table S1**. Agronomist's report from Whinton Hill detailing which pesticides were applied from August 2011-July 2012, covering the sampling period.

| Trade name | Constituents | Application date | Type | Crop |
|---|---|---|---|---|
| Oryx | 83 g/L Quinmerac; 333g/L Metazachlor | 15/08/2011 | Herbicide | Oilseed rape |
| Cirrus CS | 371.130 g /L Clomazone | 15/08/2011 | Herbicide | Combining pea, field bean, oilseed rape, vining pea |
| Grounded | Proprietary blend of aliphatic hydrocarbons, hexahydric alcohol ethoxylates and C18-C20 fatty acids and alkanolamides | 15/08/2011 | Spray application deposition aid | |
| Carakol 3 | 3 % Metaldehyde | 15/08/2011 | Molluscicide | All edible crops |
| Shogun | 100 g / L propaquizafop | 11/09/2011 | Herbicide | Bulb onion, carrot, cut log, oilseed rape, parsnip, pea (combining), potato, sugar beet, swede, turnip |
| Capitan 25 | 250 g/l Flusilazole | 23/10/2011 | Fungicide | Winter wheat, winter and spring barley, oilseed rape and sugar beet |
| Sunorg Pro | 90 g/L Metconazole | 23/10/2011 | Fungicide | Winter wheat, winter and spring barley, oilseed rape and sugar beet |
| Multitrace B | Fertilizer - trace elements including boron | 23/10/2011 | Foliar nutrient | |
| Monkey | 133 g/ L Tebuconazole; 267 g/L Prochloraz | 25/03/2012 | Fungicide | Wheat, barley, rye, oilseed rape |
| Filan | 50% Boscalid | 25/03/2012 | Fungicide | Wheat, barley, rye, oilseed rape |
| Snapper | Glyphosate | 24/07/2012 | Herbicide | Wheat, barley, rye, oilseed rape |
| Companion Gold | Aqueous solution of polymer and ammonium sulphate | 24/07/2012 | Adjuvant for use with glyphosate | |

**Table S2.** Gosner stage, snout to vent length (SVL), head width (HW), mass and body condition index (BCI = (body mass/$SVL^3$) X 100) of *Rana temporaria* tadpoles collected from two agricultural sites; one with pesticide use (WH) and one without pesticide use (CT) and an urban site (PF).

| Site | Gosner Stage | SVL (mm) | Head Width (mm) | Mass (mg) | BCI | Date collected |
|---|---|---|---|---|---|---|
| PF | 26 | 8.60 | 5.18 | 84.0 | 13.2 | 17/04/2012 |
| PF | 25 | 5.75 | 3.30 | 33.0 | 17.4 | 17/04/2012 |
| PF | 25 | 6.44 | 3.04 | 40.0 | 15.0 | 17/04/2012 |
| PF | 25 | 7.16 | 4.16 | 61.5 | 16.8 | 17/04/2012 |
| PF | 25 | 6.17 | 2.97 | 26.0 | 11.1 | 17/04/2012 |
| PF | 25 | 6.16 | 3.47 | 34.7 | 14.8 | 17/04/2012 |
| PF | 26 | 7.42 | 4.41 | 74.2 | 18.2 | 17/04/2012 |
| PF | 26 | 7.20 | 3.57 | 61.5 | 16.5 | 17/04/2012 |
| PF | 26 | 7.54 | 4.47 | 80.6 | 18.8 | 17/04/2012 |
| PF | 25 | 6.84 | 3.95 | 60.9 | 19.0 | 17/04/2012 |
| CT | 25 | 7.22 | 4.64 | 59.0 | 15.7 | 18/04/2012 |
| CT | 25 | 6.17 | 3.78 | 26.0 | 11.1 | 18/04/2012 |
| CT | 25 | 6.87 | 3.82 | 60.9 | 18.8 | 18/04/2012 |
| CT | 27 | 8.03 | 4.83 | 84.7 | 16.4 | 18/04/2012 |
| CT | 25 | 7.00 | 4.34 | 52.0 | 15.2 | 18/04/2012 |
| CT | 25 | 5.86 | 3.42 | 25.0 | 12.4 | 18/04/2012 |
| CT | 25 | 5.74 | 3.52 | 33.0 | 17.4 | 18/04/2012 |
| CT | 25 | 6.55 | 3.73 | 35.0 | 12.5 | 18/04/2012 |
| CT | 28 | 9.32 | 6.06 | 84.0 | 10.4 | 18/04/2012 |
| CT | 25 | 6.15 | 3.50 | 34.7 | 14.9 | 18/04/2012 |
| WH | 28 | 8.09 | 4.26 | 84.7 | 16.0 | 18/04/2012 |
| WH | 28 | 7.65 | 4.15 | 68.4 | 15.3 | 18/04/2012 |
| WH | 26 | 7.59 | 4.16 | 80.6 | 18.4 | 18/04/2012 |
| WH | 27 | 6.33 | 3.65 | 41.0 | 16.2 | 18/04/2012 |
| WH | 27 | 7.31 | 4.03 | 74.2 | 19.0 | 18/04/2012 |
| WH | 26 | 6.65 | 3.35 | 52.7 | 17.9 | 18/04/2012 |
| WH | 28 | 7.71 | 4.46 | 68.4 | 14.9 | 18/04/2012 |
| WH | 27 | 6.95 | 3.70 | 66.4 | 19.8 | 18/04/2012 |
| WH | 26 | 7.17 | 3.74 | 61.5 | 16.7 | 18/04/2012 |
| WH | 26 | 6.37 | 3.29 | 44.0 | 17.0 | 18/04/2012 |

**Table S3.** List of chemicals screened for in the analysis of water samples collected from CT, PF and WH. Herbicides and fungicides were measured using LC/MS/MS (triple quadrapole detector). All other compounds were measured using GC/MS with mass spectra identification screening of unknowns (NIST 02 and Wiley Mass Spectra Library).

| Chemical | Group | Detection limit (ng/L) |
|---|---|---|
| Metazachlor | Chloroacetanilide herbicide | 5 |
| Dimethachlor | Chloroacetanilide herbicide | 5 |
| Acetochlor | Chloroacetanilide herbicide | 5 |
| Chlorotoluron | Urea herbicide | 20 |
| Glyphosate | Phosphonoglycine herbicide | 25 |
| AMPA | Phosphonoglycine herbicide degradation product | 25 |
| Tebuconazole | Triazole fungicide | 20 |
| Prochloraz | Imidazole fungicide | 20 |
| Metconazole | Triazole fungicide | 10 |
| Carbendazim | Benzimidazole fungicide | 10 |
| Flusilazole | Triazole fungicide | 5 |
| Spiroxamine | Morpholine fungicide | 10 |
| Boscalid | Carboxamide fungicide | 10 |
| Aniline | Chemical intermediate/degradation product from herbicides. | 200 |
| Acetaminophen | Pharmaceutical (analgesic) | 20 |
| Gabapentin | Pharmaceutical (anti-convulsant) | 20 |
| Ketoprofen | Pharmaceutical (NSAID) | 10 |
| Erythromycin | Macrolide antibiotic | 10 |
| Benzotriazole | Heterocyclic corrosion inhibitor | 10 |
| Benzotriazole-methyl | Heterocyclic corrosion inhibitor | 10 |
| Triethyl phosphate (TEP) | Non-halogenated alkyl phosphate flame retardant | 10 |
| Tributyl phosphate (TBP) | Non-halogenated alkyl phosphate flame retardant | 10 |
| Tris(2-chloroethyl)phosphate (TCEP) | Chlorinated alkyl phosphate flame retardant | 5 |
| Tris(1-chloro-2-propyl)phosphate (TCPP) | Chlorinated alkyl phosphate flame retardant | 5 |
| Naphthalene | Polycyclic aromatic hydrocarbon (PAH) | 5 |

**Table S4.** Results from one way ANOVA of the scores generated from principal component analysis for the first ten principal components following ATR-FTIR analysis of spawn and tadpoles of *Rana temporaria*. Significant results are in bold.

| PC | Spawn | | Tadpole | |
|---|---|---|---|---|
| | **F Statistic** | ***P* value** | **F Statistic** | ***P* value** |
| 1 | 2.59 | 0.09 | **31.88** [a] | **<0.001**[a] |
| 2 | 0.64 | 0.54 | 0.35 | 0.71 |
| 3 | 0.46 | 0.64 | **11.29** | **<0.001** |
| 4 | **3.78** | **0.04** | 0.95 | 0.40 |
| 5 | 0.95 | 0.40 | **3.59** | **0.04** |
| 6 | 3.38 | 0.05 | 1.46 | 0.25 |
| 7 | **5.81**[a] | **0.002**[a] | 0.09 | 0.91 |
| 8 | 0.70 | 0.14 | 0.95 | 0.40 |
| 9 | 2.10 | 0.16 | 0.25 | 0.78 |
| 10 | 1.96 | 0.09 | 0.03 | 0.97 |

**[a] These values were generated following Welch's correction due to inequality of variances.**

# Chapter 3.

**Using biospectroscopy to monitor amphibian health: a three year study.**

**Rebecca J. Strong**, Crispin J. Halsall, Kevin C. Jones, Richard F. Shore and Francis L. Martin.

**Manuscript for submission**

Contribution:

- I acquired the samples required for the project.
- I prepared, processed and acquired data for spawn and tadpole samples including conducting computational analysis.
- I prepared the first draft of the manuscript.

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Rebecca J. Strong Prof. Francis L. Martin

**Using biospectroscopy to monitor amphibian health: a three year study**

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## Abstract

Amphibians are a class of organism vulnerable to environmental pollution due to their life history and permeable skin. As many species of amphibian show high site fidelity, there is a need for a biomonitoring approach that can assess the impacts of repeated exposure to environmental contamination over a long term period. This study assessed the use of infrared (IR) spectroscopy coupled with multivariate analysis and classification techniques in monitoring the health of common frog spawn and tadpoles over a three year period. Tadpoles and spawn were collected from ponds with differing water quality in 2012, 2013 and 2014. Comparisons were made between ponds using data collected over the three year period to determine if spawn and tadpoles could be classified based on pond of origin, despite marked annual differences. Comparisons were also made at each pond, between years to determine the effect annual factors had on the IR spectra generated. Tadpoles were readily classified based on pond of origin despite annual differences, whereas spawn showed much poorer classification rates, likely due to annual factors such as temperature masking any differences between ponds. The differences between years for tadpoles were generally in different areas of the spectrum in comparison to those between ponds, suggesting temporal effects, possibly tied to body size, as measures of body size also showed annual variation. This study highlights the use of IR spectroscopy in monitoring amphibian populations over time as a rapid and cost-effective technology, which has great promise in environmental research.

## Introduction

Amphibian numbers are declining at alarming rates around the world (Alford and Richards, 1999), with several factors implicated in such declines, among them environmental pollution, which is cited at the most important factor in such declines after habitat degradation (Davidson et al., 2002; Egea-Serrano et al., 2012; Mann et al., 2009). Several different types of pollutants occur in natural habitats, among them fertilisers and pesticides from agricultural run-off or direct application (Carpenter et al., 1998; Carter, 2000), and pharmaceutical drugs, detergents, flame retardants and plasticisers used in consumer products from urban and industrial wastewater and surface run-off (Buerge et al., 2003; Fent et al., 2006; Fries and Puttmann, 2003; Stackelberg et al., 2004). Atmospheric deposition also contributes to the overall deposition of contaminants around the world from areas of intensive use (Reemtsma et al., 2008; Wania and Mackay, 1993). The amount of pollutants in the environment is projected to increase in the future, with growing human populations and thus increased agricultural and industrial activities (Tilman et al., 2001). This may have an increasingly negative impact on amphibian populations, a group sensitive to environmental contamination as a result of their permeable skin, which is instrumental in gaseous exchange and exchange of electrolytes, particularly in larval and embryonic stages (Seymour and Bradford, 1995), and which is also thought to allow potentially toxic contaminants to be absorbed (Sparling et al., 2010). Pollution from agriculture may be of particular significance to amphibians, as breeding and larval development occurs in spring and early summer, which is often when pesticides are applied to adjacent agricultural land, and is a period sensitive to chemical exposure (Bridges, 2000).

Monitoring amphibian populations from the same areas over time is of importance in order to track any deleterious changes that occur over multiple generations. Certain contaminants may also be maternally transferred to embryos following bioaccumulation throughout development, which may lead to impaired reproductive success (Bergeron et al., 2010; Todd et al., 2011). As many anuran amphibian species show high breeding site fidelity and limited mobility between sites (Blaustein et al., 1994; Laurila and Aho, 1997), it is possible that the same populations may be monitored over time. Additionally, these factors may also mean that populations are

susceptible to local extinctions, should environmental conditions change significantly (Blaustein et al., 1994).

A technique gaining increasing use in environmental research is infrared (IR) spectroscopy. It is based on the principle that when a sample is probed with an IR beam, the functional groups within the sample and vibrate in a number of different ways: stretching, deformation or bending. There may also be combinations of these vibrational modes (Ellis and Goodacre, 2006; Stuart, 2005). This technique has previously been employed to interrogate samples of fish tissue exposed to several different environmental contaminants both under controlled laboratory conditions (Cakmak et al., 2006; Cakmak et al., 2003; Henczova et al., 2008; Henczova et al., 2006; Li et al., 2015; Palaniappan and Vijayasundaram, 2008, 2009a, b; Palaniappan et al., 2010; Palaniappan and Pramod, 2010; Palaniappan and Renju, 2009; Palaniappan and Vijayasundaram, 2009c; Palaniappan et al., 2011) and in field studies with promising results (Abdel-Gawad et al., 2012; Malins et al., 2006; Malins and Gunselman, 1994; Malins et al., 1997; Malins et al., 2004; Obinaju et al., 2014; Obinaju et al., 2015). However, this technique has yet to be applied in a field study over a more long-term period. As the spectral data sets produced are typically large and complex, multivariate feature-extraction techniques such as principal component analysis (PCA) and linear discriminant analysis (LDA) are typically employed in order to reduce the data sets into less complex and more readily interpretable formats and identify which areas of the spectrum are responsible for differences between data sets (Ellis and Goodacre, 2006; Trevisan et al., 2012). Use of machine-learning techniques for classification of data also allows unknown samples to be classified on the basis of their IR spectra, and have previously been used to identify alterations induced by different pollutant types in bird feathers (Llabjani et al., 2012), as well as the origin of oil spills from polluted beaches (Gómez-Carracedo et al., 2012).

Tadpoles at an early developmental stage are generally regarded as the stage most sensitive to environmental pollution (Cooke, 1972; Greulich and Pflugmacher, 2003; Ortiz-Santaliestra et al., 2006; Rohr et al., 2003), with embryos deemed a less sensitive stage as the jelly coat and perivitelline membrane surrounding the embryo acts as a barrier to contaminants (Edginton et al., 2007; Wagner et al., 2015). This is in agreement with the pilot study in Chapter 2, which suggested that this stage of

development is much less sensitive than the larval stage for distinguishing between ponds with different water quality using ATR-FTIR spectroscopy. However, amphibian embryos also show changes in developmental rates and subsequent deformities at metamorphosis as a result of maternal exposure (Orton and Routledge, 2011) and therefore the inclusion of amphibian embryos in a longer-term monitoring study is of merit.

The primary aim of this study was to determine if ATR-FTIR spectroscopy coupled with multivariate data analysis and classification techniques was able to distinguish embryos and tadpoles of the common frog, *Rana temporaria* collected from ponds with differing water quality over a three year period (2012-2014) on the basis of differences in their spectral signature, despite annual differences in conditions unable to be controlled for in a field study. The sites studied were in Northern England and were selected in order to give a comparison between a rural agricultural site with no pesticide input, a high pesticide-impacted agricultural site, and an urban site impacted by treated wastewater and landfill run-off. These sites are not subject to amphibian surveys and hence no time-series exists of population numbers, so the study carried out here could reveal the health-status of a given frog population. Temporal changes were also determined to ascertain which parts of the spectrum were responsible for any annual differences observed in the spectral signature of tadpoles and embryos at each pond. Within each year, separate analysis was also conducted to determine if similar areas of the spectrum were responsible for the differences detected in the spectral signature of embryos and tadpoles between ponds each year.

Current work in biomedical science aims to use IR spectroscopy to create a database of healthy individuals and those with diseases such as cancer in order to establish vibrational spectroscopy as a screening tool in disease diagnosis (Ellis and Goodacre, 2006; Gajjar et al., 2013; Hands et al., 2013; Mitchell et al., 2014). Whilst still only a relatively short-term monitoring study, the intention in this study was to ascertain a baseline level of ‘healthy’ *R.temporaria* embryos and tadpoles, defined as those collected from a pond minimally impacted by environmental contaminants and with good water quality for comparison with those from ponds with known water contamination. This approach could then potentially demonstrate the utility of

vibrational spectroscopy as an environmental monitoring tool and identify ponds with incipient water quality problems.

## Methods

### Field Sites

Sites were selected in order to give a comparison between agricultural and urban ponds and were based on site characteristics and information from landowners/land managers. The sites were:

1. Whinton Hill (WH), Plumpton, Cumbria is a farm consisting of arable and grazing land for beef and sheep, which is routinely sprayed with herbicides and fungicides.

2. Crake Trees (CT), Crosby Ravensworth is a farm used as beef grazing land and marginal arable land, which has been accepted onto Natural England's Higher Level Environmental Stewardship Scheme and uses minimal quantities of pesticides, with buffer zones to prevent pesticide run-off into water courses.

   The ponds surveyed at WH and CT are constructed wetlands created as part of the MOPS2 (Mitigation Options for Phosphorus and Sediment) project monitored by Lancaster University http://mops2.diffusepollution.info/

3. Pennington Flash Country Park (PF) located in Leigh, Lancashire is a site which receives run-off from treated wastewater and landfill, as this area was previously a landfill site.

Water quality for each pond was assessed through the measurement of key nutrients (nitrate and phosphate) as well as a range of organic chemical pollutants including pesticides. A summary of concentrations are presented in Strong *et al.* (2016). In brief, water quality with respect to these chemical parameters resulted in the ranking of the ponds as: CT highest water quality, followed by PF with WH having the lowest water quality of the three ponds.

## Collection and processing of samples

### Spawn

Samples of *R.temporaria* spawn were collected in 2012, 2013 and 2014 from all three sites (10-20 per site), at varying dates depending on the date of spawning (full details in supplementary information Table S1). Spawn was collected in solvent-rinsed glass jars and transported back to the laboratory before the jelly coat was removed with forceps and the embryo fixed in 70% ethanol overnight at 4°C. The Gosner stage of spawn samples was noted prior to fixation (Gosner, 1960). Spawn was classified as Gosner stage 10-12. Whole fixed embryos were mounted directly onto Low-E reflective glass slides (Kevley Technologies, Chesterland, OH, USA), dried overnight and stored in a desiccator before subsequent interrogation with ATR-FTIR spectroscopy.

### Tadpoles

*Rana temporaria* tadpoles were caught from all three sites in 2012, 2013 and 2014 using dip nets (ten per site, per year), euthanised using a solution of MS-222 (200mg/L) buffered with sodium bicarbonate (both from Sigma Aldrich, Poole, Dorset UK), as per Schedule 1 of the British Home Office Animals (Scientific Procedures) Act 1986. Tadpole samples were then rinsed in distilled water and fixed immediately in the field in 70% ethanol (Fisher Scientific, UK). Ethanol was replaced after 24 hours with fresh. Tadpoles were weighed and measurements taken of snout-vent length (SVL) and head width (HW) using digital callipers to the nearest 0.01mm after fixation. Tadpoles were staged according to Gosner (1960), with all tadpoles between stages 25-28 (full details of stages and tadpole SVL and HW measurements are in Table S2 of supplementary information). Body condition indices (BCI) were calculated for each tadpole as follows: (body mass/$SVL^3$) X 100 (Melvin et al., 2013).

For ATR-FTIR spectroscopy measurements, a longitudinal slice (~ 0.5 mm thick) was taken from the ventral side of the tadpole using a Stadie-Riggs tissue slicer; a simple technique previously employed for preparing tissue samples for analysis with IR spectroscopy (Maher et al., 2014; Obinaju et al., 2014; Taylor et al., 2011). Slices

were mounted skin side down onto Low-E slides, dried overnight and stored in a desiccator before interrogation with ATR-FTIR spectroscopy.

**Temperature data**

Temperature data (maximum, minimum and average air temperatures) was obtained from the Hazelrigg weather station at Lancaster University covering two week time periods beginning approximately one month prior to the start of the breeding season (~ 29$^{th}$ January) and finishing after all individuals had gone through metamorphosis (~26$^{th}$ August) for each year.. Details are provided in graphs S1A-C in the SI.

**ATR-FTIR Spectroscopy**

Between 10 and 25 spectra were taken per sample of spawn and tadpole using a Tensor 27 FTIR spectrometer with Helios ATR attachment (Bruker Optics Ltd, Coventry, UK) containing a diamond crystal (≈250 µm×250 µm sampling area). Spectra were acquired at 8 cm$^{-1}$ resolution with 2x zero-filling, giving a data-spacing of 4 cm$^{-1}$ over the range 400-4000 cm$^{-1}$. The crystal was cleaned with distilled water between the analysis of each sample and a new background reading was taken prior to the analysis of each sample in order to account for changes in atmospheric conditions.

**Data pre-processing**

A representative ATR-FTIR spectrum was obtained by taking the mean of the spectral measurements for each sample. Spectra were then cut at the biochemical cell fingerprint region (1800-900 cm$^{-1}$), baseline corrected using Savitzky-Golay 2$^{nd}$ order differentiation (2$^{nd}$ order polynomial and 9 filter coefficients), and vector normalised.

**Multivariate analysis**

Data were mean-centred before input into principal component analysis-linear discriminant analysis (PCA-LDA) with *k*-folds cross validation, where $k = 5$; this method uses a small portion of the dataset to train the model in order to prevent LDA overfitting (Trevisan et al., 2012). PCA reduces the spectra (227 wavenumbers) into a smaller number of principal components for input into LDA. In this case 9 PCs were picked for spawn analysis and 12 for analysis of tadpoles, using the PCA Pareto function in the IRootLab toolbox, as this represented ~95% of the variance in the data

and where the variance began to plateau, thus preventing noise being incorporated into the LDA algorithm. LDA maximises the differences between classes and minimises the heterogeneity within classes. The data can then be viewed as scores, to determine how the different classes separate from each other. The corresponding loadings vectors when viewed alongside the scores allow the wavenumbers which contribute maximally to the variance to be identified (Trevisan et al., 2012).

For both analysis of spawn and tadpoles, data were classed by pond (CT, PF and WH) using all of the data collected over the three year period. This was the main goal of the study; identifying differences between ponds despite annual variations. Additionally, samples of spawn and tadpoles were analysed within each year group using PCA alone due to the reduced sample size (Ellis and Goodacre, 2006; Martínez and Kak, 2001) to determine if the differences between ponds were consistently expressed each year. Within each pond, annual differences were also determined to identify which, if any areas of the spectrum corresponded to annual factors. Finally, as tadpole body size parameters showed a large variation over the course of the study (see Table S2 in SI), with significant variation found between tadpoles from PF and those from CT and WH in 2013 and 2014, separate analysis was conducted between tadpoles from CT and those from WH, excluding tadpoles from PF to try and exclude the effect of body size on the results.

All spectral pre-processing and data analysis was implemented using the IRootLab toolbox https://code.google.com/p/irootlab/ (Martin et al., 2010; Trevisan et al., 2013) in Matlab (r2012a) (The MathWorks, Inc., USA), unless otherwise stated.

**Statistical analysis**

One-way ANOVA followed by Tukey's multiple comparison tests, or two-sample t-tests where appropriate, were conducted to determine significant differences between classes using the scores from the PCA-LDA and PCA outputs. One-way ANOVA followed by Tukey's multiple comparison tests were also used to determine significant differences between body size parameters. These analyses were conducted in XLSTAT (Addinsoft, Paris).

## Classification of data

For this study, two commonly applied classifiers; principal component analysis-linear discriminant classifier (PCA-LDC) and support vector machines (SVMs) were employed for comparison of their classification ability. Both are supervised classification techniques i.e. where the classes are labelled *a priori*. The output from each classifier was a 'classification accuracy rate', which is defined as the average between sensitivity (true positives) and specificity (true negatives) (Owens et al., 2014).

## PCA-LDC Classifier

Classifiers may be generated using PCA first as a data reduction tool so that the data are not over-fitted in the subsequent LDC model; the classification version of LDA (Trevisan et al., 2012). LDC generates *n*-1 linear discriminant functions (2 in this study) which optimally discriminate *n* classes (3 classes per data set in this study). LDC uses these discriminant functions to assign unknown observations to classes. The Mahalanobis distance (the distance between a data point and a multivariate space's overall mean) is used in the classification process, as the group with the smallest distance is the one LDC classifies the observation into (Krafft et al., 2009).

Data were pre-processed as for PCA-LDA , standardised and the number of principal components for input into the classification model was selected as before using the PCA Pareto function in Matlab before input into the classifier. Five-fold cross-validation was implemented in order to prevent the model from being over-fitted during the training phase.

## SVM Classifier

SVM is a machine-learning approach, which aims to separate data classes by a hyperplane, which maximises the margin between different classes while giving a low generalisation error (Sattlecker et al., 2011). Given the labelled training dataset, the model creates an optimal hyperplane which then classifies new examples. The points determining the hyperplane are called support vectors (Fernández Pierna et al., 2005). SVMs can be linear, however they can also be useful for data that cannot be separated linearly; in this case the predictors are mapped onto a new higher-dimension space,

where they can be separated linearly, which is known as the 'kernel trick' (Subasi and Ismail Gursoy, 2010). Although originally designed to solve binary classification problems, they can also be applied to multiclass problems by creating several binary classifiers and combining them. The most common approaches for multiclass datasets are "one-against-one", which creates a separate SVM for each class or "one-against-all", which creates a separate SVM for each pair of classes (Sattlecker et al., 2011).

In this study, the SVM was set up using a "one-against-one" approach using the LibSVM library (Chang and Lin, 2011) in Matlab (software available at http://www.csie.ntu.edu.tw/~cjlin/libsvm), as there were three classes in each data set. Prior to the application of the SVM classifier, the data were pre-processed as before and then each variable was linearly scaled to the [0, 1] range. Scaling avoids variables in larger numeric ranges from dominating those in smaller ranges and prevents numerical difficulties during the calculations (Hsu et al., 2003). A radial basis kernel function (RBF) was employed and the optimum penalty parameter, C, and the kernel function parameter, $\gamma$, were found using the grid search algorithm. This approach identifies the C, $\gamma$ pairs with the best cross-validation accuracy (Huang et al., 2008). Five-fold cross-validation was conducted in order to prevent over-fitting during the training process (as in the PCA-LDC classifier for comparison).

## Results

### Body Size Measurements

As shown in figures 1A-D tadpoles did not differ in the majority of their body size measurements between ponds when all of the data were analysed together over the duration of the study, with the exception of HW, where tadpoles from PF had a significantly lower measurement of this parameter than those from CT (One-way ANOVA: $F_{2,87} = 3.97$, $P = 0.02$; Tukey's multiple comparison test, $P < 0.05$).

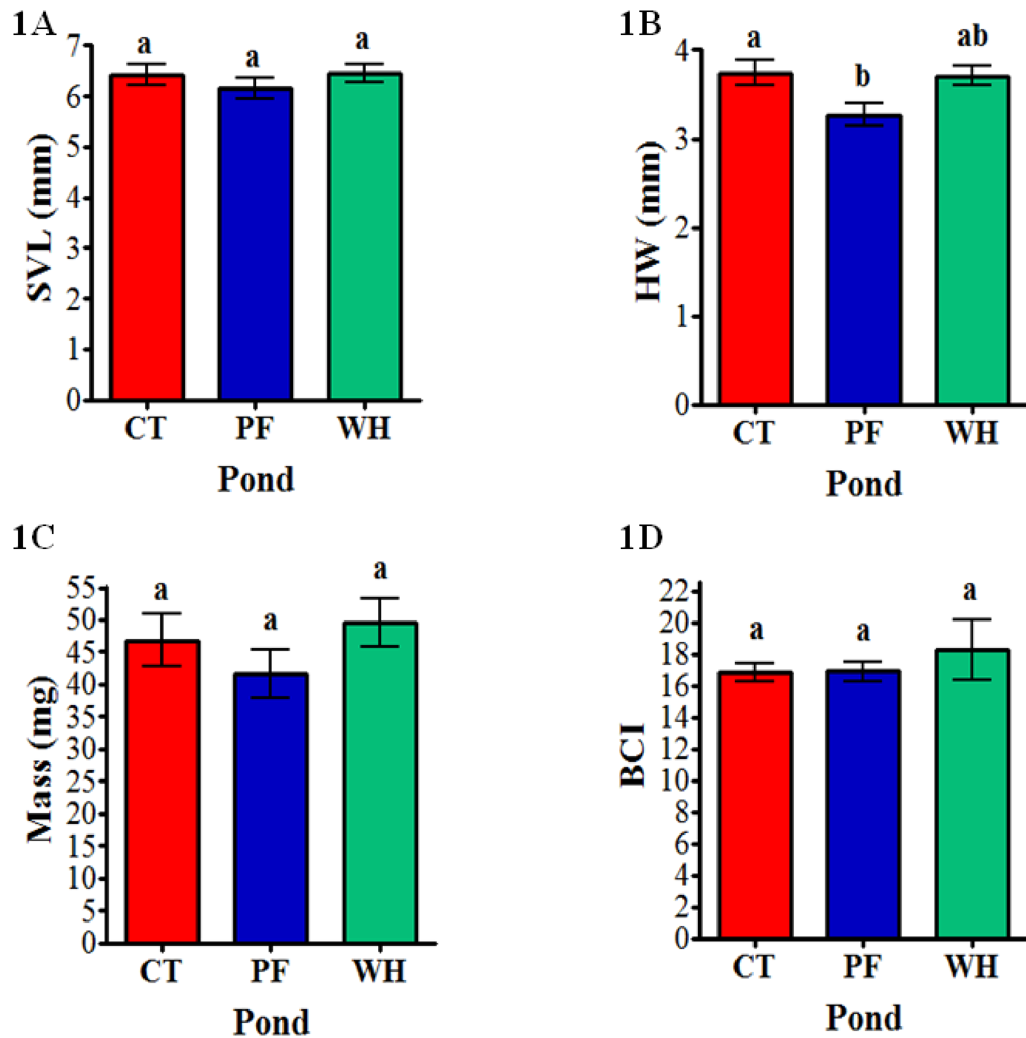


**Figure 1.** Comparison of body size parameters of *Rana temporaria* tadpoles collected over a three year period (2012-2014). Comparisons were made between tadpoles from CT: a rural agricultural pond with minimal pesticide input; PF: an urban pond impacted by wastewater and landfill run-off and WH: an agricultural pond known to be impacted by pesticides. Measurements are **(A)** snout-vent-length (SVL), **(B)** head width (HW) **(C)** body mass, and **(D)** body condition index (BCI). One-way ANOVA followed by Tukey's multiple comparison tests were used to compare each body size parameter between ponds. Different letters denote a significant difference ($P < 0.05$).

However, there were significant differences in body size measurements between ponds, within each year group for tadpoles collected in 2013 and 2014, but not 2012 (see Figs. 2A-D). Tadpoles collected in 2013 from PF were significantly smaller than those from both CT and WH on all measures of body size (One-way ANOVA: SVL: $F_{2,27} = 25.42$, $P < 0.001$; HW: $F_{2,27} = 67.08$, $P < 0.001$; Mass: $F_{2,27} = 46.07$, $P < 0.001$; Tukey's multiple comparison tests, $P < 0.05$), but not BCI (BCI: $F_{2,27} = 1.12$, $P = 0.34$); there were no significant differences between tadpoles from CT and those

from WH in the same year (Tukey's multiple comparison test, $P > 0.05$). Tadpoles collected in 2014 from CT were smaller than those collected from PF on measures of SVL and mass, (One-way ANOVA: SVL: $F_{2,27} = 3.91$, $P = 0.03$; Mass: $F_{2,27} = 6.09$, $P = 0.007$; Tukey's multiple comparison tests, $P < 0.05$) but not BCI or HW (BCI: $F_{2,27} = 3.06$, $P = 0.06$; HW: $F_{2,27} = 3.2$, $P = 0.06$). Tadpoles from WH also had a smaller mass in comparison to those from PF in this year group (Tukey's multiple comparison test, $P < 0.05$). There were no body size differences between tadpoles from CT and those from WH in 2014 (Tukey's multiple comparison test, $P > 0.05$).

There were also significant differences in body size measurements within ponds between different years, as shown in Figs. 2E-H. Comparisons of body size parameters between years within each pond revealed that tadpoles collected from CT were significantly smaller in 2014 than those collected in 2013 and 2012 on measures of SVL, HW and mass (One-way ANOVA: SVL: $F_{2,27} = 18.50$, $P < 0.001$; HW: $F_{2,27} = 19.47$, $P < 0.001$; Mass: $F_{2,27} = 19.45$, $P < 0.001$; Tukey's multiple comparison tests, $P < 0.05$). Mass differed between all years at this pond, with tadpoles collected in 2013 heaviest, followed by those collected in 2012 and then 2014. Body condition indices were lower in 2012 in comparison to those in 2013 and 2013 (BCI: $F_{2,27} = 4.80$, $P = 0.02$; Tukey's multiple comparison tests, $P < 0.05$). Tadpoles collected from PF in 2013 were smaller on all measures of body size in comparison to those collected in both 2012 and 2014 (One-way ANOVA: SVL: $F_{2,27} = 11.31$, $P < 0.001$; HW: $F_{2,27} = 15.78$, $P < 0.001$; Mass: $F_{2,27} = 10.13$, $P = 0.001$; Tukey's multiple comparison tests, $P < 0.05$), although no differences in BCI were found (BCI: $F_{2,27} = 0.65$, $P = 0.53$). No differences were found between tadpoles collected in 2013 and 2014 from PF (Tukey's multiple comparison tests, $P > 0.05$).

Tadpoles collected from WH showed significant variation in their body size measurements but not BCI (One-way ANOVA: SVL: $F_{2,27} = 8.22$, $P = 0.002$; HW: $F_{2,27} = 12.23$, $P < 0.001$; Mass: $F_{2,27} = 16.85$, $P < 0.001$; BCI: $F_{2,27} = 2.00$, $P = 0.16$). Tadpoles collected in 2014 were significantly smaller than those collected in 2013 and 2012 on measures of HW and mass; 2014 tadpoles were also smaller on measures of SVL in comparison to those collected in 2012 (Tukey's multiple comparison tests, $P < 0.05$).

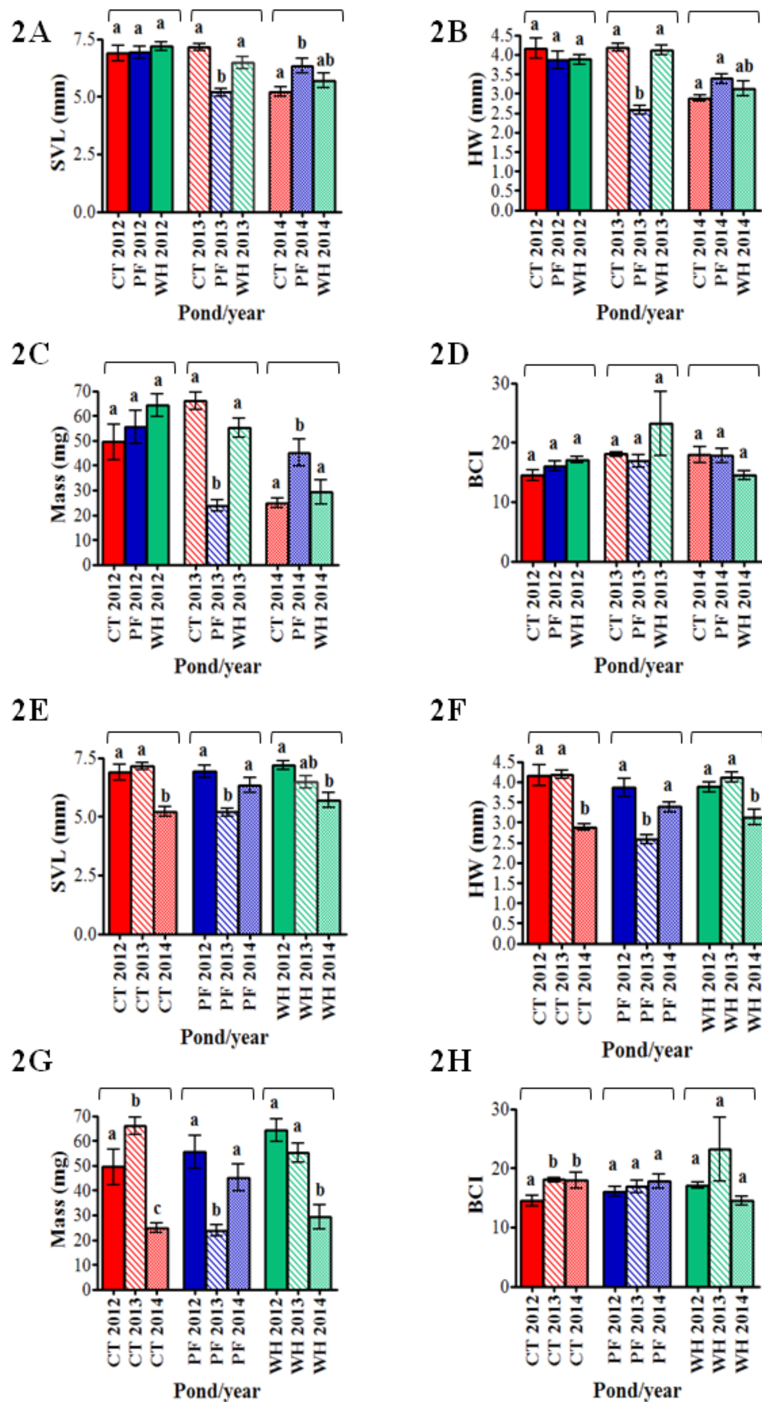


**Figure 2.** Comparison of body size parameters of *Rana temporaria* tadpoles collected from ponds with differing water quality for the years 2012, 2013 and 2014. Body size parameters were also compared at each pond, between years. Ponds are CT: a rural agricultural pond with minimal pesticide input; PF: an urban pond impacted by wastewater and landfill run-off and WH: an agricultural pond known to be impacted by pesticides. Measurements are: **(A)** snout-vent-length (SVL), **(B)** head width (HW), **(C)** body mass, and **(D)** body condition index (BCI) for comparisons between ponds each year. Within each pond, measurements are: **(E)** snout-vent-length (SVL), **(F)** head width (HW) **(G)** body mass, and **(H)** body condition index (BCI) for comparison between years. One-way ANOVA followed by Tukey's multiple comparison tests were used to compare each body size parameter between ponds and between years. Different letters denote a significant difference ($P < 0.05$).

### ATR-FTIR spectroscopy

### Spawn: differences between ponds

The mean spectra of spawn collected from each pond over the three year period are shown in figure 3A. There is very little visual difference between the mean spectra of spawn collected from each site. Analysis with PCA-LDA followed by One-way ANOVA and Tukey's multiple comparison tests demonstrated significant separation along LD1 between all three sites, but no separation along LD2 (Fig. 3B). The loadings from PCA-LDA analysis demonstrated the regions attributable to the separation of spawn between ponds was predominantly in regions associated with protein (amide I and II regions) and C=O stretching of lipids (Fig. 3C, Table 1.) The classification of spawn based on pond of origin was generally quite poor for both PCA-LDC and SVM, the latter achieving a slightly higher classification rate (see Fig. 3D and 3E), although still only achieving correct classification up to a maximum of ~65% of the time for spawn collected from WH (Fig. 3E).

**Table 1.** Distinguishing wavenumbers and proposed assignments obtained from analysis of *Rana temporaria* spawn with ATR-FTIR spectroscopy following analysis with PCA-LDA. The five largest loadings values for significant linear discriminants are shown. Comparisons were made between sites: CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides and PF: an urban pond impacted by wastewater and landfill run-off.

| Comparison | Wavenumber ($cm^{-1}$) | Tentative Assignment [¥] | Comparison [≠] |
|---|---|---|---|
| By site LD1 | 1732 | C=O stretching of lipids | CT [a] |
| | 1709 | C=O stretching (bases) | PF [b] |
| | 1616 | Amide I (carbonyl stretching vibrations in side chains of amino acids) | WH [c] |
| | 1558 | Amide II proteins | |
| | 1477 | $CH_2$ lipids | |

[¥] (Movasaghi et al., 2008; Naumann, 2001; Podrabsky et al., 2001).
[≠] Different letters denote a significant difference at the $P < 0.05$ level following one-way ANOVA and Tukey's multiple comparison tests.

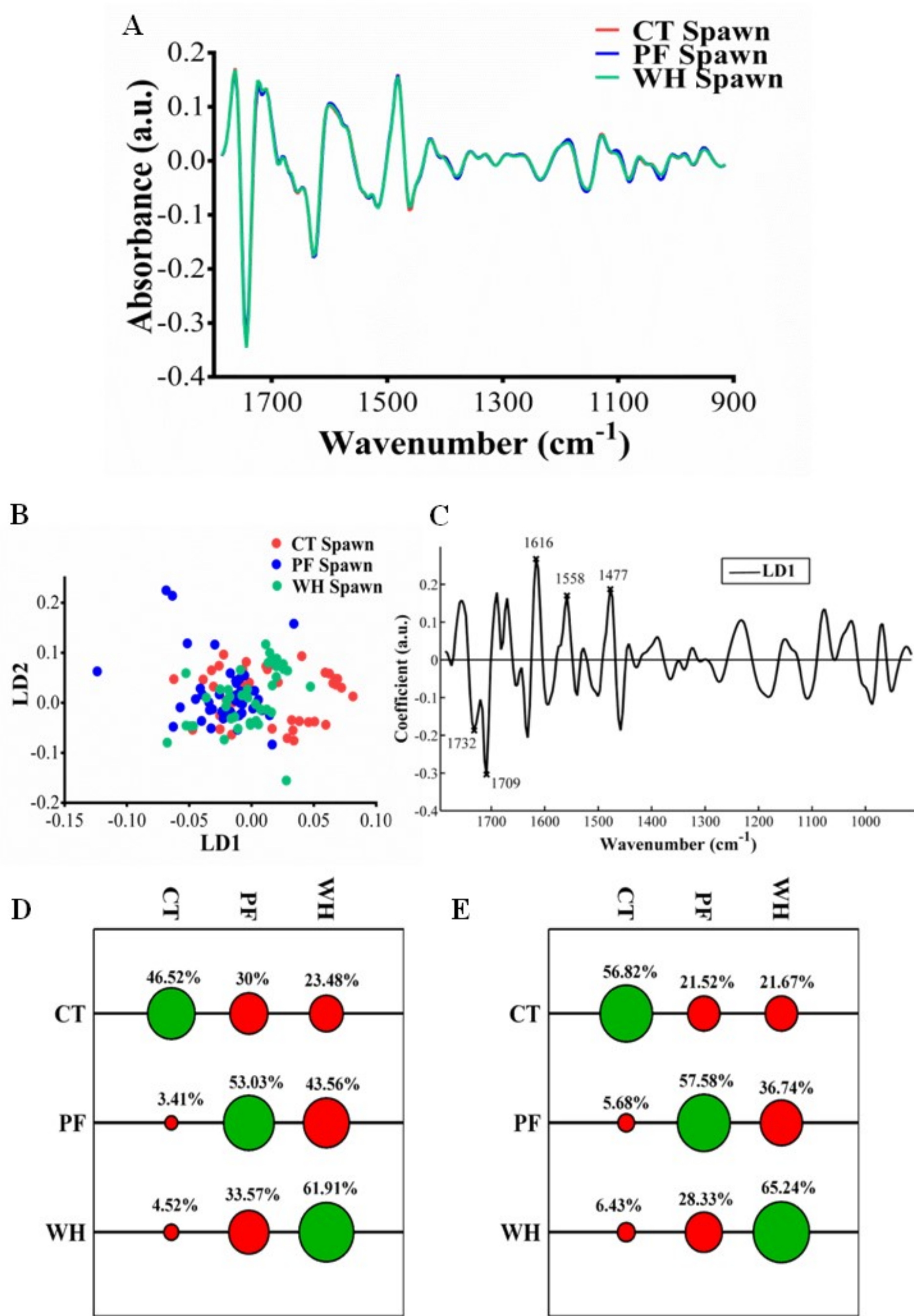


**Figure 3. A:** Mean spectra of *Rana temporaria* spawn collected over a three year period (2012-2014) from CT: a rural agricultural pond with minimal pesticide input; PF: an urban pond impacted by wastewater and landfill run-off and WH: an agricultural pond known to be impacted by pesticides. Spectra were cut at the biochemical fingerprint region (1800-900 cm$^{-1}$), processed with Savitzky-Golay second-order differentiation and vector-normalised. **B:** Two-dimensional scores plot generated following cross-validated PCA-LDA analysis of spectra. **C:** Corresponding loadings generated from PCA-LDA analysis; the five largest loadings values are highlighted. Spectra of spawn samples were classified based on pond of origin using a linear (PCA-LDC): **D** and non-linear (SVM): **E** classification method. Green circles show the % of spawn spectra correctly classified by pond of origin, with red circles showing the % of spawn spectra incorrectly classified by pond of origin**.**

Comparisons between spawn samples within each year group are shown in figs 4A-F and table S3 in the SI. Significant separation was found between spawn samples collected from CT and WH in 2012 along PC4 in areas associated with protein and lipids; and between CT and PF along PC6 (Fig. 4A) in areas associated with protein (amide I), nucleic acids and collagen as determined from the loadings plots (Fig. 4B). No differences were observed between spawn samples from PF and WH. In 2013, spawn samples from PF and WH separated along PC1 in areas associated with lipids and amide I and II proteins; PC2 revealed differences between spawn collected from CT and that from PF and WH in areas associated with lipids and symmetric phosphate stretching (Figs. 4C and 4D). Significant separation was found between spawn samples collected in 2014 from WH in comparison to those from both CT and PF along PC1 in areas associated with lipids, amide I, asymmetric phosphate stretching in RNA and glycogen; significant separation was also found along PC3 in areas associated with fatty acid esters and protein (amide I and II) between spawn collected from CT in comparison to that from both PF and WH (Figs. 4E and F).

**Spawn: differences between years**

Comparisons between spawn samples between years within each pond are shown in Figs. 5A-F and Table S4 in the SI. At CT, significant separation between spawn samples collected in 2012 and those collected in both 2013 and 2014 was apparent along PC1 in areas associated with lipids and proteins (amide I and II); along PC2, the separation was between spawn collected in 2013 and that collected in both 2012 and 2014, again in protein regions (amide I and II) as shown in Figs. 5A and 5B. Spawn collected from PF again showed separation along PC1 in samples collected in 2012 in comparison to those collected in 2013 and 2014 in lipid and protein regions. Some separation was also apparent between samples collected in 2013 and those in 2014 in regions associated with amide I and C=O stretching (Figs. 5C and 5D). In contrast, at WH, spawn collected in 2014 showed the greatest separation from that collected in 2012/3 along PC1 in regions associated with lipids and amide I/II, with some separation evident along PC2 between spawn collected in 2012 and that in 2013/4 in regions associated with amide I/II but also the phosphodiester region (Figs 5E and 5F).

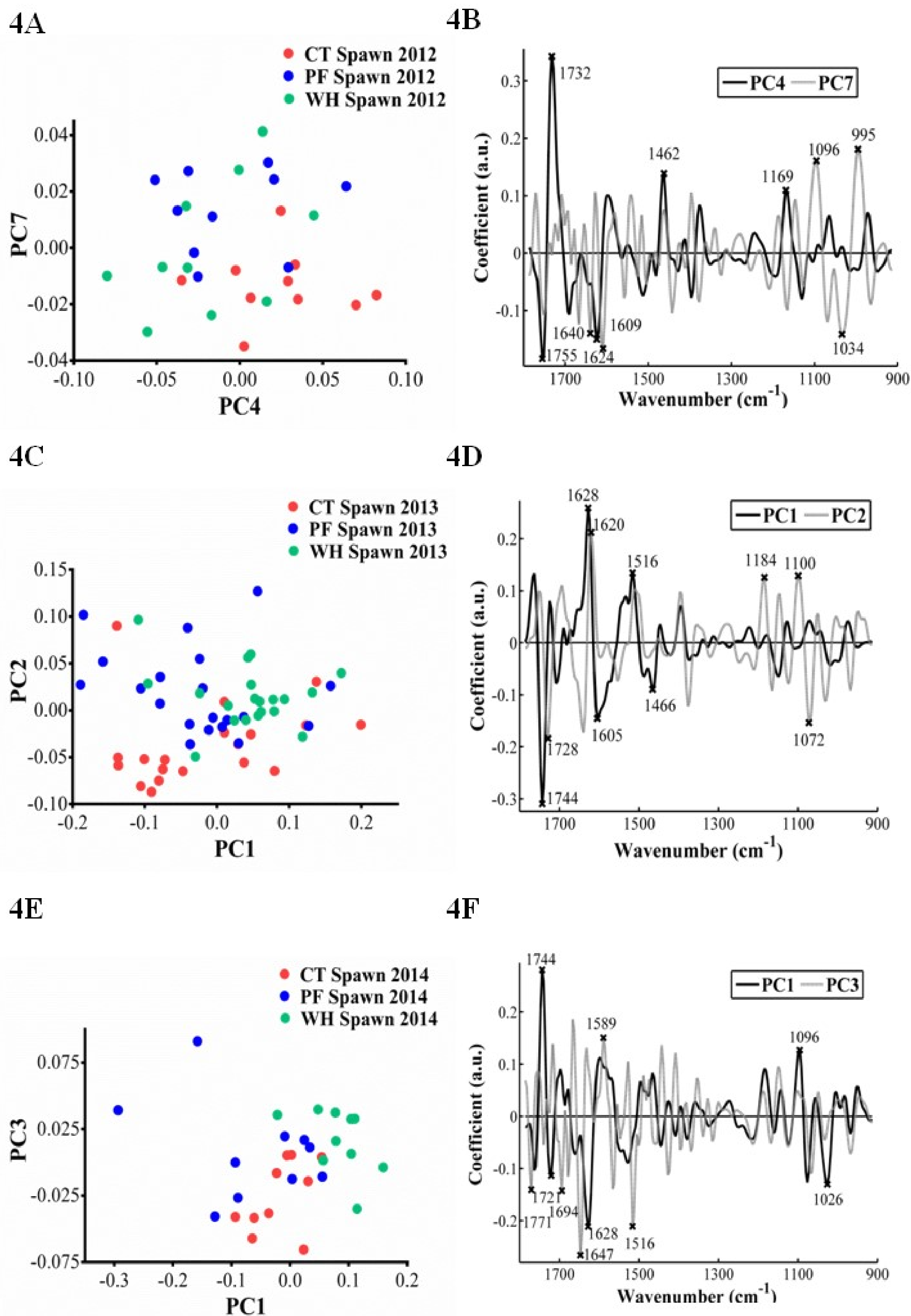


**Figure 4.** *Rana temporaria* spawn collected from three different ponds separated into year groups and analysed with PCA following interrogation with ATR-FTIR spectroscopy. **A.** Scores and **B.** Loadings plots of spawn collected in 2012; **C.** Scores and **D.** Loadings plots of spawn collected in 2013 and **E.** Scores and **F.** Loadings plots of spawn collected in 2014. Ponds are: CT: a rural agricultural pond with minimal pesticide input; PF: an urban pond impacted by wastewater and landfill run-off and WH: an agricultural pond known to be impacted by pesticides.

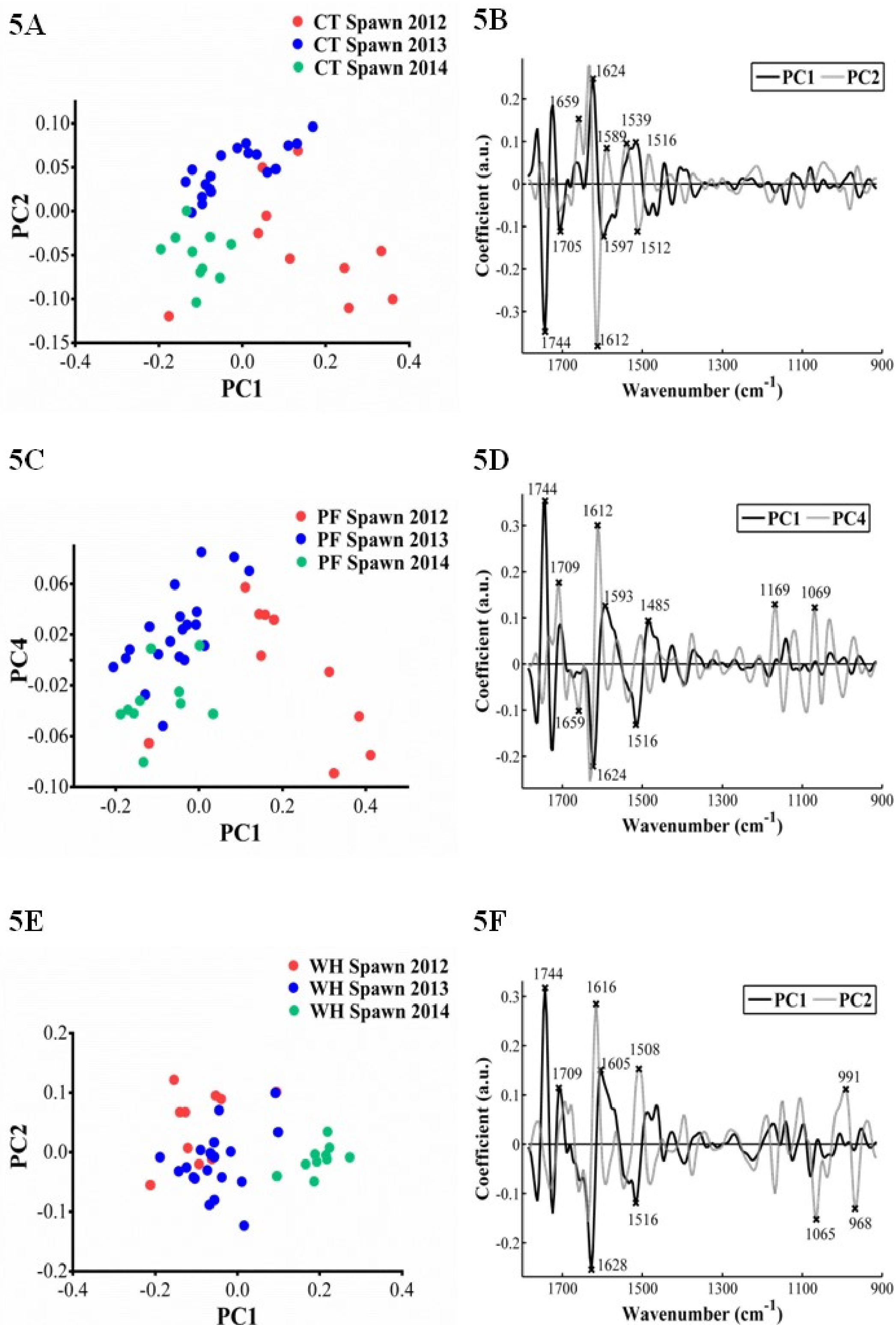


**Figure 5.** *Rana temporaria* spawn collected from three different years (2012, 2013, and 2014), separated into pond of origin and analysed with PCA following interrogation with ATR-FTIR spectroscopy. **A.** Scores and **B.** Loadings plots of spawn collected from CT; **C.** Scores and **D.** Loadings plots of spawn collected from PF and **E.** Scores and **F.** Loadings plots of spawn collected from WH. Ponds are: CT: a rural agricultural pond with minimal pesticide input; PF: an urban pond impacted by wastewater and landfill run-off and WH: an agricultural pond known to be impacted by pesticides.

**Tadpoles: differences between ponds**

The mean spectra of tadpoles collected from each pond over a three year period are shown in Fig. 6A. Visual inspection of the spectra suggests that some separation is apparent in the 1150-900 $cm^{-1}$ region. Further analysis with PCA-LDA confirmed significant separation along LD1 between all three ponds (Fig. 6B) in regions associated predominantly with symmetric phosphate stretching of DNA/glycogen (~1100-1000 $cm^{-1}$) as shown in the loadings plot in Fig. 6C. Additionally, regions associated with Amide I (proteins) also contributed to the separation along this dimension. LD2 accounts for separation between tadpoles collected from CT/PF and WH. This is again in similar regions as before: primarily symmetric phosphate stretching of DNA/glycogen (1092, 1057 $cm^{-1}$) with some contribution from Amide I. The top five loadings values and corresponding wavenumber assignments are shown in Fig. 6C and Table 2. In contrast to the poor classification results achieved for spawn cased on pond of origin, both PCA-LDC and SVM achieved high classification rates for tadpoles, both demonstrating correct classification for tadpoles collected from each pond over 85% of the time (see Figs. 6D and 6E). SVM again achieved the highest classification rates, with tadpoles collected from CT correctly identified at the highest frequency, attaining a classification rate of ~94% (Fig. 6E).

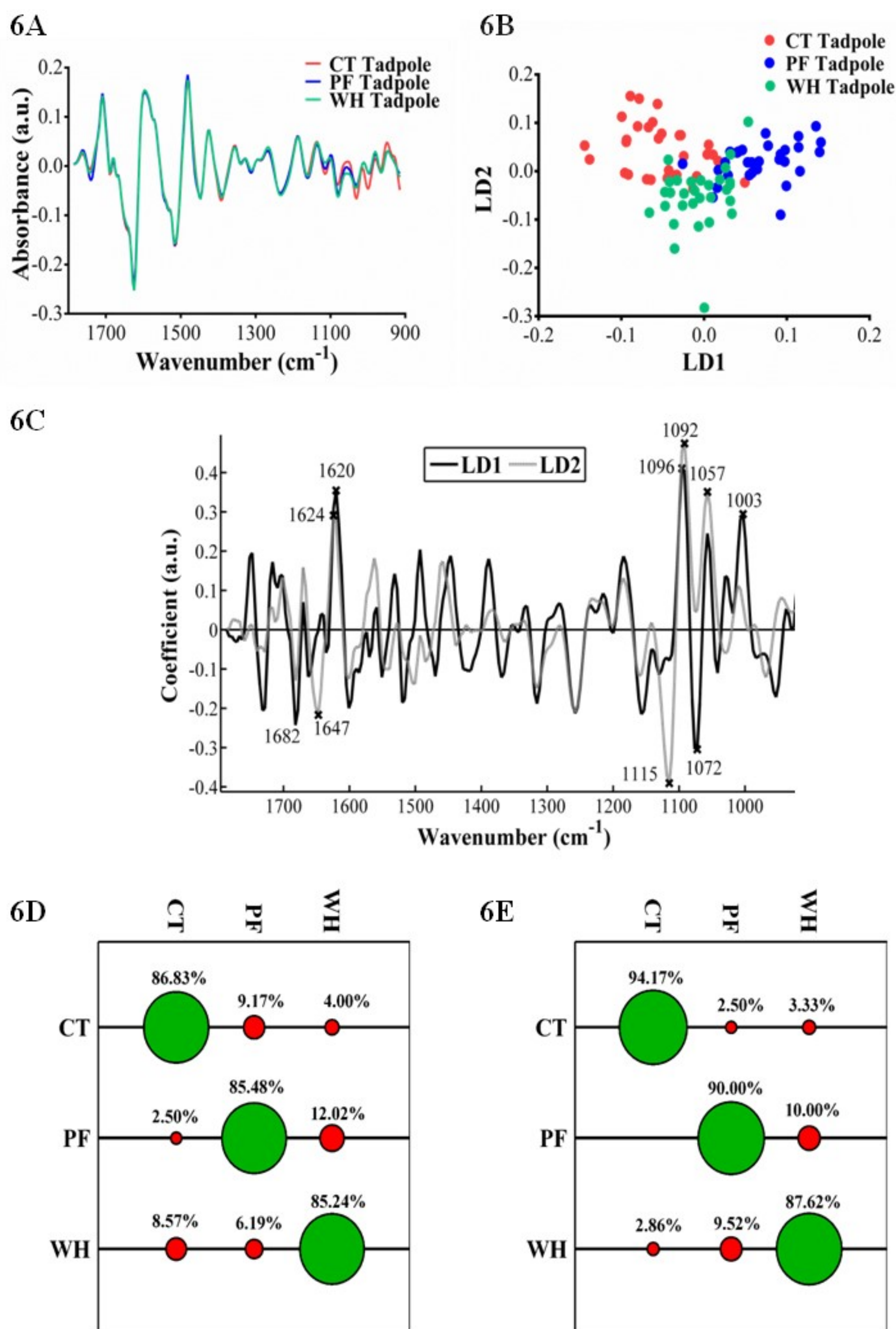


**Figure 6. A:** Mean spectra of *Rana temporaria* tadpoles collected from ponds with differing water quality over a three year period (2012-2014) from CT: a rural agricultural pond with minimal pesticide input; PF: an urban pond impacted by wastewater and landfill run-off and WH: an agricultural pond known to be impacted by pesticides. Spectra were cut at the biochemical fingerprint region (1800-900 $cm^{-1}$), processed with Savitzky-Golay second-order differentiation and vector-normalised. **B:** Two-dimensional scores plot generated following cross-validated PCA-LDA analysis of spectra. **C:** Corresponding loadings generated from PCA-LDA analysis; the five largest loadings values are highlighted. Spectra of tadpole samples were classified based on pond of origin using a linear (PCA-LDC): **D** and non-linear (SVM): **E** classification method. Green circles show the % of tadpole spectra correctly classified by pond of origin, with red circles showing the % of tadpole spectra incorrectly classified by pond of origin**.**

**Table 2.** Distinguishing wavenumbers and proposed assignments obtained from analysis of *Rana temporaria* tadpoles with ATR-FTIR spectroscopy following analysis with PCA-LDA. The five largest loadings values for the two linear discriminants are shown. Comparisons were made between sites: CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides and PF: an urban pond impacted by wastewater and landfill run-off.

| Comparison | Wavenumber ($cm^{-1}$) | Tentative Assignment ¥ | Comparison ≠ |
|---|---|---|---|
| LD1 | 1682 | Amide I deformation | CT [a] |
| | 1620 | Peak of nucleic acids due to the base carbonyl stretching and ring breathing mode | PF [b]<br>WH [c] |
| | 1096 | Stretching $PO_2^-$ symmetric | |
| | 1072 | Nucleic acid band (symmetric phosphate stretch) | |
| | 1003 | Sugar phosphate chain vibrations in nucleic acids | |
| LD2 | 1647 | Amide I | CT [a] |
| | 1624 | Amide I, $\beta$-sheet | PF [a] |
| | 1115 | Symmetric stretching P-O-C | WH [b] |
| | 1092 | Symmetric phosphate stretching | |
| | 1057 | Glycogen | |

¥ (Cakmak et al., 2006; Chu et al., 2001; Movasaghi et al., 2008; Palaniappan and Vijayasundaram, 2008).
≠ Different letters denote a significant difference at the $P < 0.05$ level following one-way ANOVA and Tukey's multiple comparison tests.

Comparisons between tadpoles within each year group are shown in Figs7A-F and table S5 in the SI. In 2012, tadpoles collected from CT showed the greatest separation between those collected from both PF and WH along PC1 in regions associated with carbohydrates (glycogen) and symmetric phosphate stretching; there was also separation along PC3 between tadpoles from WH and those from both CT and PF in regions associated with protein, lipid and glycogen (Figs. 7A and B). In 2013, tadpoles from PF separated from those from both CT and WH along PC1 in regions associated with glycogen and DNA; tadpoles from all three sites separated significantly along PC3 with the greatest separation between tadpoles from CT and those from WH, again in regions associated with glycogen and DNA, with some protein contribution (Figs. 7C and 7D). In 2014, tadpoles from PF separated from those from both CT and WH along PC1, as in 2013, although this time in regions largely associated with lipid. Along PC3, the differences were between tadpoles from

WH and those from both CT and PF, in regions largely associated with carbohydrates like glycogen, with some lipid contribution (Figs. 7E and 7F).

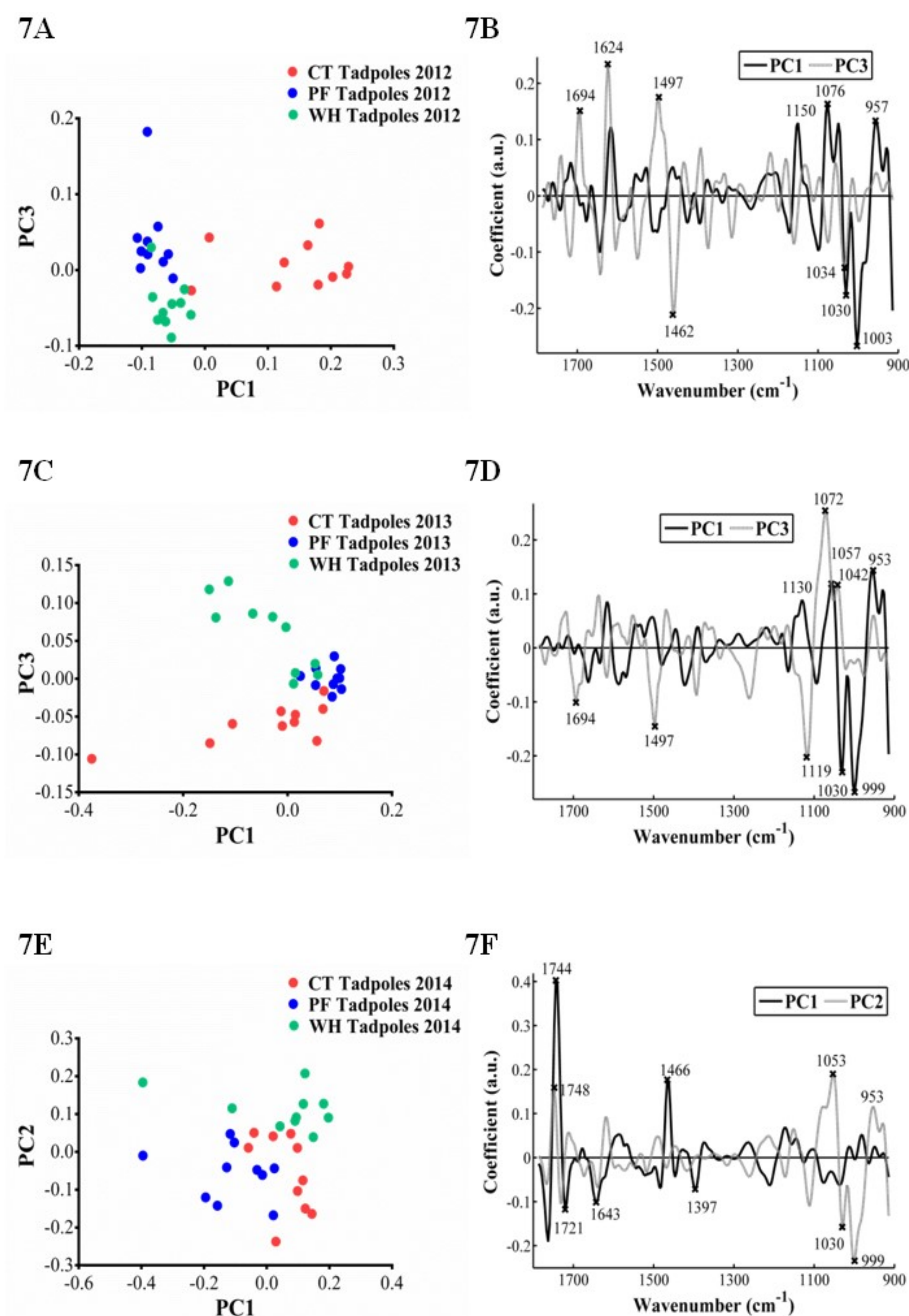


**Figure 7.** *Rana temporaria* tadpoles collected from three different ponds separated into year groups and analysed with PCA following interrogation with ATR-FTIR spectroscopy. **A.** Scores and **B.** Loadings plots of tadpoles collected in 2012; **C.** Scores and **D.** Loadings plots of tadpoles collected in 2013 and **E.** Scores and **F.** Loadings plots of tadpoles collected in 2014. Ponds are: CT: a rural agricultural pond with minimal pesticide input; PF: an urban pond impacted by wastewater and landfill run-off and WH: an agricultural pond known to be impacted by pesticides.

As significant differences in body size measurements were found between tadpoles from PF and those from CT and WH in 2013 and 2014, separate analysis was conducted with tadpoles from PF excluded from the analysis in order to remove the potentially confounding effects of body size. Significant differences were apparent between tadpoles from CT and those from WH in 2013 along PCs 2 and 3 in regions associated with C=O stretching of lipids, amide I proteins and symmetric stretching of P-O-C and nucleic acids (Table S6, Figs. 2A and B in SI). In 2014, there were differences between tadpoles from CT and those WH along PC2 only, in regions associated predominantly with carbohydrates/glycogen and sugar phosphate vibrations in nucleic acids, with some lipid contribution (Table S6, Figs 2C and 2D in SI).

**Tadpoles: differences between years**

Comparisons between tadpole samples between years within each site are shown in Figs. 8A-F and Table S7 in the SI. At CT, significant separation was seen along PC2 between tadpoles collected in 2014 and those collected in 2012/3 in regions largely associated with lipid and fatty acid esters, with some protein contribution. Along PC3, differences were confined to tadpoles collected in 2012 and those collected in 2013 in regions associated with amide I/II and lipid (Figs. 8A and 8B). At PF, like at CT, significant separation was seen between tadpoles collected in 2014 and those collected in 2012/3 in very similar regions: lipids and fatty acid esters. Along PC2, the differences at PF were confined to tadpoles collected in 2012 and those in 2013/14, again in lipid regions but mainly in amide I and II protein regions (Figs. 8C and 8D). Again at WH, significant separation was seen between tadpoles collected in 2014 and those collected in 2012/3, with lipid and protein regions indicated as before. There was also significant separation along PC2 between tadpoles collected in 2012 and those collected in 2013/14 in regions associated with amide I, but also in regions associated with symmetric phosphate stretching and glycogen (Figs. 8E and 8F).

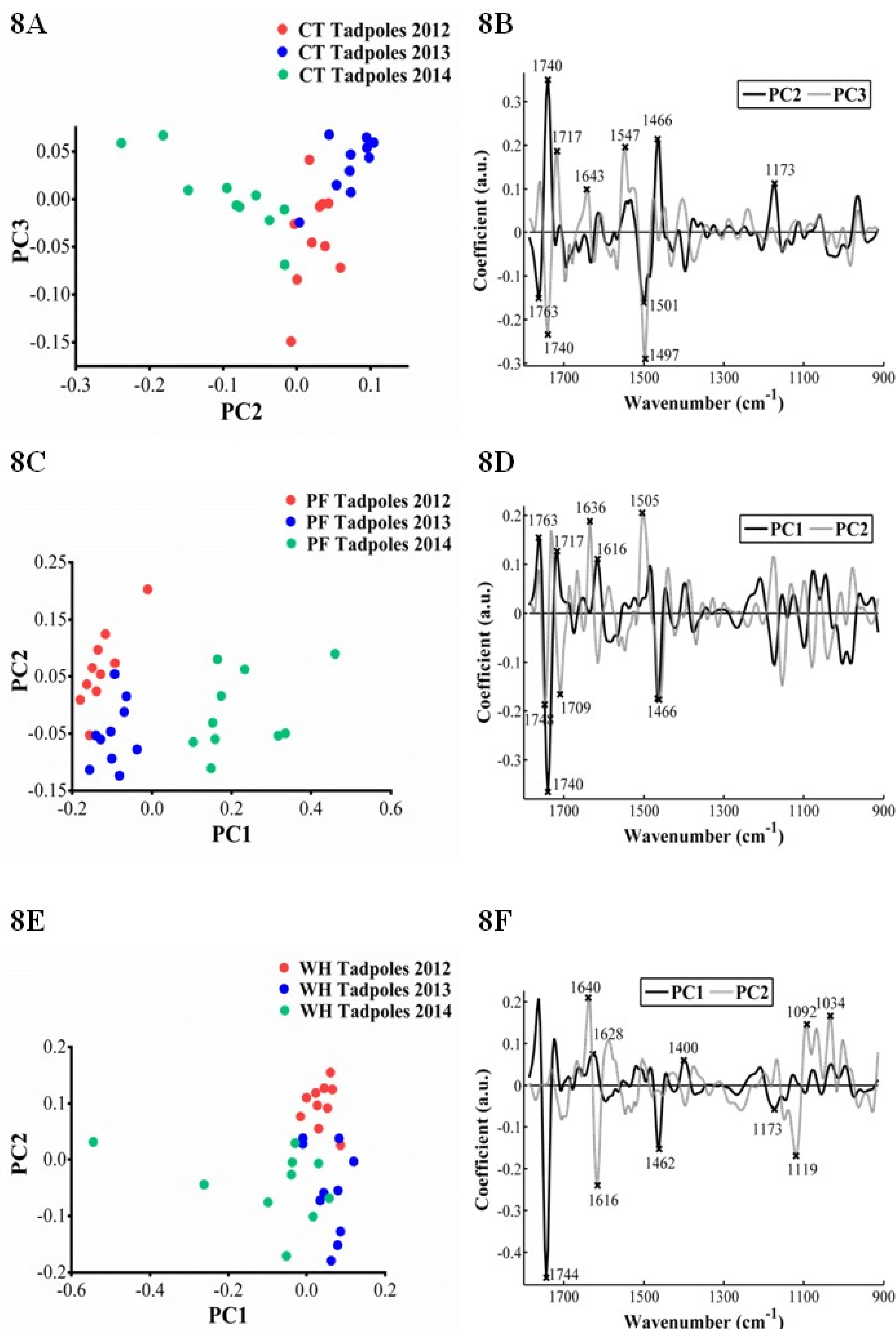


**Figure 8.** *Rana temporaria* tadpoles collected from three different years (2012, 2013 and 2014), separated into pond of origin and analysed with PCA following interrogation with ATR-FTIR spectroscopy. **A.** Scores and **B.** Loadings plots of tadpoles collected from CT; **C.** Scores and **D.** Loadings plots of tadpoles collected from PF and **E.** Scores and **F.** Loadings plots of tadpoles collected from WH. Ponds are: CT: a rural agricultural pond with minimal pesticide input; PF: an urban pond impacted by wastewater and landfill run-off and WH: an agricultural pond known to be impacted by pesticides.

## Discussion

Amphibians are sensitive to environmental pollution due to their life history and a tendency to show high site fidelity, thus allowing repeated exposure to environmental contaminants over time (Blaustein et al., 1994). Although species such as *R.temporaria* are relatively abundant (Beebee, 2014), they may serve as a useful sentinel species in environmental monitoring studies as a proxy for rarer species.

This study has demonstrated that ATR-FTIR spectroscopy in conjunction with multivariate analysis and classification techniques is able to effectively distinguish between tadpoles of the common frog, *R.temporaria* collected from ponds with differing water quality over a three year period. This was in spite of annual differences, which were also apparent when the data were analysed each year. In contrast, the differences between years for spawn were much more profound than those between ponds, suggesting that annual differences masked many of the differences detected in the IR spectra of spawn collected from each pond.

In biomedical studies involving disease screening, there naturally exists variation between individuals and possible confounding variables between samples (Bhargava et al., 2006; Lewis et al., 2010; Wood et al., 1998). Therefore screening programmes using spectroscopy must be specific enough to determine signatures attributable to a particular disease state in spite of 'noise' in the data. Chemometric processing of the data , often using multivariate methods is thus an important step in distinguishing between 'healthy' and 'diseased' tissues in these highly complex data sets. In addition, patients are matched for potentially confounding factors such as age or ethnicity where possible (Theophilou et al., 2015).

In this study, there were minimal differences in body size between tadpoles (with the exception of head width between PF and CT tadpoles), when all of the data were analysed together, thus excluding body size as a reason for the separation and high classification rates seen between ponds. The differences between ponds were largely in areas associated with glycogen/carbohydrates and symmetric phosphate stretching, with some protein contribution. Glycogen, and to a lesser extent, protein, is utilised as an energy source in amphibians and may be depleted in response to stressful situations, such as exposure to environmental contaminants, as the organism attempts to maintain homeostasis by compensatory metabolic mechanisms, thus utilising

energy reserves (Dornelles and Oliveira, 2014; Melvin et al., 2013). The differences in symmetric phosphate may be reflective of the type of contaminants tadpoles were exposed to as the ponds studied were subject to run-off from agricultural and urban environments (Strong et al., 2016), which may be associated with genotoxicity (Ralph and Petras, 1997, 1998). However caution must be exercised in interpreting the results as by the nature of the study, tadpoles were exposed to a mixture of xenobiotics as well as varying nutrient levels and no one single factor can be elucidated.

The differences between years for both tadpoles and spawn are unsurprising given the factors that may vary each year, such as temperature, and therefore date of spawning, food availability, competition and predation. Interestingly, the differences seen between tadpoles from different years were in different areas of the spectrum in comparison to the differences seen between tadpoles from different ponds. Between ponds, tadpoles varied in regions associated with carbohydrates and asymmetric and symmetric phosphate stretching with some protein contribution, whereas between years the differences were mainly confined to areas of the spectrum associated with lipids and proteins (mainly amide I and II). These differences may be tied to body size differences, as there was variability in tadpole body size parameters between years within each site. Tadpoles show developmental plasticity, where they are able to adjust their developmental rate according to environmental conditions, producing smaller individuals under conditions of low food availability and high population density (Alvarez and Nicieza, 2002; Audo et al., 1995; Beebee and Richard, 2000; Kupferberg, 1997). Although there were body size differences between tadpoles from PF and those from CT/WH in 2013/4, once tadpoles from PF were excluded from the analysis, thus excluding body size as a confounding factor, there was still significant separation between tadpoles from CT and those from WH in areas associated with amide I proteins, symmetric phosphate stretching and carbohydrates/glycogen.

In contrast, the differences between spawn samples between ponds were in similar areas of the spectrum to those between years, being predominantly in areas associated with protein and lipids. This may account for the poorer separation and classification seen in spawn samples in comparison to tadpoles. There are several factors influencing the development of spawn including temperature, oxygen levels and maternal investment (Beattie et al., 1991; Carroll et al., 2009; Gillooly et al., 2002;

Loman, 2002; Neveu, 2009; Pakkasmaa et al., 2003; Seymour et al., 2000). Unfortunately these factors cannot be controlled for in a field study of this kind. Temperature is capable of influencing egg development markedly, with date of spawning significantly correlated with ambient water temperature (Carroll et al., 2009; Neveu, 2009). Indeed, there were differences seen in this study in terms of date of spawning, with frogs spawning in early/mid March in 2012 and 2014 (between 7$^{th}$ and 16$^{th}$ March), whereas this was delayed in 2013 to late March/early April in 2013 (see Table 1 in SI), which was likely related to temperature, as average, minimum and maximum temperatures were lower around this time in 2013 (see Figs. S1A-C in the SI). Additionally in 2012, maximum temperatures were higher around the times of spawning in comparison to 2013 and 2014, which again may have influenced spawn development, with a reduction in clutch fecundity associated with extreme temperatures in the preceding year (Neveu, 2009).

## Conclusions

This study demonstrated the use of ATR-FTIR spectroscopy as a monitoring tool in assessing the health of *R.temporaria* spawn and tadpoles from ponds with differing water quality over a three year period. While spawn appeared to be more influenced by annual factors such as temperature, thus showing relatively poorer separation between ponds with differing water quality, tadpoles were correctly classified by pond of origin up to 94% of the time, despite annual differences. The annual variation between tadpoles within ponds was generally confined to different areas of the spectrum to those between ponds, and may be related to body size differences, which also showed annual differences.

As body size may influence biochemical parameters, it is recommended that any future study should ideally case-match individuals on the basis of their body size, developmental stage and where possible abiotic factors, such as temperature, pH and dissolved oxygen in order to control for such factors. In biomedical studies currently utilising IR spectroscopy in disease screening, patients are matched where possible to exclude confounding variables. With larger data sets and complementary laboratory

and mesocosm studies, IR spectroscopy could be a highly useful, cost-effective and rapid tool in monitoring amphibian health.

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**Supplementary Information:**

**Number of Figures = 2**

**Number of Tables = 7**

**Table S1**. Dates spawn samples of *Rana temporaria* were collected from the three ponds studied over a three year period. Ponds are CT: a rural agricultural pond with minimal pesticide impact, PF: an urban pond exposed to wastewater/landfill run-off and WH: an agricultural pond impacted by pesticides.

| Pond | Year | Date Collected |
|---|---|---|
| CT | 2012 | 07/03/2012 |
| CT | 2013 | 11/04/2013 |
| CT | 2014 | 07/03/2014 |
| PF | 2012 | 16/03/2013 |
| PF | 2013 | 27/03/2013 |
| PF | 2014 | 12/03/2014 |
| WH | 2012 | 07/03/2012 |
| WH | 2013 | 11/04/2013 |
| WH | 2014 | 07/03/2014 |

**Table S2.** Full details of tadpoles collected from each pond over the period 2012-2014. Ponds are CT: a rural agricultural pond with minimal pesticide impact, PF: an urban pond exposed to wastewater/landfill run-off and WH: an agricultural pond impacted by pesticides. SVL = snout-vent-length, HW = head width, GS = Gosner Stage.

| Pond | Year | Collection Date | Number | SVL (mm) | HW (mm) | Mass (mg) | BCI | GS |
|---|---|---|---|---|---|---|---|---|
| CT | 2012 | 18/04/2012 | 1 | 7.22 | 4.64 | 59.00 | 15.68 | 25 |
| CT | 2012 | 18/04/2012 | 2 | 6.87 | 3.82 | 60.90 | 18.78 | 25 |
| CT | 2012 | 18/04/2012 | 3 | 8.03 | 4.83 | 84.70 | 16.36 | 25 |
| CT | 2012 | 18/04/2012 | 4 | 7.00 | 4.34 | 52.00 | 15.16 | 27 |
| CT | 2012 | 18/04/2012 | 5 | 5.86 | 3.42 | 25.00 | 12.42 | 25 |
| CT | 2012 | 18/04/2012 | 6 | 5.74 | 3.52 | 33.00 | 17.45 | 25 |
| CT | 2012 | 18/04/2012 | 7 | 6.55 | 3.73 | 35.00 | 12.46 | 25 |
| CT | 2012 | 18/04/2012 | 8 | 9.32 | 6.06 | 84.00 | 10.38 | 25 |
| CT | 2012 | 18/04/2012 | 9 | 6.15 | 3.50 | 34.70 | 14.92 | 28 |
| CT | 2012 | 18/04/2012 | 10 | 6.17 | 3.78 | 26.00 | 11.07 | 25 |
| CT | 2013 | 31/05/2013 | 1 | 6.88 | 3.70 | 60.90 | 18.70 | 25 |
| CT | 2013 | 31/05/2013 | 2 | 6.96 | 4.47 | 66.40 | 19.69 | 26 |
| CT | 2013 | 31/05/2013 | 3 | 7.36 | 4.29 | 74.20 | 18.61 | 26 |
| CT | 2013 | 31/05/2013 | 4 | 6.63 | 3.98 | 52.30 | 17.95 | 26 |
| CT | 2013 | 31/05/2013 | 5 | 7.66 | 4.36 | 68.40 | 15.22 | 25 |
| CT | 2013 | 31/05/2013 | 6 | 7.61 | 4.64 | 80.60 | 18.29 | 26 |
| CT | 2013 | 31/05/2013 | 7 | 7.20 | 4.06 | 61.50 | 16.48 | 26 |
| CT | 2013 | 31/05/2013 | 8 | 7.77 | 4.49 | 84.70 | 18.06 | 27 |
| CT | 2013 | 31/05/2013 | 9 | 6.76 | 3.73 | 59.60 | 19.29 | 27 |
| CT | 2013 | 31/05/2013 | 10 | 6.64 | 4.08 | 52.70 | 18.00 | 25 |
| CT | 2014 | 11/04/2014 | 1 | 4.69 | 2.74 | 25.00 | 24.23 | 25 |
| CT | 2014 | 11/04/2014 | 2 | 5.13 | 2.78 | 28.00 | 20.74 | 25 |
| CT | 2014 | 11/04/2014 | 3 | 4.02 | 2.29 | 14.00 | 21.55 | 25 |
| CT | 2014 | 11/04/2014 | 4 | 5.47 | 2.89 | 22.00 | 13.44 | 25 |
| CT | 2014 | 11/04/2014 | 5 | 4.77 | 3.05 | 23.00 | 21.19 | 25 |
| CT | 2014 | 11/04/2014 | 6 | 5.09 | 2.96 | 24.00 | 18.20 | 25 |
| CT | 2014 | 11/04/2014 | 7 | 6.28 | 3.20 | 40.00 | 16.15 | 25 |
| CT | 2014 | 11/04/2014 | 8 | 5.87 | 3.15 | 25.00 | 12.36 | 25 |
| CT | 2014 | 11/04/2014 | 9 | 5.57 | 2.80 | 23.00 | 13.31 | 25 |
| CT | 2014 | 11/04/2014 | 10 | 5.15 | 2.96 | 25.00 | 18.30 | 25 |
| PF | 2012 | 17/04/2012 | 1 | 8.60 | 5.18 | 84.00 | 13.21 | 26 |
| PF | 2012 | 17/04/2012 | 2 | 6.44 | 3.04 | 40.00 | 14.98 | 25 |
| PF | 2012 | 17/04/2012 | 3 | 7.16 | 4.16 | 61.50 | 16.75 | 25 |
| PF | 2012 | 17/04/2012 | 4 | 6.17 | 2.97 | 26.00 | 11.07 | 25 |
| PF | 2012 | 17/04/2012 | 5 | 6.16 | 3.47 | 34.70 | 14.85 | 25 |
| PF | 2012 | 17/04/2012 | 6 | 7.42 | 4.41 | 74.20 | 18.16 | 25 |
| PF | 2012 | 17/04/2012 | 7 | 7.20 | 3.57 | 61.50 | 16.48 | 26 |
| PF | 2012 | 17/04/2012 | 8 | 7.54 | 4.47 | 80.60 | 18.80 | 26 |
| PF | 2012 | 17/04/2012 | 9 | 6.84 | 3.95 | 60.90 | 19.03 | 26 |
| PF | 2012 | 17/04/2012 | 10 | 6.84 | 3.95 | 60.90 | 19.03 | 25 |
| PF | 2013 | 30/05/2015 | 1 | 4.72 | 2.11 | 18.80 | 17.88 | 25 |
| PF | 2013 | 30/05/2015 | 2 | 4.81 | 2.81 | 25.20 | 22.64 | 25 |
| PF | 2013 | 30/05/2015 | 3 | 5.50 | 2.99 | 35.20 | 21.16 | 25 |
| PF | 2013 | 30/05/2015 | 4 | 6.15 | 2.95 | 34.70 | 14.92 | 26 |
| PF | 2013 | 30/05/2015 | 5 | 4.99 | 2.31 | 21.60 | 17.38 | 26 |
| PF | 2013 | 30/05/2015 | 6 | 5.26 | 2.28 | 16.40 | 11.27 | 25 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| PF | 2013 | 30/05/2015 | 7 | 5.36 | 2.64 | 20.40 | 13.25 | 25 |
| PF | 2013 | 30/05/2015 | 8 | 5.95 | 2.93 | 31.10 | 14.76 | 25 |
| PF | 2013 | 30/05/2015 | 9 | 4.58 | 2.26 | 16.20 | 16.86 | 25 |
| PF | 2013 | 30/05/2015 | 10 | 4.60 | 2.54 | 18.20 | 18.70 | 25 |
| PF | 2014 | 16/04/2014 | 1 | 6.51 | 3.94 | 60.00 | 21.75 | 26 |
| PF | 2014 | 16/04/2014 | 2 | 6.31 | 3.49 | 41.00 | 16.32 | 25 |
| PF | 2014 | 16/04/2014 | 3 | 5.71 | 3.15 | 33.00 | 17.73 | 25 |
| PF | 2014 | 16/04/2014 | 4 | 8.86 | 4.17 | 84.00 | 12.08 | 25 |
| PF | 2014 | 16/04/2014 | 5 | 6.40 | 3.50 | 44.00 | 16.78 | 26 |
| PF | 2014 | 16/04/2014 | 6 | 5.23 | 3.04 | 36.00 | 25.17 | 25 |
| PF | 2014 | 16/04/2014 | 7 | 6.67 | 3.44 | 55.00 | 18.53 | 25 |
| PF | 2014 | 16/04/2014 | 8 | 5.69 | 2.98 | 38.00 | 20.63 | 25 |
| PF | 2014 | 16/04/2014 | 9 | 5.40 | 2.91 | 26.00 | 16.51 | 25 |
| PF | 2014 | 16/04/2014 | 10 | 6.54 | 3.19 | 35.00 | 12.51 | 25 |
| WH | 2012 | 18/04/2012 | 1 | 8.09 | 4.26 | 84.70 | 16.00 | 28 |
| WH | 2012 | 18/04/2012 | 2 | 7.59 | 4.16 | 80.60 | 18.43 | 28 |
| WH | 2012 | 18/04/2012 | 3 | 6.33 | 3.65 | 41.00 | 16.16 | 26 |
| WH | 2012 | 18/04/2012 | 4 | 7.31 | 4.03 | 74.20 | 19.00 | 27 |
| WH | 2012 | 18/04/2012 | 5 | 6.65 | 3.35 | 52.70 | 17.92 | 27 |
| WH | 2012 | 18/04/2012 | 6 | 7.71 | 4.46 | 68.40 | 14.92 | 26 |
| WH | 2012 | 18/04/2012 | 7 | 6.95 | 3.70 | 66.40 | 19.78 | 28 |
| WH | 2012 | 18/04/2012 | 8 | 7.17 | 3.74 | 61.50 | 16.68 | 27 |
| WH | 2012 | 18/04/2012 | 9 | 6.37 | 3.29 | 44.00 | 17.02 | 26 |
| WH | 2012 | 18/04/2012 | 10 | 7.65 | 4.15 | 68.40 | 15.28 | 26 |
| WH | 2013 | 31/05/2013 | 1 | 6.79 | 4.33 | 50.20 | 16.04 | 27 |
| WH | 2013 | 31/05/2013 | 2 | 7.21 | 4.56 | 59.00 | 15.74 | 26 |
| WH | 2013 | 31/05/2013 | 3 | 6.99 | 3.93 | 52.00 | 15.23 | 27 |
| WH | 2013 | 31/05/2013 | 4 | 6.66 | 3.92 | 51.40 | 17.38 | 26 |
| WH | 2013 | 31/05/2013 | 5 | 4.54 | 4.37 | 66.90 | 71.49 | 26 |
| WH | 2013 | 31/05/2013 | 6 | 7.03 | 4.66 | 68.00 | 19.57 | 28 |
| WH | 2013 | 31/05/2013 | 7 | 6.92 | 4.24 | 73.60 | 22.21 | 27 |
| WH | 2013 | 31/05/2013 | 8 | 5.63 | 3.39 | 36.00 | 20.17 | 28 |
| WH | 2013 | 31/05/2013 | 9 | 6.75 | 4.08 | 52.40 | 17.04 | 26 |
| WH | 2013 | 31/05/2013 | 10 | 6.24 | 3.70 | 42.00 | 17.29 | 26 |
| WH | 2014 | 11/04/2014 | 1 | 6.18 | 3.16 | 26.00 | 11.02 | 25 |
| WH | 2014 | 11/04/2014 | 2 | 6.47 | 3.63 | 40.00 | 14.77 | 25 |
| WH | 2014 | 11/04/2014 | 3 | 4.23 | 2.39 | 11.00 | 14.53 | 25 |
| WH | 2014 | 11/04/2014 | 4 | 6.94 | 3.66 | 54.00 | 16.16 | 25 |
| WH | 2014 | 11/04/2014 | 5 | 4.33 | 2.11 | 9.00 | 11.09 | 25 |
| WH | 2014 | 11/04/2014 | 6 | 6.25 | 3.67 | 39.00 | 15.97 | 25 |
| WH | 2014 | 11/04/2014 | 7 | 5.43 | 3.05 | 28.00 | 17.49 | 25 |
| WH | 2014 | 11/04/2014 | 8 | 6.88 | 3.86 | 47.00 | 14.43 | 25 |
| WH | 2014 | 11/04/2014 | 9 | 4.92 | 2.78 | 20.00 | 16.79 | 25 |
| WH | 2014 | 11/04/2014 | 10 | 5.32 | 2.98 | 19.00 | 12.62 | 25 |

**Table S3.** Distinguishing wavenumbers and proposed assignments obtained from analysis of *Rana temporaria* spawn with ATR-FTIR spectroscopy following analysis with PCA. The five largest loadings values for the PCs which best separated the data are shown. Comparisons were made between sites for each year (2012, 2013, 2014) as follows: CT: a pond minimally impacted by pollutants; WH: a pond impacted by agricultural pollutants and PF: a pond impacted by urban pollutants.

| Comparison | Wavenumber ($cm^{-1}$) | Tentative Assignment ¥ | Differences ≠ |
|---|---|---|---|
| **Spawn 2012** | | | |
| PC4 | 1755 | Lipid fatty acids | CT [a] |
| | 1732 | Fatty acid esters | PF [ab] |
| | 1462 | $CH_2$ acyl chain of lipid | WH [b] |
| | 1624 | Amide I, $\beta$-sheet | |
| | 1169 | Asymmetric stretching CO-O-C | |
| PC6 | 1640 | Amide I protein | CT [a] |
| | 1609 | Adenine vibration DNA | PF [b] |
| | 1096 | Phosphate II stretching (asymmetric) in RNA | WH [ab] |
| | 1034 | Collagen | |
| | 995 | Stretching of phosphate groups in RNA | |
| **Spawn 2013** | | | |
| PC1 | 1744 | C=O stretching mode of lipids | CT [ab] |
| | 1628 | Amide I | PF [a] |
| | 1605 | Asymmetric stretch polysaccharides/pectin | WH [b] |
| | 1516 | Amide II | |
| | 1466 | $CH_2$ scissoring mode of the lipid acyl chain | |
| PC2 | 1728 | C=O band | CT [a] |
| | 1620 | Peak of nucleic acids due to the base carbonyl stretching and ring breathing mode | PF [b]<br>WH [b] |
| | 1184 | Amide III | |
| | 1100 | Stretching $PO_2^-$ symmetric (phosphate II) | |
| | 1072 | Nucleic acid band | |
| **Spawn 2014** | | | |
| PC1 | 1744 | C=O stretching mode of lipids | CT [a] |
| | 1721 | C=O stretching | PF [a] |
| | 1628 | Amide I | WH [b] |
| | 1096 | Asymmetric $PO_2^-$ stretching in RNA | |
| | 1026 | Glycogen | |
| PC3 | 1771 | Fatty acid esters | CT [a] |
| | 1694 | Amide I vibration | PF [b] |
| | 1647 | Amide I | WH [b] |
| | 1589 | Ring C-C stretch of phenyl | |
| | 1516 | Amide II | |

¥ (Movasaghi et al., 2008; Naumann, 2001; Podrabsky et al., 2001)

≠ Different letters denote a significant difference at the $P < 0.05$ level following one-way ANOVA and Tukey's multiple comparison tests.

**Table S4.** Distinguishing wavenumbers and proposed assignments obtained from analysis of *Rana temporaria* spawn with ATR-FTIR spectroscopy following analysis with PCA. The five largest loadings values for the two most discriminating PCs are shown. Comparisons were made between years for each site sampled. Sites are as follows: CT: a pond minimally impacted by pollutants; WH: a pond impacted by agricultural pollutants and PF: a pond impacted by urban pollutants.

| Comparison | Wavenumber ($cm^{-1}$) | Tentative Assignment [¥] | Differences [≠] |
|---|---|---|---|
| **CT Spawn** | | | |
| PC1 | 1744 | C=O stretching of lipids | 2012 [a] |
| | 1705 | C=O stretching (bases) | 2013 [b] |
| | 1624 | Amide I, $\beta$-sheet | 2014 [b] |
| | 1597 | C=N, $NH_2$ adenine | |
| | 1516 | Amide II | |
| PC2 | 1659 | Amide I | 2012 [a] |
| | 1612 | Amide I | 2013 [b] |
| | 1589 | Ring C-C stretch of phenyl | 2014 [a] |
| | 1539 | Amide II | |
| | 1512 | Amide II | |
| **PF Spawn** | | | |
| PC1 | 1744 | C=O stretching of lipids | 2012 [a] |
| | 1624 | Amide I, $\beta$-sheet | 2013 [b] |
| | 1593 | Ring C-C stretch of phenyl | 2014 [b] |
| | 1516 | Amide II | |
| | 1485 | C-H deformation | |
| PC4 | 1709 | C=O stretching (bases) | 2012 [ab] |
| | 1659 | Amide I | 2013 [a] |
| | 1612 | Amide I (carbonyl stretching vibrations in side chains of amino acids) | 2014 [b] |
| | 1169 | C-O bands from glycomaterials and proteins | |
| | 1069 | Stretching C-O ribose | |
| **WH Spawn** | | | |
| PC1 | 1744 | C=O stretching of lipids | 2012 [a] |
| | 1709 | C=O stretching (bases) | 2013 [a] |
| | 1628 | Amide I (Intramolecular $\beta$-sheet) | 2014 [b] |
| | 1605 | Asymmetric stretching polysaccharides | |
| | 1516 | Amide II | |
| PC2 | 1616 | Amide I | 2012 [a] |
| | 1508 | In-plane CH bending vibration from the phenyl rings | 2013 [b] 2014 [b] |
| | 1065 | C-O stretching of ribose and phosphodiester | |
| | 991 | C-O deoxyribose | |
| | 968 | Phosphodiester region | |

[¥] (Movasaghi et al., 2008; Naumann, 2001; Podrabsky et al., 2001)
[≠] Different letters denote a significant difference at the $P < 0.05$ level following one-way ANOVA and Tukey's multiple comparison tests.

**Table S5.** Distinguishing wavenumbers and proposed assignments obtained from analysis of *Rana temporaria* tadpoles with ATR-FTIR spectroscopy following analysis with PCA. The five largest loadings values for the PCs which best separated the data are shown. Comparisons were made between sites for each year (2012, 2013, 2014) as follows: CT: a pond minimally impacted by pollutants; WH: a pond impacted by agricultural pollutants and PF: a pond impacted by urban pollutants.

| Comparison | Wavenumber ($cm^{-1}$) | Tentative Assignment ¥ | Differences ≠ |
|---|---|---|---|
| **Tadpole 2012** | | | |
| PC1 | 1150 | C-O stretching of carbohydrates | CT [a] |
| | 1076 | Symmetric phosphate stretching | PF [b] |
| | 1030 | Glycogen | WH [b] |
| | 1003 | Sugar phosphate chain vibrations in nucleic acids | |
| | 957 | Symmetric stretching vibration of phosphate | |
| PC3 | 1694 | Amide I vibration | CT [a] |
| | 1624 | Amide I, $\beta$-sheet | PF [a] |
| | 1497 | C=C, deformation C-H | WH [b] |
| | 1462 | $CH_2$ acyl chain of lipid | |
| | 1034 | Glycogen/collagen | |
| **Tadpole 2013** | | | |
| PC1 | 1130 | Polysaccharides | CT [a] |
| | 1057 | Stretching C-O deoxyribose | PF [b] |
| | 1030 | Glycogen | WH [a] |
| | 999 | Ring stretching vibration | |
| | 953 | Phosphodiester region | |
| PC3 | 1694 | Amide I vibration | CT [a] |
| | 1497 | C=C, deformation C-H | PF [b] |
| | 1119 | Symmetric stretching P-O-C | WH [c] |
| | 1072 | Nucleic acid band | |
| | 1042 | Glycogen | |
| **Tadpole 2014** | | | |
| PC1 | 1744 | C=O stretching mode of lipids | CT [a] |
| | 1721 | C=O | PF [b] |
| | 1643 | Amide I band (from C=O stretching) | WH [a] |
| | 1466 | $CH_2$ scissoring mode of the acyl chain of lipid | |
| | 1397 | $CH_3$ bending/deformation | |
| PC2 | 1748 | Lipids/fatty acids (C=C) | CT [a] |
| | 1053 | C-O stretching carbohydrates | PF [a] |
| | 1030 | Glycogen | WH [b] |
| | 999 | Ring stretching vibration | |
| | 953 | Phosphodiester region | |

¥ (Cakmak et al., 2006; Chu et al., 2001; Movasaghi et al., 2008; Palaniappan and Vijayasundaram, 2008).

≠ Different letters denote a significant difference at the $P < 0.05$ level following one-way ANOVA and Tukey's multiple comparison tests.

**Table S6.** Distinguishing wavenumbers and proposed assignments obtained from analysis of *Rana temporaria* tadpoles with ATR-FTIR spectroscopy following analysis with PCA. The five largest loadings values for the principal components which best separated the data are shown. Tadpoles from PF were removed from analysis due to body size differences between tadpoles from this site and those from CT and WH; the loadings therefore represent the areas of the spectrum attributable to differences between CT and WH only.

| Comparison | Wavenumber ($cm^{-1}$) | Tentative Assignment [¥] | Differences [≠] |
|---|---|---|---|
| **Tadpole 2013** | | | |
| PC2 | 1744 | C=O stretching mode of lipids | CT [a] |
| | 1497 | C=C, deformation C-H | WH [b] |
| | 1466 | $CH_2$ scissoring mode of the acyl chain of lipid | |
| | 1119 | Symmetric stretching P-O-C | |
| | 1072 | Nucleic acid band | |
| PC3 | 1744 | C=O stretching mode of lipids | CT [a] |
| | 1667 | Amide I | WH [b] |
| | 1636 | Amide I | |
| | 1119 | Symmetric stretching P-O-C | |
| | 1072 | Nucleic acid band | |
| **Tadpole 2014** | | | |
| PC2 | 1744 | C=O stretching mode of lipids | CT [a] |
| | 1115 | Symmetric stretching P-O-C | WH [b] |
| | 1053 | C-O stretching carbohydrates | |
| | 1030 | Glycogen | |
| | 1003 | Sugar phosphate chain vibrations in nucleic acids | |

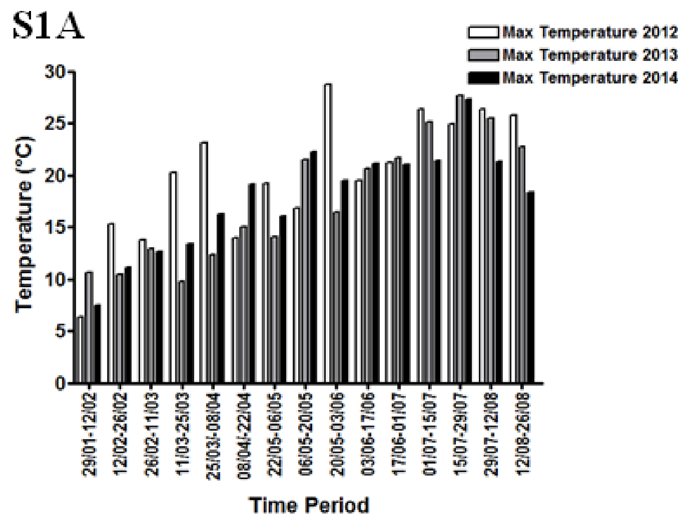
[¥] (Cakmak et al., 2006; Chu et al., 2001; Movasaghi et al., 2008; Palaniappan and Vijayasundaram, 2008).
[≠] Different letters denote a significant difference at the $P < 0.05$ level following analysis with two-sample t-tests.

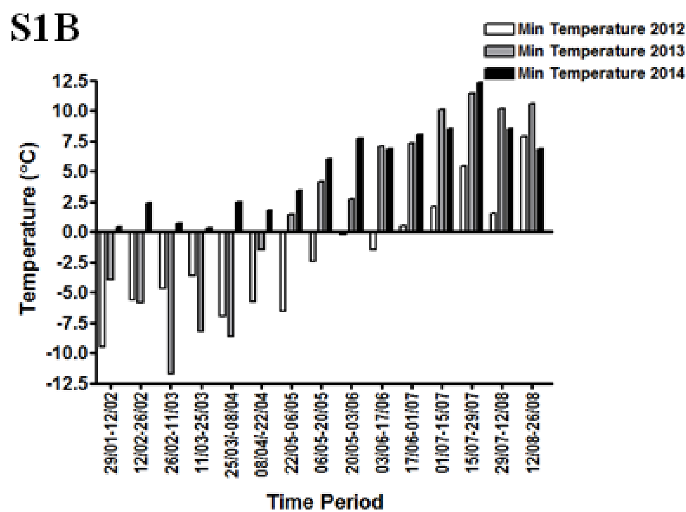
**Table S7.** Distinguishing wavenumbers and proposed assignments obtained from analysis of *Rana temporaria* tadpoles with ATR-FTIR spectroscopy following analysis with PCA. The five largest loadings values for the two most discriminating PCs are shown. Comparisons were made between years for each site sampled. Sites are as follows: CT: a pond minimally impacted by pollutants; WH: a pond impacted by agricultural pollutants and PF: a pond impacted by urban pollutants.

| Comparison | Wavenumber ($cm^{-1}$) | Tentative Assignment [¥] | Differences [≠] |
|---|---|---|---|
| **CT Tadpole** | | | |
| PC2 | 1763 | Lipid | 2012 [a] |
| | 1740 | C=O stretching (lipids) | 2013 [a] |
| | 1501 | Amide II bending | 2014 [b] |
| | 1466 | $CH_2$ scissoring mode of the acyl chain of lipid | |
| | 1173 | C-O stretching of protein and carbohydrate | |
| PC3 | 1740 | C=O stretching (lipids) | 2012 [a] |
| | 1717 | C=O stretching vibration | 2013 [b] |
| | 1643 | Amide I | 2014 [ab] |
| | 1547 | Amide II | |
| | 1497 | C=C, deformation C-H | |
| **PF Tadpole** | | | |
| PC1 | 1763 | Fatty acid esters | 2012 [a] |
| | 1740 | C=O stretching (lipids) | 2013 [a] |
| | 1717 | C=O stretching vibration | 2014 [b] |
| | 1616 | Amide I | |
| | 1466 | $CH_2$ scissoring mode of the acyl chain of lipid | |
| PC2 | 1748 | Lipids/fatty acids (C=C) | 2012 [a] |
| | 1709 | C=O stretching (bases) | 2013 [b] |
| | 1636 | Amide I | 2014 [b] |
| | 1505 | In-plane CH bending vibration from the phenyl rings | |
| | 1466 | $CH_2$ scissoring mode of the acyl chain of lipid | |
| **WH Tadpole** | | | |
| PC1 | 1744 | C=O stretching (lipids) | 2012 [a] |
| | 1628 | Amide I | 2013 [a] |
| | 1462 | $CH_2$ scissoring mode of the acyl chain of lipid | 2014 [b] |
| | 1400 | Symmetric bending/stretching of methyl groups in proteins | |
| | 1173 | C-O stretching of protein and carbohydrate | |
| PC2 | 1640 | Amide I | 2012 [a] |
| | 1616 | Amide I | 2013 [b] |
| | 1119 | Symmetric stretching P-O-C | 2014 [b] |
| | 1092 | Stretching $PO_2^-$ symmetric | |
| | 1034 | Glycogen | |

[¥] (Cakmak et al., 2006; Chu et al., 2001; Movasaghi et al., 2008; Palaniappan and Vijayasundaram, 2008).

[≠] Different letters denote a significant difference at the $P < 0.05$ level following one-way ANOVA and Tukey's multiple comparison tests.



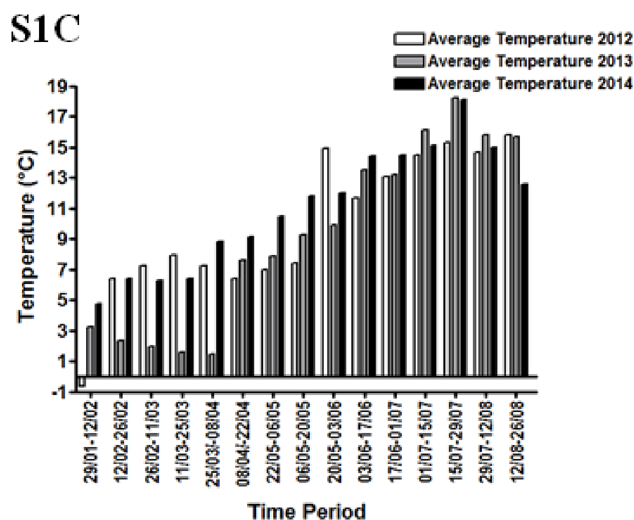
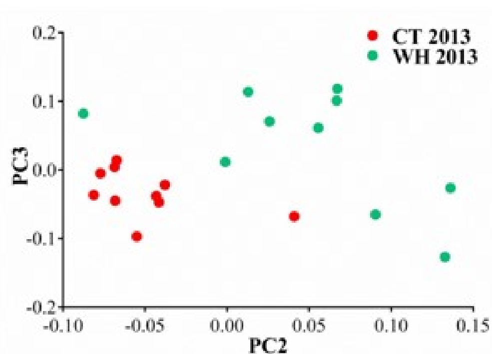




**Figure S1.** Maximum (A), minimum (B) and average (C) air temperatures collected from Hazelrigg Weather station at Lancaster University over two week time periods from a month before the beginning of the common frog breeding season to after metamorphosis of tadpoles.

**S2A**

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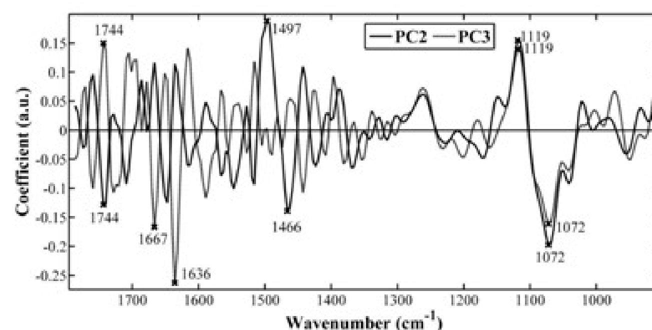
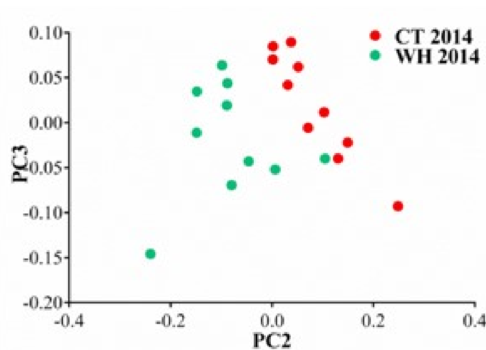



**S2C**




**S2D**

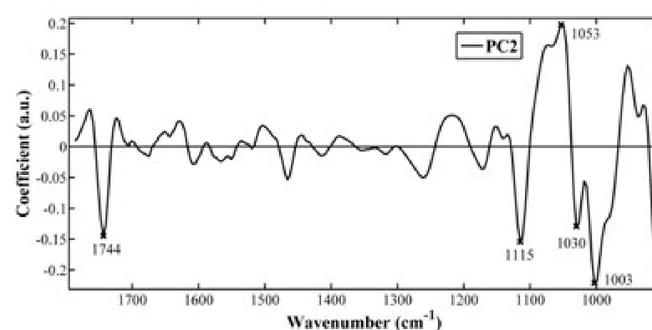



**Figure S2.** *Rana temporaria* tadpoles collected from two different ponds separated into year groups and analysed with PCA following interrogation with ATR-FTIR spectroscopy. **A.** Scores and **B.** Loadings plots of tadpoles collected in 2013; **C.** Scores and **D.** Loadings plots of tadpoles collected in 2014. Ponds are: CT: a rural agricultural pond with minimal pesticide input and WH: an agricultural pond known to be impacted by pesticides. Tadpoles from PF were excluded from analysis due to body size differences.

# Chapter 4.

**Biospectroscopy reveals the effect of varying water quality on tadpole tissues of the common frog (*Rana temporaria*).**

**Rebecca J. Strong**, Crispin J. Halsall, Martin Ferenčík, Kevin C. Jones, Richard F. Shore and Francis L. Martin.

**Environmental Pollution 213 (2016): 322-337.**

Contribution:

- I acquired the samples required for the project.
- I prepared, processed and acquired data for tadpole samples including conducting computational analysis.
- Water analysis for organic contaminants was acquired by Martin Ferenčík at Povodí Labe, Czech Republic.
- Nutrient analysis was carried out by the Centre for Ecology and Hydrology, Lancaster.
- I prepared the first draft of the manuscript.

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Rebecca J. Strong Prof. Francis L. Martin

**Biospectroscopy reveals the effect of varying water quality on tadpole tissues of the Common Frog (*Rana temporaria*)**

Rebecca J. Strong [a], Crispin J. Halsall [a,*], Martin Ferenčík [b,c], Kevin C. Jones [a], Richard F. Shore [d] and Francis L. Martin [a,*]

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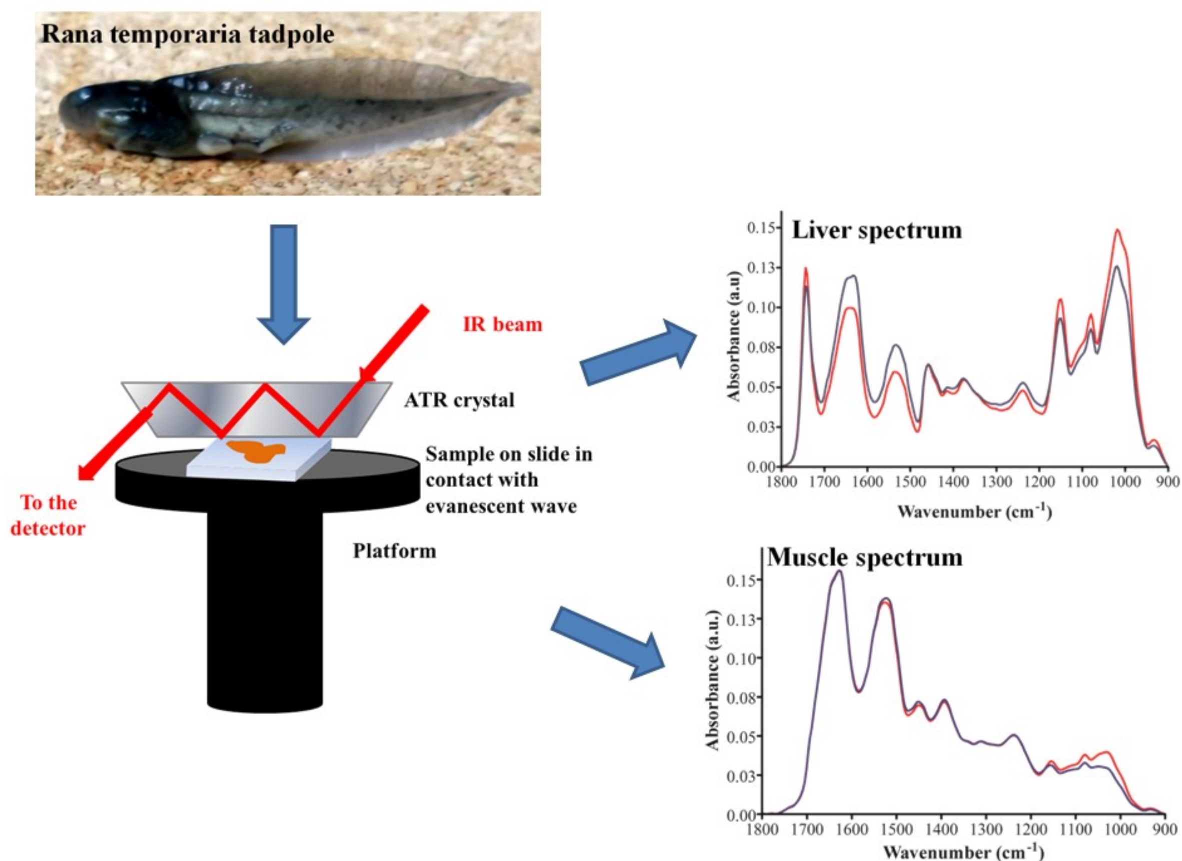
## Graphical abstract




## Highlights

- Comparison of ponds with differing water quality in the UK
- ATR-FTIR spectroscopy detects spectral alterations in common frog tadpoles
- Spectral alterations detected in several tissues; liver is most sensitive
- Liver size also potentially affected by agricultural pollutant exposure

## Abstract

Amphibians are undergoing large population declines in many regions around the world. As environmental pollution from both agricultural and urban sources has been implicated in such declines, there is a need for a biomonitoring approach to study potential impacts on this vulnerable class of organism. This study assessed the use of infrared (IR) spectroscopy as a tool to detect changes in several tissues (liver, muscle, kidney, heart and skin) of late-stage common frog (*Rana temporaria*) tadpoles collected from ponds with differing water quality. Small differences in spectral signatures were revealed between a rural agricultural pond and an urban pond receiving wastewater and landfill run-off; these were limited to the liver and heart, although large differences in body size were apparent, surprisingly with tadpoles from the urban site larger than those from the rural site. Large differences in liver spectra were found between tadpoles from the pesticide and nutrient impacted pond compared to the rural agricultural pond, particularly in regions associated with lipids. Liver mass and hepatosomatic indices were found to be significantly increased in tadpoles from the site impacted by pesticides and trace organic chemicals, suggestive of exposure to environmental contamination. Significant alterations were also found in muscle tissue between tadpoles from these two ponds in regions associated with glycogen, potentially indicative of a stress response. This study highlights the use of IR spectroscopy, a low-cost, rapid and reagent-free technique in the biomonitoring of a class of organisms susceptible to environmental degradation.

**Keywords:** Amphibian declines; Environmental pollution; IR spectroscopy; Liver; Tadpoles

**Capsule:** Infrared spectroscopy was used as a tool to detect contaminant-induced alterations in pro-metamorphic tadpoles of the common frog in a range of tissues.

## Introduction

Amphibians are facing large declines globally, with a number of hypotheses proposed to explain such declines, including habitat destruction, disease, climate change, UV radiation, predation and environmental contamination (Beebee and Griffiths, 2005; Blaustein et al., 2003; Mann et al., 2009; Stuart et al., 2004). Whilst no one factor is likely to be the sole cause of population decreases (Blaustein et al., 2011), it is known that amphibians may be particularly vulnerable to environmental contamination as their reproduction and larval development occurs in aquatic habitats, often adjacent to surface run-off from agricultural and urban sources (Mann et al., 2009; Ralph and Petras, 1997). This coupled with the permeable skin of amphibians, offering little protection against toxic contaminants (Blaustein et al., 2003), means that they are regarded as indicators of environmental stress (Blaustein and Wake, 1995).

While amphibians are considered to be most vulnerable to environmental stress during early tadpole development (Bridges, 2000; Greulich and Pflugmacher, 2003), the effects of such exposure may have consequences in later development (Bridges, 2000; Orton and Routledge, 2011; Orton and Tyler, 2014). This could include a smaller size at metamorphosis, exposing the juvenile amphibian to an increased risk of predation, or delayed development and metamorphosis, which could mean that the ephemeral ponds dry up before metamorphosis occurs (Altwegg and Reyer, 2003; Egea-Serrano et al., 2012; Hayes et al., 2006; Venturino et al., 2003). Thus, it is useful to determine the effects in the later stages of development prior to metamorphic climax.

A technique increasingly being employed to derive detailed information from biological samples is Fourier-transform infrared (FTIR) spectroscopy, which is used in three major sampling modes: transmission, reflectance or attenuated total reflection (ATR) (Kazarian and Chan, 2006). FTIR spectroscopy has been widely used in several biological applications including the diagnosis of disease states (Baker et al., 2014; Bellisola and Sorio, 2012; Ellis and Goodacre, 2006; Kazarian and Chan, 2006; Movasaghi et al., 2008; Toyran et al., 2006), imaging of tissue composition (Greve et al., 2008; Purna Sai and Babu, 2001), identifying microorganisms (Mariey et al., 2001; Naumann et al., 1991) and for analysing the effects of environmental contaminants at the cellular and tissue level (Abdel-Gawad et al., 2012; Cakmak et

al., 2006; Cakmak et al., 2003; Corte et al., 2010; Holman et al., 2000; Llabjani et al., 2012; Malins et al., 2006; Obinaju et al., 2014; Palaniappan and Vijayasundaram, 2008, 2009; Palaniappan et al., 2011; Ukpebor et al., 2011).

The basic principle of FTIR spectroscopy is that when a sample is analysed with an IR beam, the functional groups within the sample vibrate in different ways in the mid-IR region: stretching (asymmetric or symmetric) or deformations (mainly asymmetric and symmetric bending) (Bellisola and Sorio, 2012). The absorption can then be correlated to particular biochemical entities (*e.g.*, DNA/RNA, carbohydrate, proteins and lipids) and the resultant spectrum viewed as an infrared fingerprint (Ellis and Goodacre, 2006). Using IR spectroscopy is advantageous as this technique is label-free, thus allowing samples to be used subsequently for other applications, rapid, reagent-free, and cost-effective as minimal sample preparation is required (Baker et al., 2014; Kazarian and Chan, 2006).

FTIR spectroscopy generates large detailed datasets so is often coupled with a multivariate approach such as principal component analysis (PCA) or linear discriminant analysis (LDA) to extract useful information from the resulting IR absorbance spectrum (Ellis and Goodacre, 2006). Used in this manner, FTIR spectroscopy is able to distinguish between different groups on the basis of their biochemical fingerprint and also identifies which wavenumbers, and therefore which chemical bonds are altered between samples (Trevisan et al., 2012). Additionally, use of derivative spectra may allow more detailed examination of overlapping peaks in the spectrum, thus allowing the quantification of particular biochemical constituents (Rieppo et al., 2012).

The aim of this study was to determine whether tadpoles of the Common frog, *Rana temporaria*, at a pro-metamorphic stage in development, *i.e.*, following the emergence and development of hindlimbs [Gosner stage 38-40 (Gosner, 1960)] collected from ponds with varying water quality could be distinguished on the basis of their ATR-FTIR spectral fingerprint. Detection of underlying differences may suggest the possible application of IR spectroscopy as an environmental monitoring tool. Liver and muscle samples were taken from individual tadpoles and analysed with ATR-FTIR spectroscopy; previous studies in amphibians using other techniques have demonstrated changes in metabolic constituents such as lipid, protein and glycogen

following exposure to environmental contaminants in these tissues (Dornelles and Oliveira, 2014; Gendron et al., 1997; Gurushankara et al., 2007; Melvin et al., 2013). Other tissues less routinely used in assessing amphibian health (heart, kidney and skin) were also analysed in this study, thus providing spectral fingerprints of several different tissues of an amphibian species. Although applied to fish in several studies (Cakmak et al., 2006; Henczova et al., 2008; Malins et al., 2006; Malins et al., 2004; Obinaju et al., 2014), this is the first time to our knowledge that IR spectroscopy has been used to characterise amphibian tissue.

## Materials and Methods

### Pond Selection

Sites were selected in order to give a comparison between agricultural and urban ponds and were based on site characteristics and information from landowners/land managers.

1. Whinton Hill (WH), Plumpton, Cumbria is a farm consisting primarily of arable land, which is routinely sprayed with herbicides and fungicides. The pond surveyed was the shallow pond of a pair of deep and shallow ponds (32 m long × 8 m wide x 0.5 m deep), located in a boggy field, and fed by a field drain from approximately 30 ha ($3\times10^5$ $m^2$) of farmland.

2. Crake Trees (CT), Crosby Ravensworth is a farm used as beef grazing land and marginal arable land, which has been accepted onto Natural England's Higher Level Environmental Stewardship Scheme and uses minimal quantities of pesticides, with buffer zones to prevent pesticide run-off into water courses. The pond surveyed was the second pond of a pair of shallow ponds (each 17 m long × 6 m wide x 0.5 m deep), located in a field corner, and fed by surface runoff from approximately 20 ha ($2\times10^5$ $m^2$) of farmland.

   The ponds surveyed at WH and CT are part of the MOPS2 (Mitigation Options for Phosphorus and Sediment) project monitored by Lancaster University (http://mops2.diffusepollution.info/).

3. Pennington Flash Country Park (PF) located in Leigh, Lancashire is a site managed by Wigan and Leigh Culture Trust. The 'Flash' is a large lake formed over time by mining subsidence. The southern part of the Flash was filled with domestic waste during the 1950s to prevent the regular flooding of nearby St Helen's Road. The pond sampled is adjacent to Westleigh Brook, which receives treated wastewater from Leigh wastewater treatment works.

**Water sampling**

Samples of surface water (15-20 cm depth) were collected over the amphibian breeding season (March-August) in 2012. Water samples for organics analysis were only available from PF for March and April, and March, April and June for nutrient analysis. Water samples were collected in methanol-rinsed amber bottles for organics analysis and acid-washed bottles for nutrient analysis and then stored at 4°C until analysis.

**Chemical analysis**

The concentrations of trace metals (Al, Fe, Mg, Ca, K and Na) were determined in filtered acidified water samples (1% $HNO_3$) using ICP-OES (Perkin Elmer DV 7300) while concentrations of major anions (Cl, $NO_3$-N, $SO_4$-S) as well as phosphate, ammonium and total organic N (TON) were determined using colorimetric methods performed by the Centre for Ecology and Hydrology (Lancaster) in a quality-assured, previously published method (Neal et al., 2000). For trace organic chemical analysis, 800 mL of sample water (adjusted to pH 9.5 with borate buffer) underwent liquid-liquid (1:1) extraction using dichloromethane (DCM) on a laboratory shaker (Gerhardt Shaker LS-500) followed by separation and evaporation of the DCM on a rotary evaporator (rotavapor Büchi R-210). The concentrated DCM extracts (700 µL) underwent initial qualitative screening using GC-MS (Agilent 6890N GC and Agilent 5973 single quad MS) operated by ChemStation software (D.02.00.275) with subsequent mass spectral identification using Mass Hunter software and comparison to the NIST spectral library. The following chemicals were detected: aniline, metazachlor, acetochlor, dimetachlor, triethylphosphate, tributylphosphate, tris(2-chloroethyl)phosphate, tris(1-chloro-2-propyl)phosphate and flusilazole. These compounds were quantitatively analysed using authentic standards using a 7-point

calibration, with standards ranging from 0-2000 ng/L for each analyte. Internal standards comprising of $^{13}C$-labelled aniline, acetochlor and metalochlor were added to sample extracts and calibration standards prior to analysis. Limits of quantification (LOQ) ranged from 5-10 ng/L (aniline 200 ng/L) with recoveries based on spiked water samples ranging from 80-120%. Water samples were also analysed for more polar, water-soluble compounds. For this analysis, 10 mL of a water sample was filtered (using a 0.2 µm RC syringe filter), spiked with internal standards and analysis performed on a Waters Acquity Binary Ultra Performance Liquid Chromatograph (UPLC) (Waters Corporation, Milford, USA) coupled to a Waters Premier XE triple quadrupole mass spectrometer (LC-MS/MS) operated by MassLynx software V 4.1.The MS was operated in electrospray positive (ESI+) ionisation mode with multiple reaction monitoring (MRM). A 250 µL aliquot was injected via an autosampler, with analyte separation performed under a $MeOH/H_2O$ (with 5 mmol/L ammonium acetate added to both phases) mobile gradient eluted through an Acquity BEH $C_{18}$ column (1.7 µm, 2.1 mm × 50 mm) fitted with a VanGuard Acquity precolumn. The following compounds, including pesticides and pharmaceuticals, were qualified/quantified: chlorotoluron, isproturon, caffeine, tebuconazole, prochloraz, carbendazim, gabapentin, acetaminophen, benzotriazole, benzotriazole-methyl, ketoprofen, dimethyl-chlorotoluron, metconazole, spiroxamine, boscalid, and erythromycin. Samples were analysed separately for glyphosate and its degradation by-product, aminomethylphosphonic acid (AMPA), using LC-MS/MS. For the analysis of glyphosate and AMPA, 8 mL of a water sample was acidified to pH 1 (addition of 160 µL of 6 M HCl) and subject to derivatisation using 9-fluorenylmethyl chloroformate using a previously published method (Ibáñez et al., 2006). Analytes were separated using the same LC-MS/MS instrument and method above. Internal standards comprised of 1,2-$^{13}C_2$ $^{15}N$ Glyphosate and $^{13}C$ $^{15}N$ AMPA with a 7-point calibration with standards ranging from 0 to 2000 ng/L. Ionisation was through ESI+ (precursor ions) and MRM (product ions). LOQs were 10 ng/L for both glyphosate and AMPA with recoveries ranging from 70-130% (water spiked with internal standards).

## Tadpole collection

Tadpoles of *R. temporaria* were collected over a two-year period. In 2012, tadpoles were collected from CT and PF (five from each pond), and in 2013 tadpoles were collected from CT and WH (ten from each pond). Tadpoles were collected at Gosner stage 38-40, when hindlimbs were fully emerged and toes developed. Stages 30-40 are considered to be relatively stable regarding key traits, before the more dramatic changes in metamorphosis occur after stage 41 (Gosner, 1960). Tadpoles were caught using dip nets, euthanized using a solution of MS-222 (400 mg/L) buffered with sodium bicarbonate (both from Sigma Aldrich, Poole, Dorset UK) in accordance with Schedule 1 of the British Home Office Animals (Scientific Procedures) Act 1986. Tadpoles were then rinsed in distilled water and fixed immediately in the field in 70% ethanol (Fisher Scientific, UK). A small slit was made into the abdomen of each tadpole to allow the fixative to penetrate all of the tissues adequately. Ethanol was replaced after 24 hours with fresh solution. Measurements were taken of snout-to-vent length (SVL), head width (HW), body mass and tail length for all tadpoles; liver weights were also taken for tadpoles collected from CT and WH in 2013. After fixation, the following organs were excised: liver, kidney, heart, muscle, and skin, and slices (~0.5 mm thick) taken using a Stadie-Riggs tissue slicer; a technique previously employed for preparing tissue samples for spectroscopy studies (Maher et al., 2014; Obinaju et al., 2014; Taylor et al., 2011). Slices of each organ were mounted onto Low-E reflective glass slides (Kevley Technologies, Chesterland, OH, USA), dried overnight and stored in a desiccator before subsequent interrogation with ATR-FTIR spectroscopy.

## ATR-FTIR spectroscopy

Spectra of each sample were obtained using a Tensor 27 FTIR spectrometer with Helios ATR attachment (Bruker Optics Ltd, Coventry, UK) containing a diamond crystal (≈250 μm × 250 μm sampling area). Spectra were acquired at 8 $cm^{-1}$ resolution with 2× zero-filling, giving a data-spacing of 4 $cm^{-1}$ over the range 400-4000 $cm^{-1}$. Ten-25 spectra were acquired from each sample; these were averaged in order to give a representative spectrum per organ/tadpole. Distilled water was used to clean the crystal in between analysis of each sample. A new background reading was taken

prior to the analysis of each sample in order to account for changes in atmospheric conditions.

**Spectral pre-processing**

Spectra were cut at the biochemical cell fingerprint region (1800-900 $cm^{-1}$), baseline corrected using Savitzky-Golay (SG) $2^{nd}$ order differentiation ($2^{nd}$ order polynomial and 9 filter coefficients), and vector normalised. Processing the data with second derivative spectroscopy allows overlapping peaks in the absorbance spectrum to be resolved, thus allowing more detailed analysis of particular peaks. By taking second derivatives, constant and linear components of baseline errors are also removed (Rieppo et al., 2012). For broad spectra the derivative intensity decreases with increasing derivative order, whereas for sharp spectra, the reverse is true. Therefore the underlying shape of the spectrum determines the intensity of the derivative spectrum, with flat peaks decreasing in intensity with each derivative order, and sharp peaks increasing in intensity, thus allowing small sharp peaks overlapped by broad flat peaks to be exposed (Kus et al.).

SG derivation is applied by fitting a simple polynomial to a small section of given size to the spectrum and calculates the derivative of the polynomial in the centre point of this section (Rinnan et al., 2009). In this study, a $2^{nd}$ order polynomial and nine smoothing points were employed in the SG algorithm. This resulted in the loss of 4 wavenumbers from each end of the spectrum as a symmetric window smoothing is used requiring the number of data points on each side of the centre point to be the same, and the number of wavenumbers lost equals the number of smoothing points minus one (Rinnan et al., 2009). The polynomial order and number of smoothing points was selected based upon a compromise between noise removal and signal distortion as no method exists which is able to eliminate all noise without losing important information. A small number of smoothing points and a high polynomial degree can give a noisy spectrum, whereas a large number of smoothing points and a low polynomial degree can distort the spectrum (Vivó-Truyols and Schoenmakers, 2006).

## Multivariate analysis

### Principal component analysis (PCA)

Spectral data for each tissue were analysed using principal components analysis (PCA) for exploratory analysis. PCA is a technique, which allows the large amount of data generated by IR spectroscopy to be reduced into a smaller number of principal components while retaining the majority of the variance in the dataset. PCA is an unsupervised technique which looks for inherent similarities in the data and groups them the way the data ‘naturally’ cluster and is useful for small data sets (Ellis and Goodacre, 2006). PCA generates scores and loadings: scores represent each spectrum as a single data point and allow one to see if the points cluster together, suggesting similarity, or away from each other, suggesting differences. Corresponding loadings from PCA demonstrate which wavenumbers are responsible for the separation of the scores in a dataset (Trevisan et al., 2012).

After the data were mean-centred, PCA was employed to reduce the 227 absorbance values into 10 principal components (PCs), which represented >95% of the variance in the datasets. The most statistically significant PCs were retained, as these represented the best separation in the data (see Table S1 in Electronic Supplementary Information [ESI] for *P*-values of scores for each PC for each tissue) (Malins et al., 2006; Malins et al., 2004). Loadings from the most significant PCs were used to identify wavenumbers accounting for the separation between ponds. A peak detecting algorithm was employed to determine the five largest loadings values (constrained by a minimum of 20 $cm^{-1}$ spacing between values).

### Linear discriminant analysis (LDA)

In addition to PCA, linear discriminant analysis (LDA) was also employed to improve the discrimination between the spectra of tissues between ponds. LDA is a supervised technique (the class groupings are known *a priori*) which maximises the differences between classes, while minimising within-class heterogeneity (Martínez and Kak, 2001). For small datasets, like the ones in this study, LDA alone can over-fit the data, resulting in good data separation by chance, as the number of variables (wavenumbers) are much larger than the number of samples, therefore a data

reduction technique is necessary to overcome this (Gromski et al., 2015). In this case, PCA was used prior to LDA to reduce the variables to a smaller number of PCs, which still represented ~95% of the variance in the data (see SI table S2 for the number of PCs selected for each data set). PCA also removes colinearity between variables (Gromski et al., 2015).

Data were standardised prior to the application of PCA-LDA and leave-one-out cross validation, where a small portion of the data set is used to train the model was used, again to prevent over-fitting and so as to prevent bias in the output (Trevisan et al., 2012). The output from PCA-LDA again generates scores and loading plots, however this technique generates $n$-1 linear discriminants (LDs), which optimally separate $n$ classes; in the case of this study a one-dimensional scores plot and one loading is generated per data set. To aid with the interpretation of the scores plots, a linear discriminant classifier (LDC) was also employed, which uses the same principle as LDA but fits a Gaussian classifier to separate the data and provides a % classification rate for each data set (Trevisan et al., 2012). Data were standardised and cross-validated as before.

**Comparison of absorbance values**

Detailed quantification of differences between samples at specific wavenumbers was also implemented using absorbance values from the second derivatives; this has previously been used to quantify the biochemical entities in biological samples following analysis with vibrational spectroscopy (Rieppo et al., 2012). The second derivative has its maximum value at the same wavelength as the underlying absorbance peak, but in the opposite (negative) direction (Mark and Workman Jr, 2010).

All spectral pre-processing and data analysis was implemented using the IRootLab toolbox https://code.google.com/p/irootlab/ (Martin et al., 2010; Trevisan et al., 2013) in Matlab (r2012a) (The MathWorks, Inc., USA), unless otherwise stated.

### Statistical analysis

Body condition indices (BCI) were calculated for each tadpole as follows: (body mass/$SVL^3$) × 100 (Melvin et al., 2013). Hepatosomatic indices (HSI) were also calculated for tadpoles collected from CT and WH in 2013 as follows (liver mass/body mass) × 100.

Two-sample *t*-tests were used in order compare SVL, HW, tail length, body mass, BCI, and where indicated, liver mass and HSI between tadpoles collected from the two ponds within each year group. Tadpoles were not compared between years in order to control for the differences present due to annual factors, rather than factors due to the pond itself. Data were tested for normality and homogeneity of variances, the results of which indicated that parametric analysis was appropriate.

Two-sample *t*-tests were also used to compare absorbance values for each organ from second-derivatives between ponds within each year group and to compare the statistical significance of the scores for each PC and each LD. All statistical analyses were carried out in XL Stat (Addinsoft, Paris, France).

## Results and discussion

### Water quality analysis

Water samples were collected from March-August to cover the amphibian-breeding period and to determine water quality status given the classification of the ponds based on their land-use data. Data for the major anions and cations are presented in Table 1. Nitrate concentrations remained low (<3 mg/L) at all sites throughout the sampling period reaching the highest levels in August at CT, March at PF and April at WH. Phosphate concentrations were low at all three sites during the March sampling period (<0.08 mg/L) but were higher in April at PF and WH, at levels of 0.3 and 0.6 mg/L respectively, which are considered relatively high for UK surface waters (UKTAG, 2013; Williams et al., 2004). Phosphate levels remained high at WH during June (0.58 mg/L), coinciding with the start of metamorphosis, whereas phosphate levels were much lower at both CT and PF during this time (0.12 and 0.17 mg/L respectively).

Results from the analysis of water samples for trace organic chemicals are shown in Table 2. Screening of the water samples collected from CT, PF and WH revealed

large differences in the organic contaminants detected. CT and PF appeared to be the least contaminated sites; xenobiotics detected in water samples from these sites included caffeine, several OP flame retardants and the pharmaceutical drugs acetaminophen and gabapentin, commonly found in surface waters (Mompelat et al., 2009). Both sites also had detectable levels of aminomethylphosphonic acid (AMPA), the degradation product of glyphosate. Aminomethylphosphonic acid may also form following the degradation of other phosphonate compounds including detergents, so is not necessarily indicative of glyphosate residue (Botta et al., 2009; Van Stempvoort et al., 2014). However, as glyphosate was also detected at PF and AMPA levels were higher here than at CT, this suggests that glyphosate was the likely source. Interestingly, relatively high levels of benzotriazole and benzotriazole-methyl were detected at CT. These compounds are commonly used as corrosion inhibitors so may have leached from farm machinery etc and they were frequently detected in a recent European-wide survey of river water (Loos et al., 2009). Water samples collected from PF also showed detectable levels of naphthalene, which has previously been associated with detrimental effects in aquatic species, although at much higher concentrations than those found in this study (Farré et al., 2008; Pillard et al., 2001).

Water samples collected from WH demonstrated relatively high levels of aniline, a compound generated during the degradation of several herbicides and pesticides (Xiao et al., 2007) early in the season. In contrast to CT, the other agricultural site, several pesticides, particularly fungicides were detected at WH during April and June: these included carbendazim, flusilazole, tebuconazole, boscalid, dimethachlor, chlorotoluron, metconazole and glyphosate. Carbendazim and flusilazole displayed the highest concentrations in April, with much lower levels in June and August. Glyphosate and boscalid showed the highest concentrations in June, coinciding with tadpole metamorphosis, but with much lower levels by August. Like CT, WH showed detectable levels of the corrosion inhibitors benzotriazole and benzotriazole-methyl.

**Table 1.** Analysis of water samples for inorganic anions and cations collected from CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides and PF: an urban pond impacted by wastewater and landfill run-off. Water samples were collected during the breeding season of *Rana temporaria* (March-August). Values marked < LD were below limit of detection.

| Anion/Cation (mg/L) | CT Mar | CT Apr | CT Jun | CT Aug | PF Mar | PF Apr | PF Jun | WH Mar | WH Apr | WH Jun | WH Aug |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Al** | < LD | < LD | < LD | < LD | < LD | < LD | < LD | < LD | < LD | < LD | < LD |
| **Ca** | 84.4 | 77.6 | 64.8 | 105 | 46.3 | 36.8 | 30.7 | 53.2 | 56.6 | 33.3 | 47.9 |
| **Cl** | 9.06 | 10.4 | 2.98 | 8.44 | 21.6 | 11.6 | 11.4 | 64.1 | 47.8 | 36.1 | 15.1 |
| **Fe** | 0.47 | 0.007 | 0.019 | 0.014 | 0.008 | 0.76 | 0.095 | 0.026 | 0.03 | 0.15 | 0.016 |
| **K** | 1.97 | 1.57 | 0.507 | 0.811 | 3.88 | 4.01 | 5.27 | 11.3 | 18.1 | 11.5 | 10.4 |
| **Mg** | 2.95 | 4.37 | 5.09 | 4.74 | 10.0 | 7.99 | 6.00 | 9.35 | 10.7 | 5.54 | 8.96 |
| **Na** | 4.88 | 4.97 | 3.03 | 4.95 | 15.0 | 9.82 | 9.27 | 38.8 | 37.2 | 24.6 | 12.6 |
| **$NH_4$-N** | 0.028 | 0.412 | 1.47 | 0.014 | 0.06 | 0.128 | 1.28 | 0.303 | 0.282 | 5.50 | 0.033 |
| **$NO_3$-N** | < 0.001 | 0.219 | 0.012 | 1.62 | 0.427 | 1.18 | 0.016 | 0.01 | 2.49 | 0.017 | 0.912 |
| **$PO_4$-P** | 0.029 | 0.033 | 0.121 | 0.15 | 0.068 | 0.304 | 0.17 | 0.006 | 0.639 | 0.584 | 0.089 |
| **$SO_4$-S** | 0.706 | 0.195 | 0.124 | 0.225 | 6.59 | 2.61 | 1.30 | 9.92 | 12.7 | 2.70 | 11.6 |

**Table 2.** Organic contaminant analysis of water samples collected from CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides and PF: an urban pond impacted by wastewater and landfill run-off. Water samples were collected to coincide with the breeding season of *Rana temporaria* (March-August). Values marked < LD were below limit of detection.

| Chemical (ng/L) | CT Mar | CT Apr | CT Jun | CT Aug | PF Mar | PF Apr | WH Mar | WH Apr | WH Jun | WH Aug |
|---|---|---|---|---|---|---|---|---|---|---|
| Naphthalene | <LD | <LD | <LD | <LD | 10 | <LD | <LD | <LD | <LD | <LD |
| Aniline | <LD | <LD | <LD | <LD | <LD | <LD | 1100 | <LD | <LD | <LD |
| Dimethachlor | <LD | <LD | <LD | <LD | <LD | <LD | <LD | 26 | 49 | <LD |
| Chlorotoluron | <LD | <LD | <LD | <LD | <LD | <LD | <LD | 23 | 52 | <LD |
| Caffeine | <LD | 441 | <LD | <LD | <LD | 107 | <LD | <LD | 200 | 103 |
| Glyphosate | <LD | <LD | <LD | <LD | 40 | <LD | <LD | 50 | 2310 | 50 |
| AMPA | <LD | 150 | <LD | 45 | 130 | 658 | <LD | 1470 | 1040 | 39 |
| Tebuconazole | <LD | <LD | <LD | <LD | <LD | <LD | 76 | <LD | 34 | 109 |
| Carbendazim | <LD | <LD | <LD | <LD | <LD | <LD | <LD | 866 | 76 | <LD |
| TEP | <LD | <LD | <LD | <LD | 11 | 11 | <LD | <LD | 160 | <LD |
| TBP | <LD | 13 | <LD | <LD | <LD | <LD | <LD | <LD | <LD | <LD |
| TCEP | <LD | 26 | <LD | <LD | 190 | 12 | <LD | 7.2 | 42 | 5.7 |
| TCPP | 15 | 125 | <LD | 20 | 142 | 314 | 25 | 1600 | 539 | 187 |
| Flusilazole | <LD | <LD | <LD | <LD | <LD | <LD | <LD | 552 | 30 | 26 |
| Gabapentin | <LD | <LD | 23 | 25 | 75 | <LD | 21 | <LD | 56 | <LD |
| Acetaminophen | <LD | <LD | 34 | 35 | 20 | <LD | 50 | 33 | 41 | 29 |
| Benzotriazole | <LD | <LD | <LD | <LD | <LD | <LD | <LD | 85 | 206 | 47 |
| Benzotriazole-methyl | <LD | <LD | 1520 | 53 | <LD | <LD | <LD | 268 | 263 | 60 |
| Ketoprofen | <LD | <LD | <LD | <LD | <LD | <LD | <LD | <LD | 13 | <LD |
| Desmethyl-chlrotoluron | <LD | <LD | <LD | 35 | <LD | <LD | <LD | <LD | <LD | <LD |
| Metconalzole | <LD | <LD | <LD | <LD | <LD | <LD | <LD | <LD | 14 | <LD |
| Spiroxamin | <LD | <LD | <LD | <LD | <LD | <LD | 30 | <LD | <LD | <LD |
| Boscalid | <LD | <LD | <LD | <LD | <LD | <LD | <LD | <LD | 122 | 19 |
| Erythromycin | <LD | <LD | <LD | <LD | <LD | <LD | <LD | 181 | 24 | <LD |

All three sites showed detectable levels of OP flame retardants, the particular type varying between each site: TEP present at PF and WH but absent from CT; TBP only present at CT. TCPP and TCEP were detected at all three sites, with TCPP generally detected at the highest levels, particularly at WH, where it reached a maximum level of 1600 ng/L, which is similar to that found in other studies, where it is the dominant OP flame retardant (van der Veen and de Boer, 2012). These compounds are frequently detected in surface waters due to their lack of biodegradability in wastewater treatment (Fries and Puttmann, 2003; Regnery and Püttmann, 2010) . As PF receives treated wastewater as well as run-off from landfill, this may explain the higher levels found here.

**Body measurements**

As shown in Figure 1, tadpoles from PF (2012) were significantly larger than those from CT (2012) on all measures of body size (Fig. 1A-D); tadpoles from PF also had a significantly higher BCI (Fig. 1E), as determined by two sample t-tests (SVL: $t_8$ =4.02, *P* =0.004; HW: $t_8$ =2.83, *P* =0.022; tail length: $t_8$ =4.67, *P* =0.002; body mass: $t_8$ =5.28, *P* =0.0007; BCI: $t_8$ =3.08, *P* =0.015). This finding is somewhat unexpected considering that CT is regarded as the pond with better water quality. However, there were many factors not measured in this study that could account for the differences. Such factors include selection pressures such as predation/presence of competing species, population density, food availability, abiotic factors (pH, temperature and dissolved oxygen), and changes in pond depth.

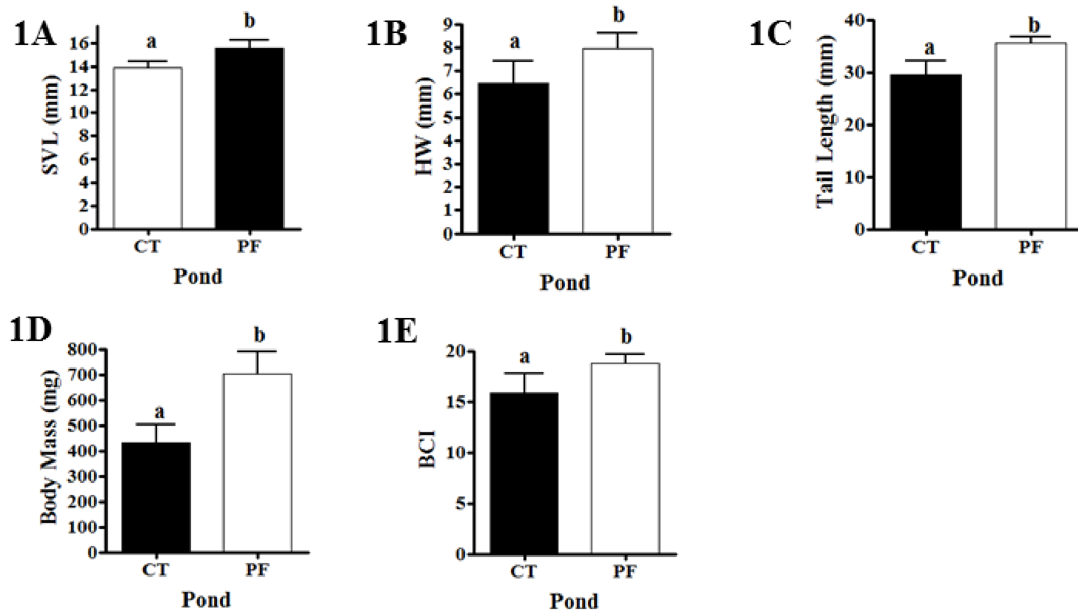



**Figure 1.** Comparison of body size parameters of pro-metamorphic *Rana temporaria* tadpoles collected in 2012 from CT: a rural agricultural pond with no pesticide input and PF: an urban pond impacted by wastewater and landfill run-off. Measurements are snout-vent-length (SVL), **(A)**, head width (HW), **(B)**, tail length, **(C)**, body mass, **(D)** and body condition index (BCI), **(E)**. Two-sample *t*-tests were used to compare each body size parameter. Different letters denote a significant difference ($P$ <0.05).

In contrast, tadpoles collected from CT (2013) only differed from those collected from WH (2013) on measures of tail length and body mass (Fig. 2A-E); tadpoles from CT were significantly larger on these two measures (Two sample t-test: SVL: $t_{18}$ =1.41, $P$ =0.17; HW: $t_{18}$ =0.57, $P$ =0.57; tail length: $t_{18}$ =2.40, $P$ =0.027; body mass: $t_{18}$ =2.22, $P$ =0.04; BCI: $t_{18}$ =1.16, $P$ =0.26). Additional measurements were made for tadpoles from CT (2013) and those from WH (2013) of liver mass and HSI (Fig. 2F and 2G), with the finding that tadpoles from WH had significantly larger values of liver mass and HSI than those from CT (Two sample t-test: liver mass: $t_{18}$ =2.31, $P$ =0.033; LSI: $t_{18}$ =4.23, $P$ =0.0005). Again, the differences in body mass and tail length could simply be due to uncontrolled factors such as food availability and pond size (Vences et al., 2002). However, the greater liver mass and HSI of tadpoles from WH in comparison to those from CT is indicative of liver inflammation or growth abnormalities (Olivares et al., 2010). Larger livers may be reflective of biochemical changes that occur as an organism attempts to maintain homeostasis and have been associated with exposure to environmental contaminants in aquatic species, including

amphibians (Edwards et al., 2006; Kim et al., 2013; Lowe-Jinde and Niimi, 1984; Melvin et al., 2013; Tetreault et al., 2003). Therefore the larger HSI seen in tadpoles from WH, coupled with their smaller mass and tail length is a clear indicator of environmental stress most likely attributable to poor water quality and marked by environmental contamination through agricultural run-off at this site.

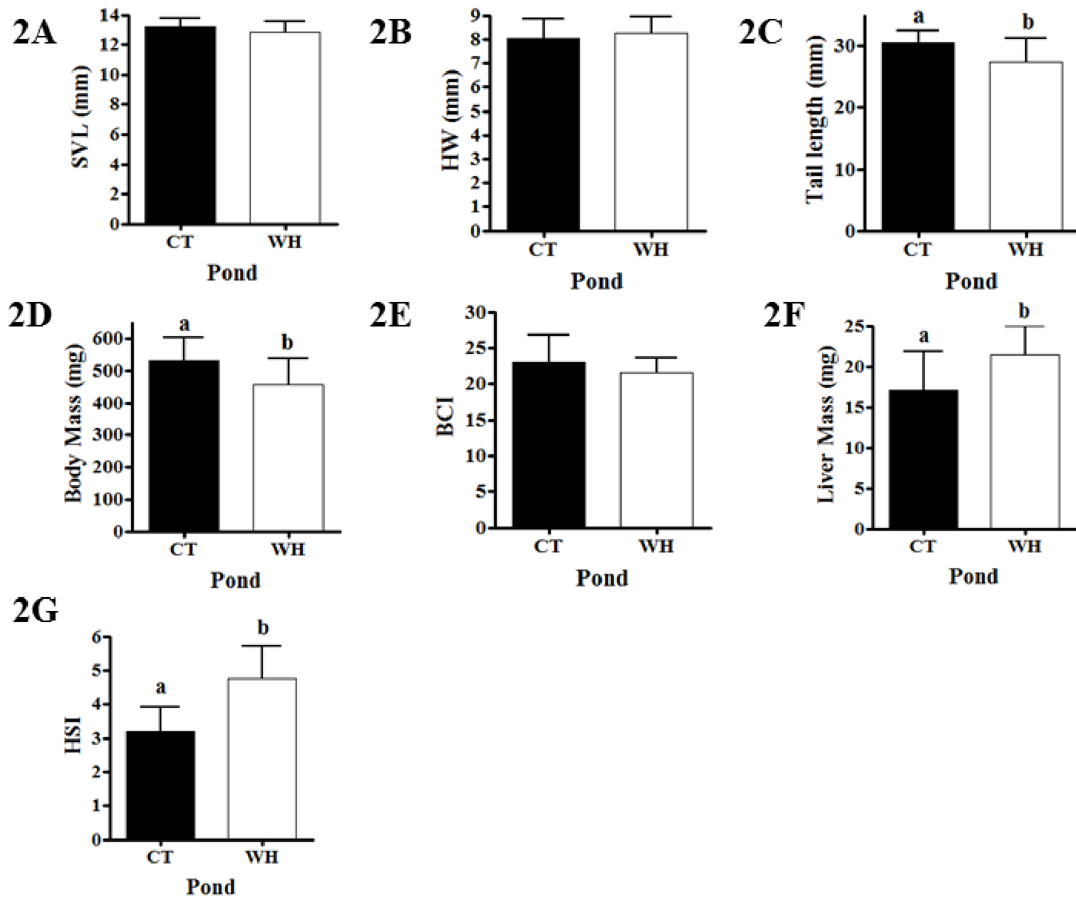



**Figure 2.** Comparison of body size parameters of pro-metamorphic *Rana temporaria* tadpoles collected in 2013 from a CT: a rural agricultural pond with no pesticide input and WH: an agricultural pond known to be impacted by pesticides. Measurements are snout-vent-length (SVL), **(A)**, head width (HW), **(B)**, tail length, **(C)**, body mass, **(D)**, body condition index (BCI), **(E)**, liver mass **(F)**, and hepatosomatic index (HSI), **(G)**. Two-sample *t*-tests were used to compare each body size parameter. Different letters denote a significant difference ($P < 0.05$).

*Rana temporaria* tadpoles, like other species, are able to show developmental plasticity, where developmental rate is adjusted according to environmental conditions, producing smaller metamorphs under conditions of low food availability and high population density. Food availability and quality may also affect

metamorphic performance and body size, with higher protein diets associated with a larger size at metamorphosis, (Alvarez and Nicieza, 2002; Audo et al., 1995; Beebee and Richard, 2000; Kupferberg, 1997) although this can vary with species (Castañeda et al., 2006). Therefore there is uncertainty regarding the effect of these uncontrolled factors and their interactions on body size parameters and a future study would aim to control such factors. Tadpoles collected in this study were at stages 38-40; a stage of development regarded as pro-metamorphic and defined as when the hindlimbs emerge and differentiate (Chambers et al., 2011; Gosner, 1960). Whilst slight differences in developmental stage can impact on size, the stages between 30-40 are considered to be one of stability in the development of key traits (Gosner, 1960). The body size of tadpoles peaks in late pro-metamorphosis before forelimb emergence (stage 42) and declines during metamorphic climax (Alvarez and Nicieza, 2002). Therefore as the tadpoles collected in this study were at a late stage in pro-metamorphosis, but before metamorphic climax, the differences in size are unlikely to be due to this factor.

**ATR-FTIR spectroscopy**

Figures 3A-F show the 2-dimensional scores plots and corresponding loadings following PCA for tissues which separated significantly in tadpoles collected from CT (2012) and PF (2012) (tentative assignments in Table S3 in ESI); 1-dimensional scores plots are shown in Figures S1A-E in ESI, with corresponding statistical and classifier analysis shown in Tables S4 and S5 respectively with tentative assignments in Table S6. Figures 4A-E show the mean spectra for each tissue type following second derivative analysis. Figures 5A-E show the 2-dimensional scores plots following PCA for tissues analysed from tadpoles collected from CT (2013) and WH (2013); corresponding loadings are shown in figure 6A-D (tentative assignments in Table S3 in ESI), with 1-dimensional scores plots following analysis with PCA-LDA shown in Fig S2A-E in ESI; corresponding statistical and classifier analysis are shown in Tables S4 and S5, respectively, with tentative assignments in Table S6. Figures 7A-E show the mean spectra for each tissue type following second derivative analysis. Table 3 shows a list of all the major second derivative peaks from each tissue and their corresponding tentative assignments. Raw spectra are shown in Figures S3 and S4 in ESI.

### Liver samples

Results from ATR-FTIR spectroscopy demonstrated that the liver was the tissue which best-distinguished tadpoles collected from CT or PF in 2012, and also tadpoles collected from CT or WH in 2013. This is perhaps expected, as the liver is the organ responsible for metabolism of xenobiotics in vertebrates, including amphibians; therefore any changes induced by environmental contamination may be detected here (Fenoglio et al., 2011). In addition, the liver is an energy store in tadpoles, and lipids, protein and glycogen are utilised for the completion of metamorphosis (Sheridan and Kao, 1998); thus changes in the levels of these constituents may be reflective of the energy status and thus condition of the tadpole (Melvin et al., 2013). However, other factors such as food availability and composition and predation may also impact the stress status of amphibians in synergy with chemical-insult (Relyea and Mills, 2001). This must be taken into account in the interpretation of the results and is a limitation of this study.

Comparison of liver samples from tadpoles collected from CT (2012) and those from PF (2012) demonstrated significant separation along PC1 (Fig. 3A), following PCA which was associated with alterations in C-O ribose (991 $cm^{-1}$), carbohydrate (1153 $cm^{-1}$), Amide II (1516 $cm^{-1}$), C=N cytosine (1601 $cm^{-1}$) and Amide I $\beta$-sheets (1624 $cm^{-1}$) as seen in the loadings plot in Figure 3B and Table S3 (see ESI). Further analysis with PCA-LDA led to improved separation in the scores plot (see ESI Fig. S1A), with a correct classification rate of 99 and 90% for CT and PF respectively (see Tables S4 and S5 in ESI). Loadings were in regions associated with carbohydrates and proteins as before, as well as some lipid contribution (Table S6 in ESI). Analysis of the second derivative peak heights also showed significant differences between tadpole livers from CT (2012) and those from PF (2012) in regions associated with protein (Amide I and II), with the finding that peak heights in these regions were larger in tadpole livers from PF (2012) in comparison to those from CT (2012) (Table 3, Fig. 4A).

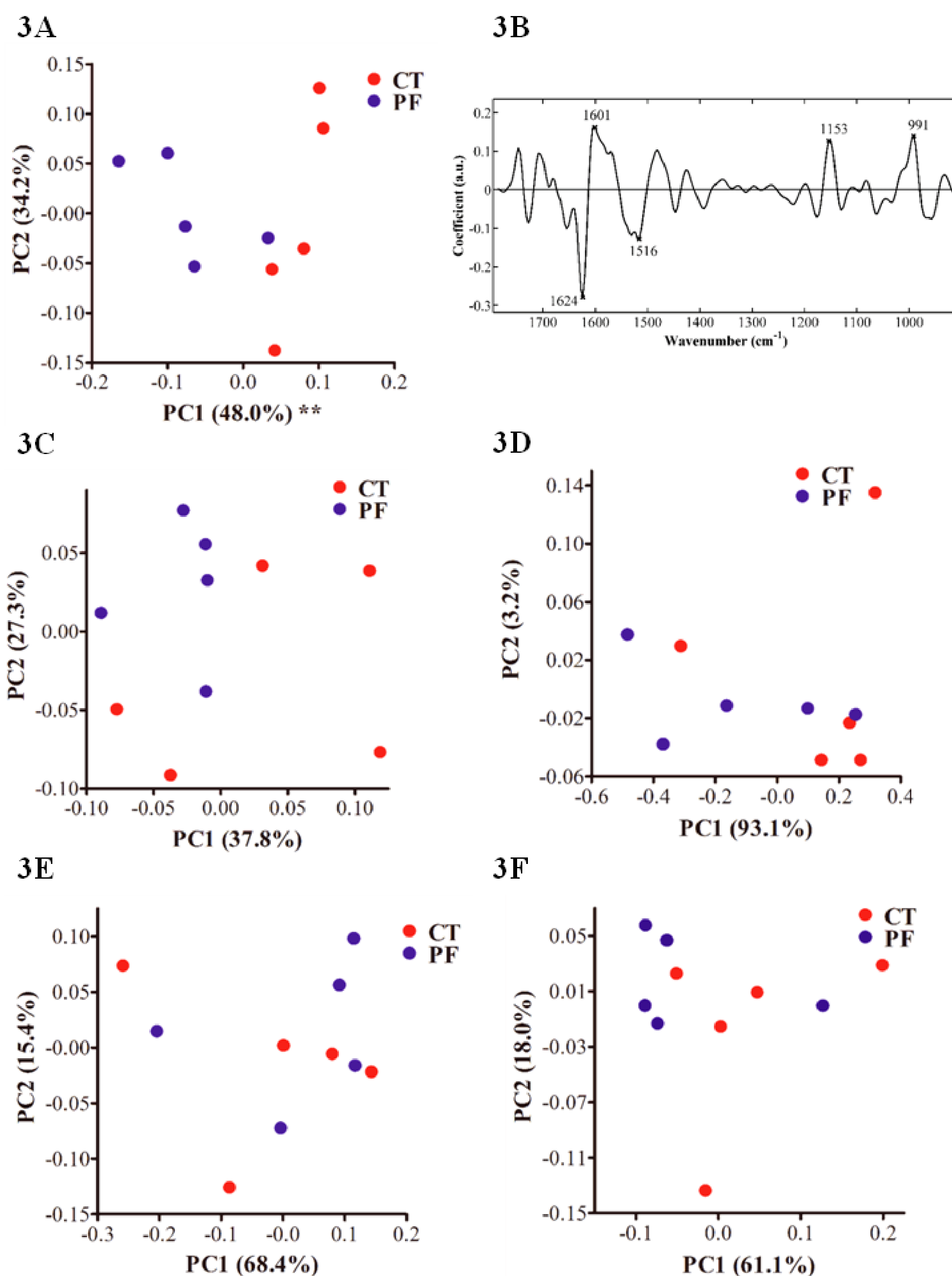



**Figure 3.** Two-dimensional scores plots and significant loadings following principal components analysis (PCA) of ATR-FTIR spectra obtained from several different tissues taken from *Rana temporaria* pro-metamorphic tadpoles. Tissues are liver **(A: scores, B: loadings)**, muscle **(B)**, heart **(C)**, kidney **(D)** and skin **(E)**. Tadpoles were collected in 2012 from CT: a rural agricultural pond with no pesticide input or PF: an urban pond impacted by wastewater and landfill run-off ($n$ = 10). Two sample $t$-tests were employed to detect differences in the PC scores between ponds within each year. Asterisks indicate a $P$-value of <0.05 (*) or <0.01 (**). Values in parentheses show the contribution of each principal component to the overall variance.

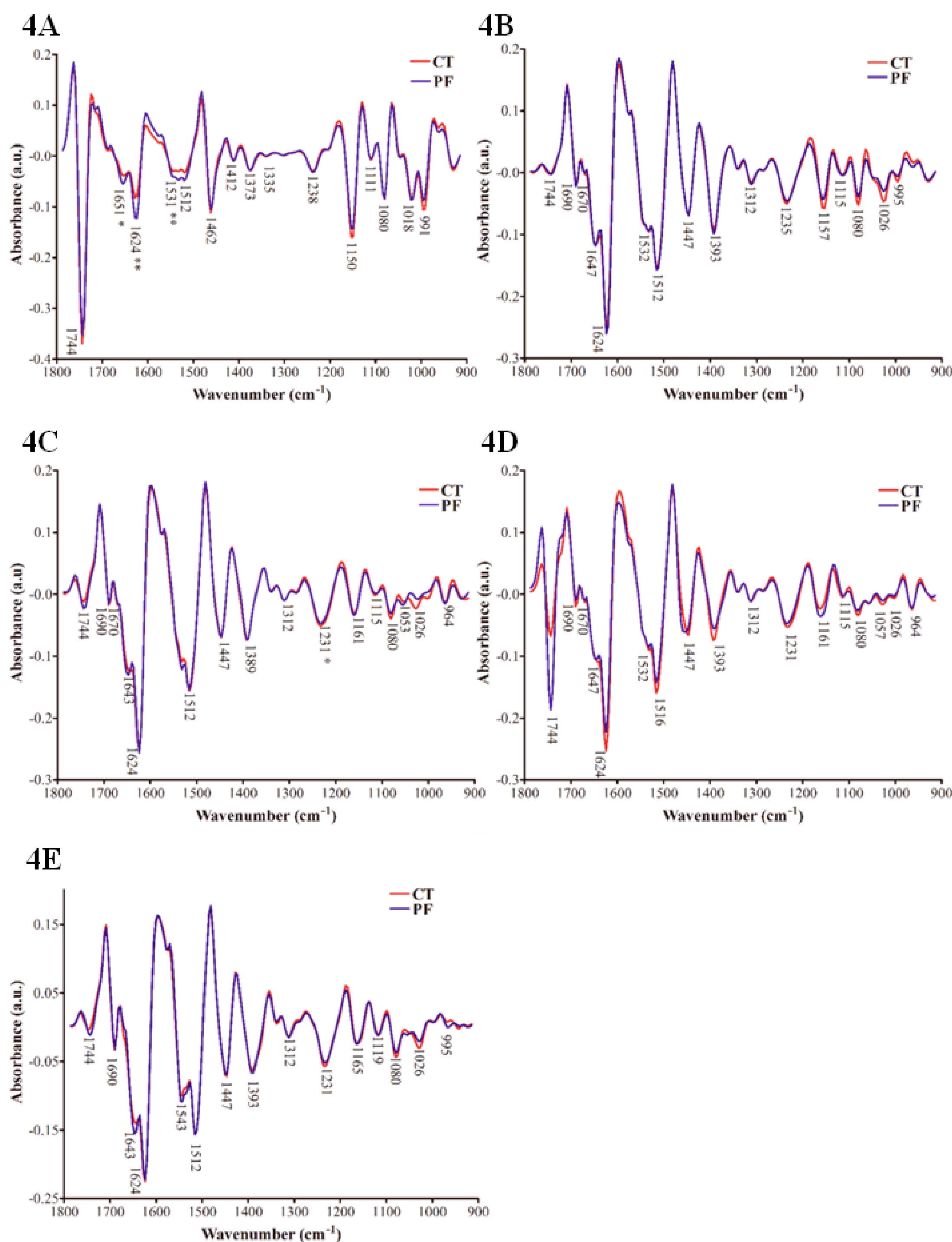


**Figure 4.** Second derivative mean spectra of tissues taken from *Rana temporaria* pro-metamorphic tadpoles. Spectra were cut at the biochemical fingerprint region (1800-900 $cm^{-1}$), processed with Savitzky-Golay second-order differentiation and vector-normalised. Tissues are liver **(A)**, muscle **(B)**, heart **(C)**, kidney **(D)** and skin **(E)**. Tadpoles were collected in 2012, from CT: a rural agricultural pond with no pesticide input or PF: an urban pond impacted by wastewater and landfill run-off ($n$=10). Peaks are labelled with the corresponding wavenumbers. Two sample $t$-tests were employed to detect differences in the second derivative peak height at each labelled peak between ponds within each year. Asterisks indicate a $P$-value of <0.05 (*) or <0.01 (**).

Results from PCA demonstrated that tadpole livers from CT (2013) segregated from tadpole livers from WH (2013) along PCs 1 and 4 (Fig. 5A); the major loadings accounting for this separation were in regions assigned as C-O ribose, (988-991 $cm^{-1}$), glycogen (1022 $cm^{-1}$), symmetric phosphate stretching vibrations (1080 $cm^{-1}$), Amide I (1616, 1624, 1639 and 1697 $cm^{-1}$) and stretching of triglycerides (1744 $cm^{-1}$), as shown in Fig. 6A and Table S3 (see ESI). Supervised analysis with PCA-LDA showed an improvement in the separation of the data in the scores plot with a high classification accuracy of 98% and 100% for CT and WH respectively, as shown in Fig. S2A and Table S4 in ESI. Loadings associated with this separation were again in regions assigned as carbohydrates, proteins and lipids (Table S6 in ESI). Analysis of the second derivative peak heights showed larger peak heights in regions associated with proteins (both Amide I and II) and symmetric phosphate stretching vibrations in tadpole livers from WH (2013) in comparison to CT (2013); however, in regions associated with lipids, peak heights were larger in tadpole livers from CT (2013) (Table 3 and Fig. 7A).

Lipid levels are generally low in pre-metamorphic tadpoles, rising during pro-metamorphosis, as lipids are the main energy source metabolised during metamorphic climax (Sheridan and Kao, 1998). As the tadpoles in this study were at the pro-metamorphic stage of development (emergence of hindlimbs), it was expected that a clear lipid peak would be present in the liver (Figs. 4A and 7A, see ESI Figs. S3A and S4A). Previous studies have demonstrated changes in lipid levels in the livers of tadpoles and adult amphibians exposed to pesticides, with some reporting a decrease (Dornelles and Oliveira, 2014; Gurushankara et al., 2007), while others report an increase (Melvin et al., 2013) or no change (Zaya et al., 2011). Although no differences in hepatic lipid levels were detected between tadpoles collected in 2012 from CT and PF, tadpoles collected in 2013 from WH had significantly lower levels of hepatic lipid than those from CT in the same year. This coupled with the finding that tadpoles from WH had significantly larger livers than those from CT is suggestive of exposure to an environmental stressor, which may have resulted in the tadpoles using the lipid stored in the liver as an energy source to overcome the noxious stimuli and maintain homeostasis.

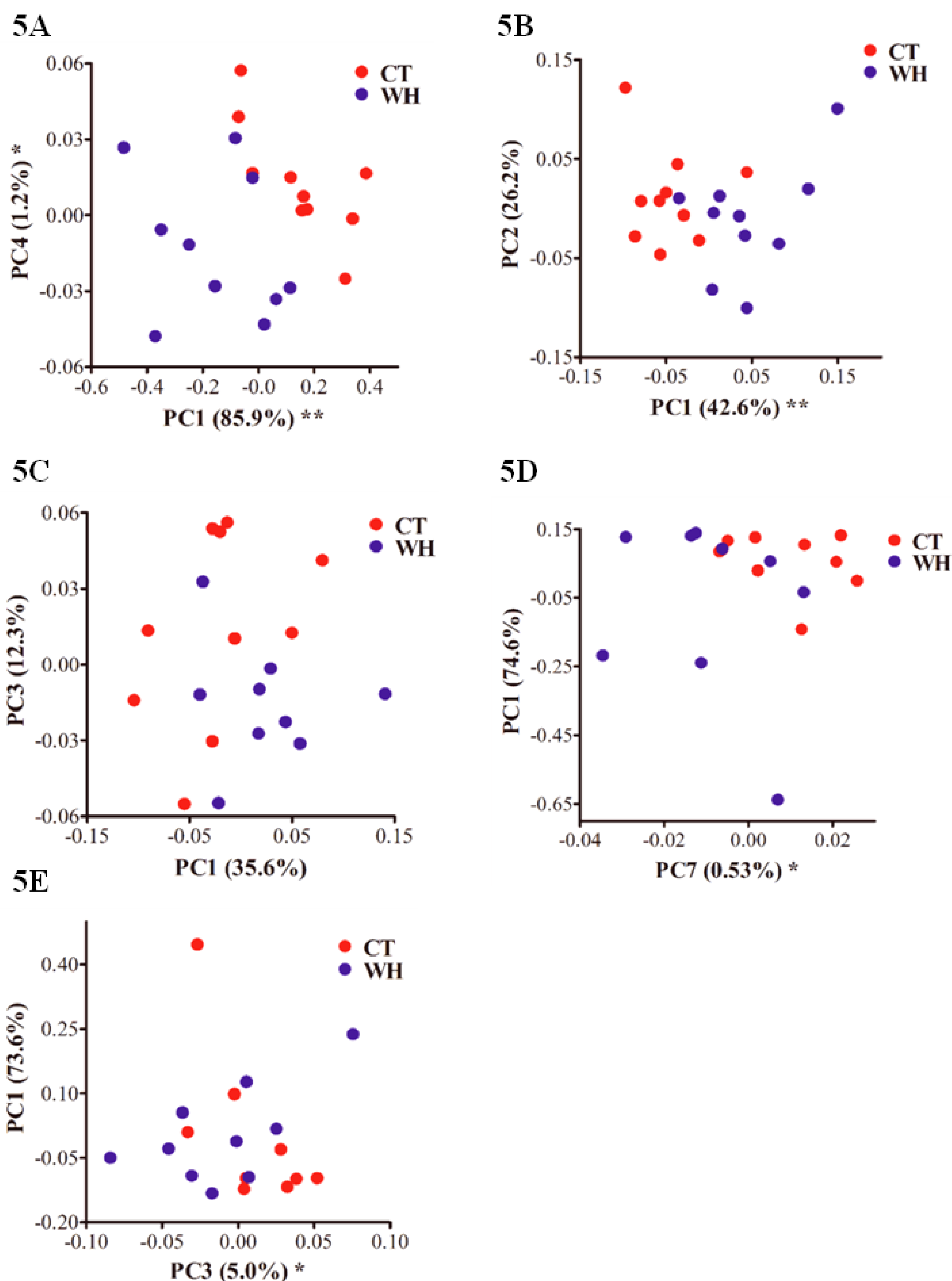



**Figure 5.** Two-dimensional scores plots following principal components analysis (PCA) of ATR-FTIR spectra obtained from several different tissues taken from *Rana temporaria* pro-metamorphic tadpoles. Tissues are liver **(A)**, muscle **(B)**, heart **(C)**, kidney **(D)** and skin **(E)**. Tadpoles were collected in 2013 from CT: a rural agricultural pond with no pesticide input or WH: an agricultural pond known to be impacted by pesticides ($n$=20). Two sample $t$-tests were employed to detect differences in the PC scores between ponds within each year. Asterisks indicate a $P$ value of <0.05 (*) or <0.01 (**). Values in parentheses show the contribution of each principal component to the overall variance.

Glycogen levels may be altered in amphibian livers exposed to environmental stress due to contaminant exposure or hypoxia (Gendron et al., 1997; Loumbourdis and Kyriakopoulou-Sklavounou, 1991); levels may decrease as the organism utilises this energy source in order to overcome the stressful situation. Results from PCA demonstrated separation between tadpole livers from CT (2012) and those from PF (2012) along PC1, with one of the largest identified loadings associated with carbohydrates including glycogen (1153 $cm^{-1}$). Separation along PC4 between tadpoles from CT (2013) and those from WH (2013) also had some contribution from glycogen (1022 $cm^{-1}$).

Protein levels were also found to be altered in tadpole livers collected from CT (2012) in comparison to those from PF (2012), and between tadpole livers from CT (2013) and those from WH (2013), with the finding that tadpoles from CT had lower protein levels than those from the other two sites. This is unexpected as CT is considered to have the best water quality based on the analysis conducted in this study. Reduced protein levels have previously been associated with pesticide exposure/hypoxia in amphibian livers (Dornelles and Oliveira, 2014). However, increased protein levels have also been associated with pesticide exposure in the livers of fish, with the suggestion that higher protein synthesis is initiated to compensate for protein loss, leading to a higher protein turnover (Oruç and Üner, 1999).

**Muscle samples**

No significant differences were detected between tadpole muscle samples from CT (2012) and those from PF (2012) following analysis with either PCA or PCA-LDA (Figs. 3C, 4B and S1B in ESI). In contrast, the comparison of muscle tissue from tadpoles collected from CT (2013) and WH (2013) with PCA demonstrated separation along PC1 (Fig. 5B) in regions associated with the $OCH_3$ band of polysaccharides (972 $cm^{-1}$) glycogen (1022 $cm^{-1}$), C-O stretching of the phosphodiester and ribose (1065 $cm^{-1}$), carbohydrates (1154 $cm^{-1}$) and Amide II (1501 $cm^{-1}$), as shown in the loadings plot in Figure 6B and Table S3 (see ESI). Analysis with PCA-LDA led to some improvement in the separation of the data in the scores plot, with a reasonable classification accuracy of 71% and 80% for tadpoles from CT and WH respectively (Fig. S2B and Tables S4 and S5 in ESI). Loadings confirmed separation based upon changes in the phosphodiester and protein regions, with additional contributions from

lipids (Table S6 in ESI). Second derivative peak heights show greater absorbance in muscle samples from CT (2013) in regions associated with glycogen, carbohydrates, symmetric phosphate and Amide I and II; in regions associated with asymmetric phosphate stretching vibrations, and Amide III, peaks heights were larger in tadpole muscle samples from WH (2013) (Fig. 7B, Table 3).

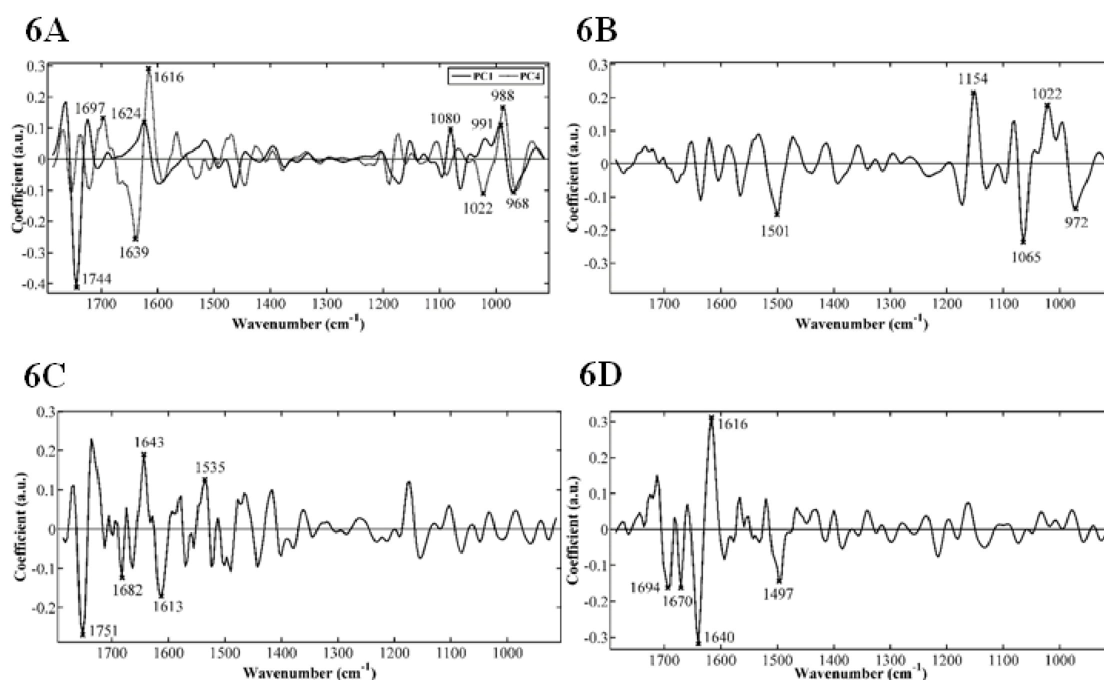



**Figure 6.** Loadings plots following PCA of ATR-FTIR spectra obtained from several different tissues taken from *Rana temporaria* pro-metamorphic tadpoles. **A**: Liver; **B**: Muscle; **C**: Kidney; **D**: Skin. Ponds are as follows: CT: a rural agricultural pond with no pesticide input; WH: an agricultural pond known to be impacted by pesticides.

Lower levels of both glycogen and protein have previously been found in muscle samples from tadpoles exposed to pesticides (Dornelles and Oliveira, 2014). Reduced glycogen levels in muscle tissues have also been associated with pesticide-induced stress in several species of fish, where glycogenolysis and glycolysis occur in order to provide more energy so that the organism can overcome stressful stimuli (Ferrando and Andreu-Moliner, 1991; Gluth and Hanke, 1985; Oruç and Üner, 1999).

**Other Tissues: heart, kidney and skin**

Differences between the other tissues analysed: heart, kidney and skin were small in comparison to the differences in liver and muscle tissue. Whilst analysis with PCA

showed no significant differences between hearts from tadpoles collected from CT (2012) and those from PF (2012) (Fig. 3D), the use of PCA-LDA led to an improvement in data separation, as shown in Fig. S1C, and Tables S4 and S5 in the ESI. The largest loadings values accounting for the separation were in regions associated with symmetric phosphate stretching vibrations (1088 $cm^{-1}$) as well as carbohydrates (1138 $cm^{-1}$) and collagen (1196 $cm^{-1}$), as shown in Table S6 (see ESI). Analysis of the second derivative peak heights revealed a significant difference at the peak associated with asymmetric phosphate stretching; where it was larger at CT (2012) than PF (2012) as shown in Figure 4C. Tadpole hearts collected from CT (2013) and WH (2013) demonstrated some separation along PC3 following PCA (Fig. 5C), but this was not statistically significant ($P$ =0.06); however a significant improvement in data separation was seen when PCA-LDA was employed, with a classification accuracy of 76% and 71% for tadpoles from CT and WH respectively (Fig S2C and Table S4 and S5 in ESI) Loadings values confirmed the separation in regions assigned as collagen and protein (Amide I) as shown in Table S6 (see ESI). Analysis of the second derivative peak heights demonstrated significant differences between CT (2013) and WH (2013) in the region associated with $CH_3$ bending of lipids, where peak height was smaller at CT than WH, and in the Amide I region, where the peak height was larger at CT in comparison to WH (Fig 7C, Table 3). Previous work in fish has also shown differences in heart tissue in fish collected from polluted rivers, in regions associated with Amide I and lipids, as measured with ATR-FTIR spectroscopy (Obinaju et al., 2014). Whilst no previous spectroscopic measurements of tadpole hearts have been published, it is known that cardiac output in tadpoles may be altered in response to stressful situations induced by xenobiotics (Costa et al., 2008). Future work could attempt to correlate differences in cardiac output with the spectral signature.

No significant differences were found in the spectral signature of tadpole kidney samples collected from CT (2012) or PF (2012) when analysed with either PCA or PCA-LDA (Figs. 3E and 4D, Fig. S1D in ESI). However, PCA revealed significant separation between kidney samples from tadpoles from CT (2013) and those from WH (2013) as shown in Fig. 5D. Loadings from PCA revealed that these differences were attributable to protein (Amide I and II) and lipid alterations (Fig. 6C, see ESI Table S2).

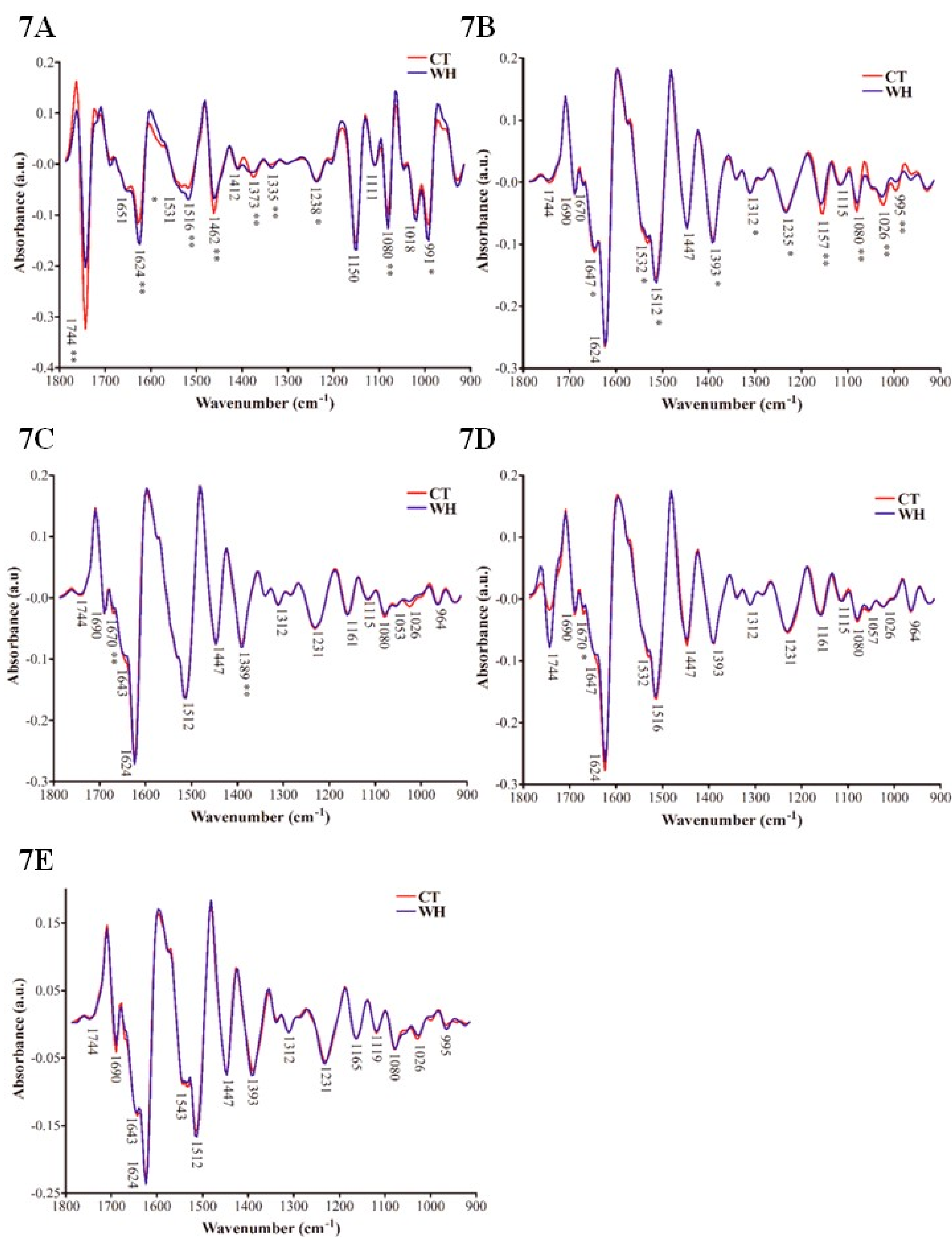



**Figure 7.** Second derivative mean spectra of tissues taken from *Rana temporaria* pro-metamorphic tadpoles. Spectra were cut at the biochemical fingerprint region (1800-900 cm$^{-1}$), processed with Savitzky-Golay second-order differentiation and vector-normalised. Tissues are liver **(A)**, muscle **(B)**, heart **(C)**, kidney **(D)** and skin **(E)**. Tadpoles were collected in 2013, from CT: a rural agricultural pond with no pesticide input or WH: an agricultural pond known to be impacted by pesticides ($n$=20). Peaks are labelled with the corresponding wavenumbers. Two sample $t$-tests were employed to detect differences in the second derivative peak height at each labelled peak between ponds within each year. Asterisks indicate a $P$-value of <0.05 (*) or <0.01 (**).

Analysis with PCA-LDA actually led to poorer data separation, as shown in Fig S2D and Tables S4 and S4 in ESI, which may occur when working with small datasets, as in this study (Martínez and Kak, 2001). Second derivative peak analysis also confirmed alterations associated with Amide I/stretching of fatty acids at 1670 $cm^{-1}$, with kidney samples from CT having a larger peak height than those from WH (Fig 7D, Table 3). The kidney, like the liver is susceptible to the effects of several toxicants, with a previous study in amphibians finding differences in the structure and histochemistry of kidney samples from adult frogs collected from polluted compared to unpolluted sites (Fenoglio et al., 2011). Alterations in the kidneys of fish from polluted sites have previously been detected using ATR-FTIR spectroscopy; these were also in regions associated with Amide I and II of proteins, as in this study; however, no alterations in lipids were detected, in contrast to that found in this study (Obinaju et al., 2014).

There were no differences detected in the tadpole skin samples from CT (2012) in comparison to those from PF (2012) when the data were analysed with PCA, PCA-LDA or using the peak absorbances (Fig. 3F and 4E, Table 3, Fig. S1E; Tables S4 and S5 in ESI). In contrast, skin samples taken from tadpoles from CT (2013) and WH (2013) showed some separation along PC3 following PCA (Fig. 5E); this was mainly in regions associated with Amide I (1616, 1640 $cm^{-1}$), with some contribution from lipids (1497, 1694 $cm^{-1}$), as shown in the loadings plot in Fig. 6D and Table S3 (see ESI). The use of PCA-LDA led to improved data separation as shown in the scores plot in Fig. S2E and Tables S4 and S5, associated with Amide I proteins as before, with some contributions from collagen and C-O stretching of carbohydrates (Table S6 in ESI). Analysis of second derivative peak heights showed no significant differences between skin samples from CT (2013) and WH (2013) (Fig. 7E, Table 3). That some separation was apparent between skin samples is of note, given that the skin is the first organ that environmental contaminants come into contact with in amphibian species. The skin of amphibians is permeable to water, where it plays a vital role in respiration and osmoregulation; therefore the skin provides a significant exposure route to chemicals in addition to that from ingestion and has previously been proposed as a bioindicator of deleterious environmental conditions, with structural changes detected following exposure to environmental contaminants (Bernabò et al., 2013; Fenoglio et al., 2009; Fenoglio et al., 2006; Haslam et al., 2014). The skin of larval

amphibians may also be more susceptible to chemical insult than that of adults due to the lack of specialised cells and many of the detoxifying enzymes, which are present in adults (Fenoglio et al., 2009).

**Table 3.** Wavenumbers and assigned bands of infrared peaks following ATR-FTIR analysis of several organs of pro-metamorphic *Rana temporaria* tadpoles. Absorbance values of second derivatives were compared between CT: a rural agricultural pond with no pesticide input and PF: an urban pond impacted by wastewater and landfill run-off in 2012 and between CT and WH: an agricultural pond known to be impacted by pesticides in 2013.

| Tissue | Wavenumber ($cm^{-1}$) | Proposed Assignment [a] | CT vs. PF (2012) | CT vs. WH (2013) |
|---|---|---|---|---|
| **Liver** | 991 | C-O ribose[1] | NS | CT < WH * |
| | 1018 | Glycogen[1] | NS | NS |
| | 1080 | $PO_2$− symmetric stretching: nucleic acids and phospholipids [2, 3] C-O stretch: glycogen [2, 3] | NS | CT < WH ** |
| | 1111 | *v*(CO), *v*(CC) ring (polysaccharides, cellulose) [1] | NS | NS |
| | 1150 | CO-O-C asymmetric stretching: glycogen and nucleic acids [2, 3] | NS | NS |
| | 1238 | $PO_2$- asymmetric stretch: mainly nucleic acids with the little contribution from phospholipids [2, 3] | NS | CT < WH * |
| | 1335 | δ(CH), ring (polysaccharides, pectin) | NS | CT < WH ** |
| | 1373 | Deformation N-H, C-H [1] | NS | CT > WH ** |
| | 1412 | COO symmetric stretch: fatty acids and amino acids [4] | NS | NS |
| | 1462 | $CH_2$ bending of lipids [2, 3] | NS | CT > WH ** |
| | 1516 | Amide II [1] | NS | CT < WH ** |
| | 1531 | Amide II [1] | CT < PF ** | CT < WH ** |
| | 1624 | Amide I *β*-sheets [5] | CT < PF ** | CT < WH ** |
| | 1651 | Amide I protein α-helix [2, 3, 5] | CT < PF * | NS |
| | 1744 | Ester C-O stretch: triglycerides, cholesterol esters [2, 3] | NS | CT > WH ** |
| **Muscle** | 995 | C-O ribose, C-C [1] | NS | CT > WH** |
| | 1026 | Glycogen [1] | NS | CT > WH ** |
| | 1080 | $PO_2^-$ symmetric stretch: nucleic acids and phospholipids C–O stretch: glycogen [6] | NS | CT > WH ** |
| | 1115 | Symmetric stretching P-O-C [1] | NS | NS |
| | 1157 | C-O stretching of protein and carbohydrates [1] | NS | CT > WH ** |
| | 1235 | $PO_2^-$ asymmetric stretch: mainly nucleic acids with little contribution from phospholipids [6] | NS | CT < WH * |
| | 1312 | Amide III of proteins [1] | NS | CT < WH * |
| | 1393 | $COO^-$ symmetric stretch: fatty acids and amino acids [6] | NS | CT < WH * |

| | | | | |
|---|---|---|---|---|
| | 1447 | $CH_2$ bending mainly lipids [6] | NS | NS |
| | 1512 | Amide II, C-H bending [1] | NS | CT < WH * |
| | 1532 | Amide II stretching C=N, C=C [1] | NS | CT > WH * |
| | 1624 | Amide I $\beta$-sheets [5] | NS | NS |
| | 1647 | Amide I [1] | NS | CT > WH * |
| | 1670 | Amide I (anti-parallel $\beta$-sheet) $v$(C=C) trans, lipids, fatty acids [1] | NS | NS |
| | 1690 | Peak of nucleic acid due to ring breathing mode and base carbonyl stretching [1] | NS | NS |
| | 1744 | C=O stretching lipids [1, 4] | NS | NS |
| **Heart** | 964 | C-O deoxyribose, C-C [1] | NS | NS |
| | 1026 | Glycogen [1] | NS | NS |
| | 1053 | $V$ C-O and $\delta$ C-O of carbohydrates [1] | NS | NS |
| | 1080 | $PO_2^-$ symmetric stretching: nucleic acids and phospholipids [7] C-O stretch: glycogen [2, 3] | NS | NS |
| | 1115 | Symmetric stretching P-O-C [1] | NS | NS |
| | 1161 | C-O asymmetric stretching of glycogen [7, 8] | NS | NS |
| | 1231 | $PO_2^-$ asymmetric stretching: phospholipids, nucleic acids [2] | CT > PF * | NS |
| | 1312 | Amide III band of proteins [1] | NS | NS |
| | 1389 | $CH_3$ bending: lipids [7] | NS | CT < WH ** |
| | 1447 | $CH_2$ bending mainly lipids [6] | NS | NS |
| | 1512 | Amide II, C-H bending [1] | NS | NS |
| | 1624 | Amide I $\beta$-sheets [5] | NS | NS |
| | 1643 | Amide I, C=O stretching vibrations [1] | NS | NS |
| | 1670 | Amide I (anti-parallel $\beta$-sheet) $v$(C=C) trans, lipids, fatty acids [1] | NS | CT > WH ** |
| | 1690 | Peak of nucleic acid due to ring breathing mode and base carbonyl stretching [1] | NS | NS |
| | 1744 | C=O stretching lipids [1, 4] | NS | NS |
| **Kidney** | 964 | C-O deoxyribose, C-C [1] | NS | NS |
| | 1026 | Glycogen [1] | NS | NS |
| | 1057 | C-O stretching, polysaccharides 8 | NS | NS |
| | 1080 | $PO_2$- symmetric stretching of nucleic acids | NS | NS |
| | 1115 | Symmetric stretching P-O-C [1] | NS | NS |
| | 1161 | C-O asymmetric stretching of glycogen [8] | NS | NS |
| | 1231 | $PO_2$- asymmetric stretching of mainly phospholipids [8] | NS | NS |
| | 1312 | Amide III band of proteins [1] | NS | NS |
| | 1393 | $COO^-$ symmetric stretch of fatty acids and amino acids [8] | NS | NS |
| | 1447 | Asymmetric CH3 bending of the methyl groups of proteins [1] | NS | NS |
| | 1516 | Amide II [1] | NS | NS |
| | 1532 | Amide II stretching C=N, C=C | NS | NS |

| | | | | |
|---|---|---|---|---|
| | 1624 | Amide I $\beta$-sheets [5] | NS | NS |
| | 1647 | Amide I [1] | NS | NS |
| | 1670 | Amide I (anti-parallel $\beta$-sheet)<br>$v$(C=C) trans, lipids, fatty acids [1] | NS | CT > WH * |
| | 1690 | Peak of nucleic acid due to ring breathing mode and base carbonyl stretching [1] | NS | NS |
| | 1744 | C=O stretching of lipids [1] | NS | NS |
| **Skin** | 964 | C-O deoxyribose, C-C [1] | NS | NS |
| | 1030 | Collagen [1]<br>$v$(CC), lipid cis [9] | NS | NS |
| | 1080 | $v$(CC), lipid trans [9] | NS | NS |
| | 1119 | $v$(CC), lipid trans [9] | NS | NS |
| | 1165 | $v$(CC), $\delta$(COH) [9] | NS | NS |
| | 1231 | Amide III protein [1, 10] | NS | NS |
| | 1312 | Amide II protein [1] | NS | NS |
| | 1393 | $\delta[C(CH_3)_2]$ symmetric [1,9] | NS | NS |
| | 1447 | $\delta[C(CH_3)_2]$ symmetric [1] | NS | NS |
| | 1512 | Amide II[1] [1] | NS | NS |
| | 1543 | Amide II [1] | NS | NS |
| | 1624 | $v$(C=O), amide I, $\beta$ [9] | NS | NS |
| | 1643 | Collagen [10]<br>$v$(C=O), amide I, $\alpha$ [9] | NS | NS |
| | 1690 | Amide I[1] [1] | NS | NS |
| | 1744 | Lipid [1] | NS | NS |

$v$: stretching; δ: deformation

[a] Sources 1. Movasaghi et al. (2008) 2. Cakmak et al. (2003) 3. Cakmak et al. (2006) 4. Abdel-Gawad et al. (2012) 5. Palaniappan et al. (2011) 6. Palaniappan et al. (2008) 7. Toyran et al. (2006) 8. Palaniappan et al. (2009). 9. Greve et al. 2008 10. Purna Sai et al. (2001). Asterisks denote significance at the $P$ <0.05 level (*), and $P$ <0.01 level (**). NS = not significant.

## Conclusions

ATR-FTIR spectroscopy is capable of detecting differences in a range of tissue samples from tadpoles of the Common frog collected from ponds with varying water quality and different types of environmental contamination. Interestingly, despite the unexpected finding that tadpoles from the urban pond were on average larger than those from the rural pesticide-free agricultural pond, the differences in tissues detected by ATR-FTIR spectroscopy were relatively small and mainly found in the liver. In contrast, the differences between tadpoles from the rural pesticide-free agricultural and pesticide-impacted agricultural pond were detected in multiple tissues, most notably the liver and muscle.

The liver was the organ that consistently distinguished tadpoles collected from the relatively unpolluted agricultural pond, and ponds with pollutants associated with

urban and agricultural activity. Tadpoles collected from the pesticide-impacted agricultural pond also had relatively larger livers and reduced lipid levels; a finding associated with exposure to environmental contaminants such as pesticides and other trace organic pollutants, although the effect of raised nutrient levels (such as nitrate and phosphate), possibly in synergy with other pollutants, needs to be investigated. Interactions with other factors such as food availability and predation may also affect these parameters; therefore any future study should attempt to control these conditions. Clear differences were also apparent in the muscle tissue of tadpoles from a pond with no pesticide input and those from a pond impacted by several pesticides. This finding was also apparent to a lesser extent in the kidney, heart and skin of these tadpoles.

This study is the first to characterise a range of tissues from an amphibian species with ATR-FTIR spectroscopy. Additionally, this study demonstrates the possible use of this technique as a rapid and cost-effective environmental monitoring tool. This technology could be of great promise as an early warning for assessing the health of amphibian populations exposed to varying or diminished water quality.

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# Electronic Supplementary Information

**Biospectroscopy reveals the effect of varying water quality on tadpole tissues of the Common Frog (*Rana temporaria*)**

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**Table S1.** Results from two-sample *t*-tests of the scores generated from principal component analysis for the first ten principal components following ATR-FTIR spectrochemical analysis of several tissues of *Rana temporaria* tadpoles. Significant results are highlighted in bold.

| PC | Liver *P*-value | | Muscle *P*-value | | Heart *P*-value | | Kidney *P*-value | | Skin *P*-value | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CT *vs.* PF | CT *vs.* WH | CT *vs.* PF | CT *vs.* WH | CT *vs.* PF | CT *vs.* WH | CT *vs.* PF | CT *vs.* WH | CT *vs.* PF | CT *vs.* WH |
| **1** | **< 0.01** | **< 0.01** | 0.25 | **< 0.01** | 0.20 | 0.10 | 0.18 | 0.17 | 0.62 | 0.51 |
| **2** | 0.89 | 0.38 | 0.31 | 0.33 | 0.15 | 0.25 | 0.65 | 0.36 | 0.49 | 0.25 |
| **3** | 0.20 | 0.61 | 0.86 | 0.55 | 0.58 | 0.06 | 0.20 | 0.15 | 0.21 | **0.03** |
| **4** | 0.95 | **0.04** | 0.29 | 0.69 | 0.81 | 0.59 | 0.98 | 0.64 | 0.40 | 0.26 |
| **5** | 0.51 | 0.08 | 0.19 | 0.19 | 0.22 | 0.15 | 0.18 | 0.99 | 0.57 | 0.18 |
| **6** | 0.77 | 0.58 | 0.32 | 0.71 | 0.82 | 0.70 | 0.66 | 0.28 | 0.08 | 0.36 |
| **7** | 0.61 | 0.87 | 0.19 | 0.22 | 0.95 | 0.95 | 0.21 | **0.02** | 0.70 | 0.71 |
| **8** | 1.00 | 0.26 | 0.94 | 0.87 | 0.95 | 0.75 | 0.39 | 0.23 | 0.17 | 0.12 |
| **9** | 0.83 | 0.63 | 0.56 | 0.93 | 0.51 | 0.18 | 0.58 | 0.21 | 0.78 | 0.76 |
| **10** | 0.87 | 0.50 | 0.50 | 0.61 | 0.70 | 0.78 | 0.50 | 0.89 | 0.41 | 0.57 |

**Table S2.** Number of principal components (PCs) retained for input into a cross-validated linear discriminant analysis (LDA) model for optimum discrimination and classification. The Pareto tool function in Matlab was used to determine the number of PCs that represented 95% of the variance in each data set.

| Tissue | Number of PCs | |
|---|---|---|
| | CT *vs.* PF 2012 | CT *vs.* WH 2013 |
| Heart | 14 | 17 |
| Kidney | 9 | 16 |
| Liver | 6 | 11 |
| Muscle | 11 | 16 |
| Skin | 11 | 18 |

**Table S3.** Distinguishing wavenumbers and proposed assignments obtained from analysis of several organs of pro-metamorphic *Rana temporaria* tadpoles with ATR-FTIR spectroscopy following analysis with principal component analysis (PCA). The five largest loadings values for the most discriminating principal components are shown. Comparisons were made between tadpoles from CT: a rural agricultural pond with no pesticide input and PF: an urban pond impacted by wastewater and landfill run-off in 2012 and between tadpoles from CT and those from WH: an agricultural pond known to be impacted by pesticides in 2013. Sources: (Abdel-Gawad et al., 2012; Cakmak et al., 2006; Cakmak et al., 2003; Greve et al., 2008; Movasaghi et al., 2008; Palaniappan and Vijayasundaram, 2008, 2009; Palaniappan et al., 2011; Purna Sai and Babu, 2001; Toyran et al., 2006).

| Site Comparison | Organ | Wavenumber ($cm^{-1}$) | Proposed Assignment |
|---|---|---|---|
| CT *vs.* PF 2012 PC1 | Liver | 991 | C-O ribose |
| | | 1153 | CO-O-C asymmetric stretching: glycogen and nucleic acids |
| | | 1516 | Amide II |
| | | 1601 | C=N cytosine |
| | | 1624 | Amide I $\beta$-sheets |
| CT *vs.* WH 2013 PC1 | Liver | 968 | C-O deoxyribose, C-C |
| | | 991 | C-O ribose |
| | | 1080 | $PO_2^-$ symmetric stretching: nucleic acids and phospholipids |
| | | 1624 | Amide I $\beta$-sheets |
| | | 1744 | Ester C-O stretch: triglycerides, cholesterol ester |
| CT *vs.* WH 2013 PC4 | | 988 | C-O ribose |
| | | 1022 | Glycogen |
| | | 1616 | Amide I |
| | | 1639 | Amide I |
| | | 1697 | Base region |
| CT *vs.* WH 2013 PC1 | Muscle | 972 | $OCH_3$ band of polysaccharides |
| | | 1022 | Glycogen |
| | | 1065 | C-O stretching of the phosphodiester and ribose |
| | | 1154 | Carbohydrates |
| | | 1501 | Amide II |
| CT *vs.* WH 2013 PC7 | Kidney | 1535 | Amide II stretching |
| | | 1613 | Amide I |
| | | 1643 | Amide I |
| | | 1682 | Amide I random coils |
| | | 1751 | Lipid |
| CT *vs.* WH 2013 PC3 | Skin | 1497 | Lipid |
| | | 1616 | Amide I |
| | | 1640 | Amide I |
| | | 1670 | Amide I (antiparallel $\beta$-sheet) $\nu$(C=C) *trans*, lipids, fatty acids |
| | | 1694 | Amide I (antiparallel $\beta$-sheet) |

**Table S4.** Results from two-sample *t*-tests of the scores generated from principal component analysis-linear discriminant analysis (PCA-LDA) for the first linear discriminant (LD1) following ATR-FTIR spectrochemical analysis of several tissues of *Rana temporaria* tadpoles. Significant results are highlighted in bold.

| **Tissue** | ***P* value** | |
|---|---|---|
| | **CT *vs.* PF 2012** | **CT *vs.* WH 2013** |
| Heart | **0.03** | **< 0.001** |
| Kidney | 0.57 | 0.39 |
| Liver | **0.02** | **< 0.001** |
| Muscle | 0.81 | **< 0.001** |
| Skin | 0.54 | **0.01** |

**Table S5.** Results of classification accuracy (± standard deviation) for ATR-FTIR spectra of several tissues of *Rana* temporaria tadpoles with principal component analysis-linear discriminant classifier (PCA-LDC).

| **Tissue** | **Classification Rate (%)** | | | |
|---|---|---|---|---|
| | **CT (2012)** | **PF (2012)** | **CT (2013)** | **WH (2013)** |
| Heart | 68.40 ± 22.20 | 64.40 ± 32.81 | 76.00 ± 26.33 | 71.11 ± 36.89 |
| Kidney | 60.40 ± 37.41 | 44.00 ± 37.84 | 68.00 ± 31.20 | 51.11 ± 38.73 |
| Liver | 99.20 ± 1.79 | 90.00 ± 15.14 | 98.00 ± 6.32 | 100 ± 0 |
| Muscle | 34.98 ± 24.97 | 64.80 ± 39.44 | 71.00 ± 34.79 | 80.00 ± 25.39 |
| Skin | 55.20 ± 16.83 | 54.40 ± 23.60 | 66.00 ± 26.33 | 66.67 ± 34.64 |

**Table S6.** Distinguishing wavenumbers and proposed assignments obtained from analysis of several organs of pro-metamorphic *Rana temporaria* tadpoles with ATR-FTIR spectroscopy following analysis with principal component analysis-linear discriminant analysis (PCA-LDA). The five largest loadings values are shown. Comparisons were made between tadpoles from CT: a rural agricultural pond with no pesticide input and PF: an urban pond impacted by wastewater and landfill run-off in 2012 and between tadpoles from CT and those from WH: an agricultural pond known to be impacted by pesticides in 2013. Sources: (Abdel-Gawad et al., 2012; Cakmak et al., 2006; Cakmak et al., 2003; Greve et al., 2008; Movasaghi et al., 2008; Palaniappan and Vijayasundaram, 2008, 2009; Palaniappan et al., 2011; Purna Sai and Babu, 2001; Toyran et al., 2006).

| Site comparison | Organ | Wavenumber ($cm^{-1}$) | Proposed Assignment |
|---|---|---|---|
| CT *vs*. PF 2012 | Liver | 1115 | Symmetric stretching P-O-C |
| | | 1138 | Carbohydrates |
| | | 1192 | Phosphodiester stretching |
| | | 1624 | Amide I $\beta$-sheets |
| | | 1732 | C=O stretch lipids |
| CT *vs*. PF 2012 | Heart | 941 | Phosphodiester region |
| | | 1088 | Symmetric phosphate stretching vibrations |
| | | 1138 | Carbohydrates |
| | | 1196 | Collagen |
| | | 1273 | CH rocking |
| CT *vs*. WH 2013 | Liver | 1161 | Stretching vibrations of hydrogen-bonding C-OH groups |
| | | 1454 | $CH_2$ bending of lipids with some protein contribution |
| | | 1651 | Amide I |
| | | 1690 | Peak of nucleic acids due to carbonyl stretching |
| | | 1724 | C=O stretching of fatty acid esters |
| CT *vs*. WH 2013 | Muscle | 988 | Phosphodiester region |
| | | 1111 | Symmetric stretching P-O-C |
| | | 1605 | DNA vibration |
| | | 1651 | Amide I |
| | | 1755 | C=C lipids |
| CT *vs*. WH 2013 | Heart | 1161 | Stretching vibrations of hydrogen-bonding C-OH groups |
| | | 1339 | Collagen |
| | | 1624 | Amide I $\beta$-sheets |
| | | 1647 | Amide I |
| | | 1697 | Base region |
| CT *vs*. WH 2013 | Skin | 1165 | C-O stretching of proteins and carbohydrates |
| | | 1327 | Collagen |
| | | 1354 | C-O stretching |
| | | 1578 | Base stretching |
| | | 1667 | Amide I (antiparallel $\beta$-sheet)<br>$\nu$(C=C) *trans*, lipids, fatty acids |

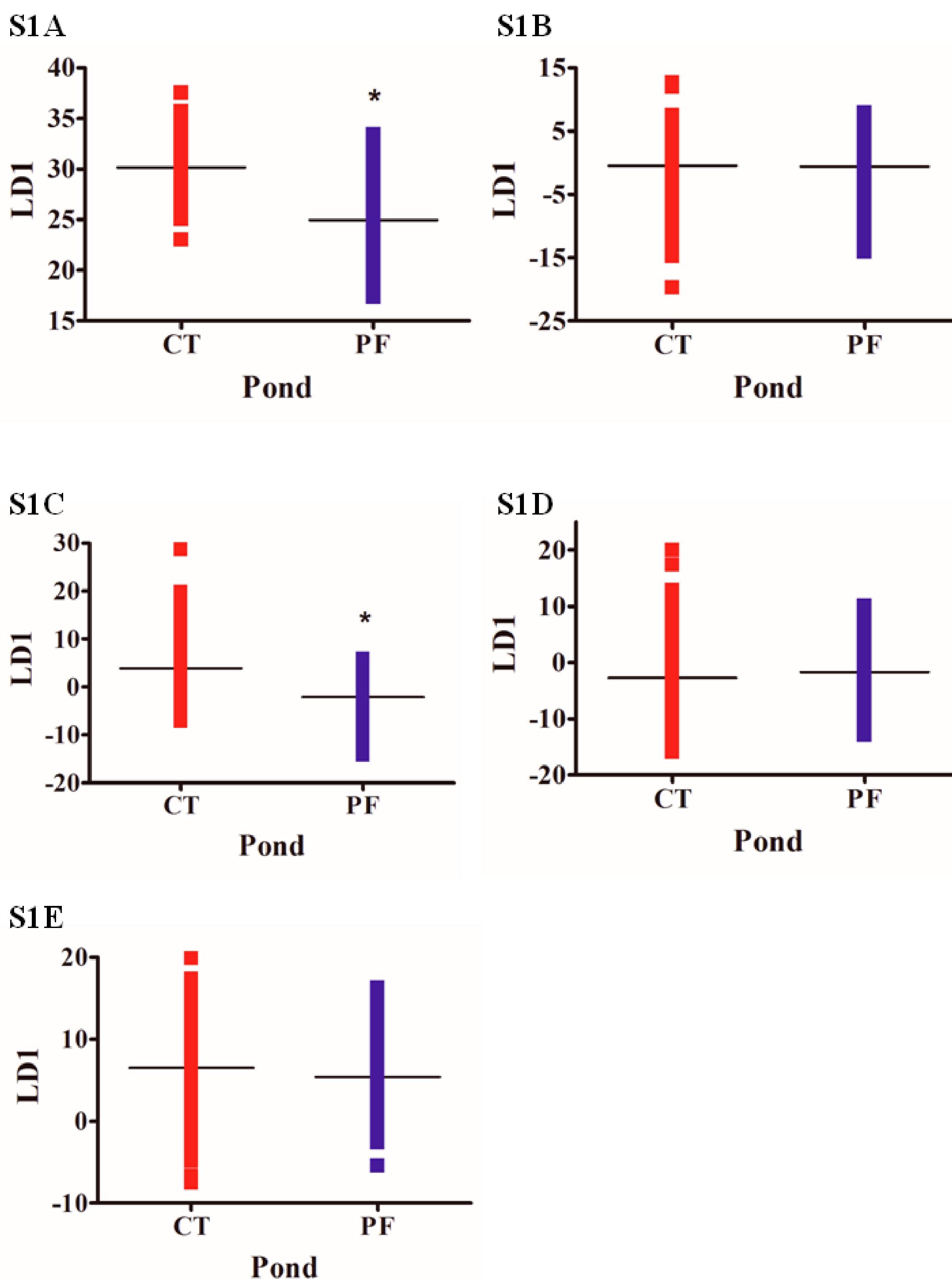


**Figure S1.** One dimensional scores plots following principal components analysis-linear discriminant analysis (PCA-LDA) of ATR-FTIR spectra obtained from several different tissues taken from *Rana temporaria* pro-metamorphic tadpoles. Tissues are liver **(A)**, muscle **(B)**, heart **(C)**, kidney **(D)** and skin **(E)**. Tadpoles were collected in 2012 from CT: a rural agricultural pond with no pesticide input or PF: an urban pond impacted by wastewater and landfill run-off ($n$=10). Two sample $t$-tests were employed to detect differences in the scores between ponds within each year. Asterisks indicate a $P$-value of <0.05 (*) or <0.01 (**).

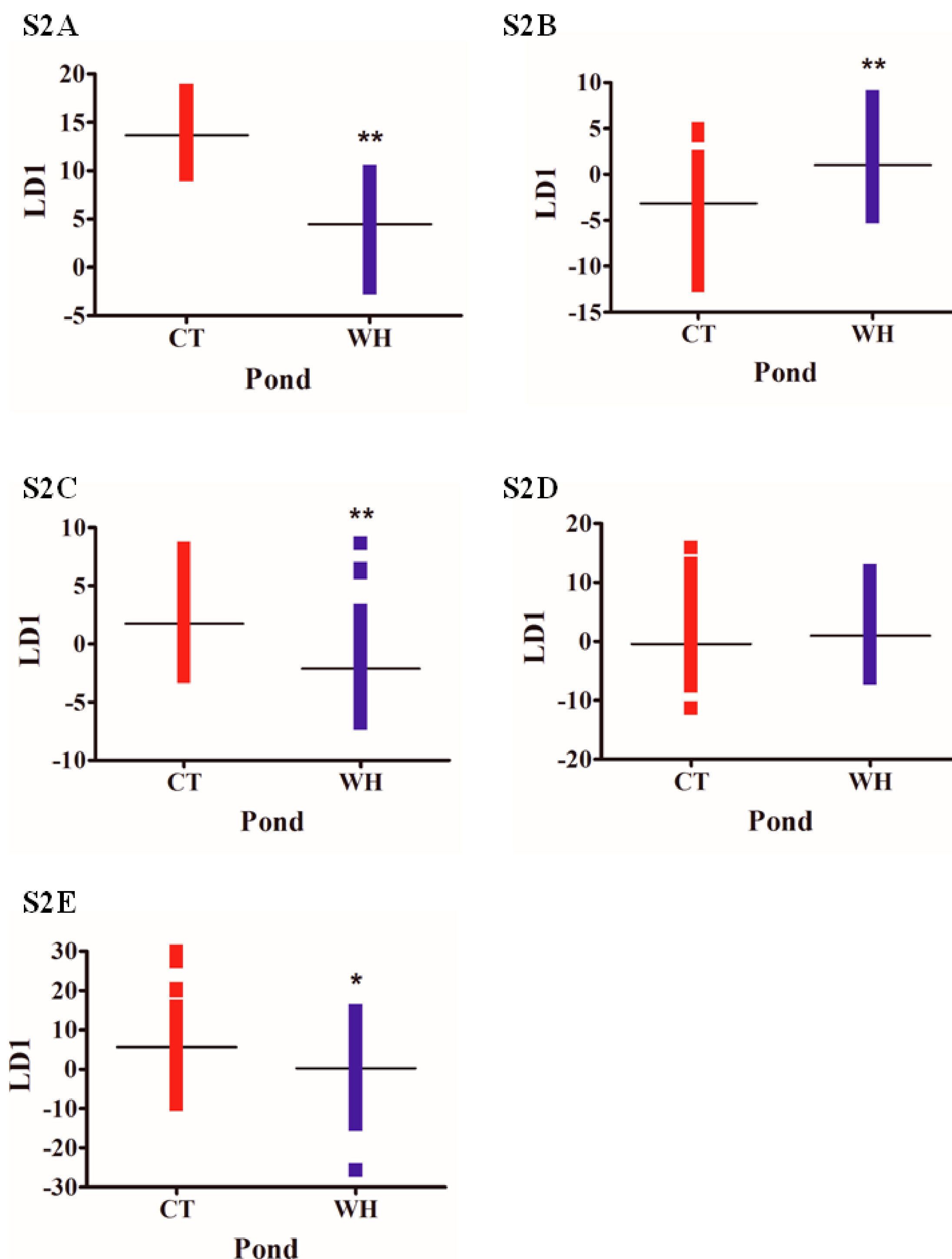


**Figure S2.** One dimensional scores plots following principal components analysis-linear discriminant analysis (PCA-LDA) of ATR-FTIR spectra obtained from several different tissues taken from *Rana temporaria* pro-metamorphic tadpoles. Tissues are liver **(A)**, muscle **(B)**, heart **(C)**, kidney **(D)** and skin **(E)**. Tadpoles were collected in 2013 from CT: a rural agricultural pond with no pesticide input or WH: an agricultural pond known to be impacted by pesticides ($n$=20). Two sample $t$-tests were employed to detect differences in the scores between ponds within each year. Asterisks indicate a $P$-value of <0.05 (*) or <0.01 (**).

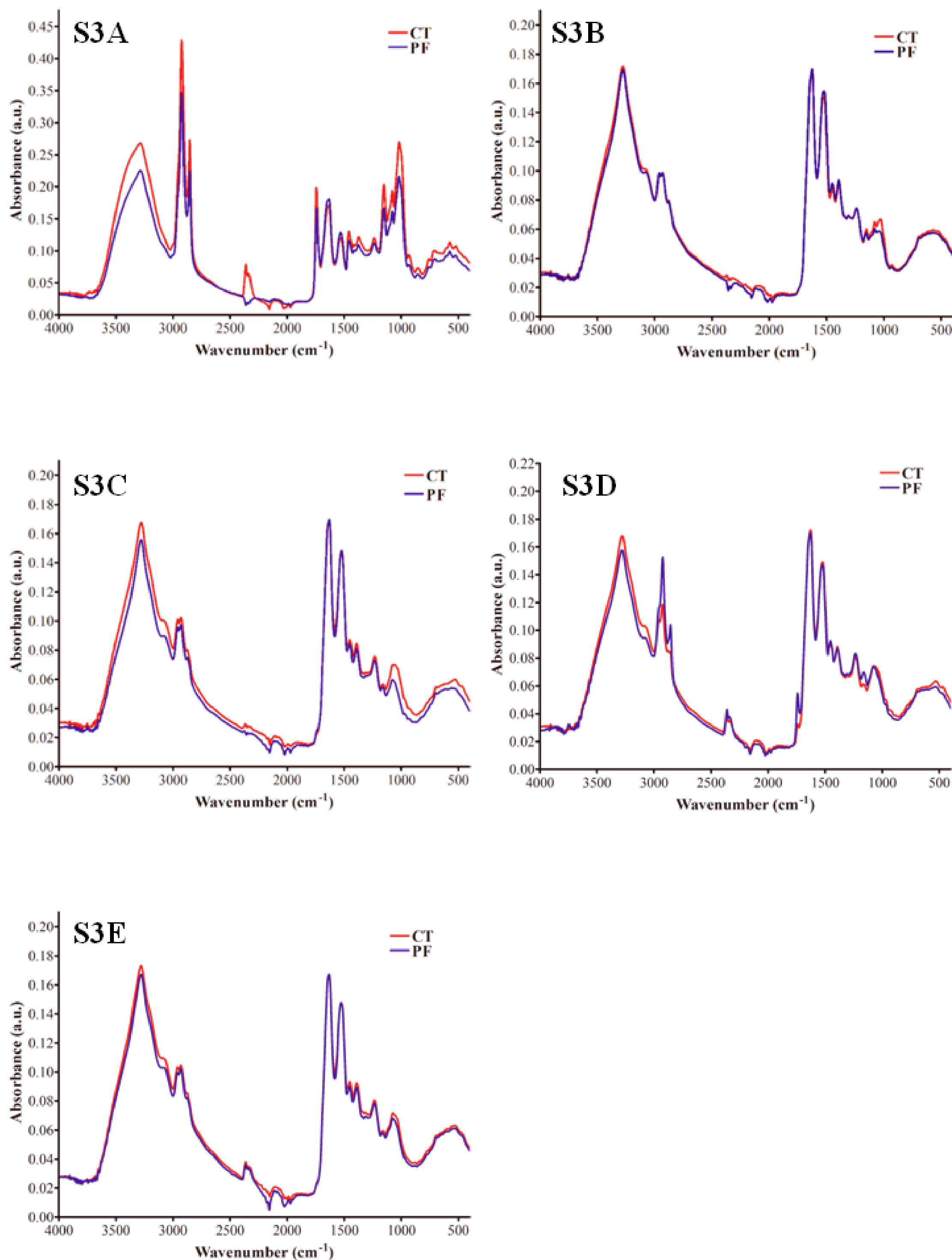


**Figure S3.** Raw mean ATR-FTIR spectra of tissue samples taken from *Rana temporaria* pro-metamorphic tadpoles. Tissues are liver **(A)**, muscle **(B)**, heart **(C)**, kidney **(D)** and skin **(E)**. Tadpoles were collected in 2012, from CT: a rural agricultural pond with no pesticide input or PF: an urban pond impacted by wastewater and landfill run-off (*n*=10).

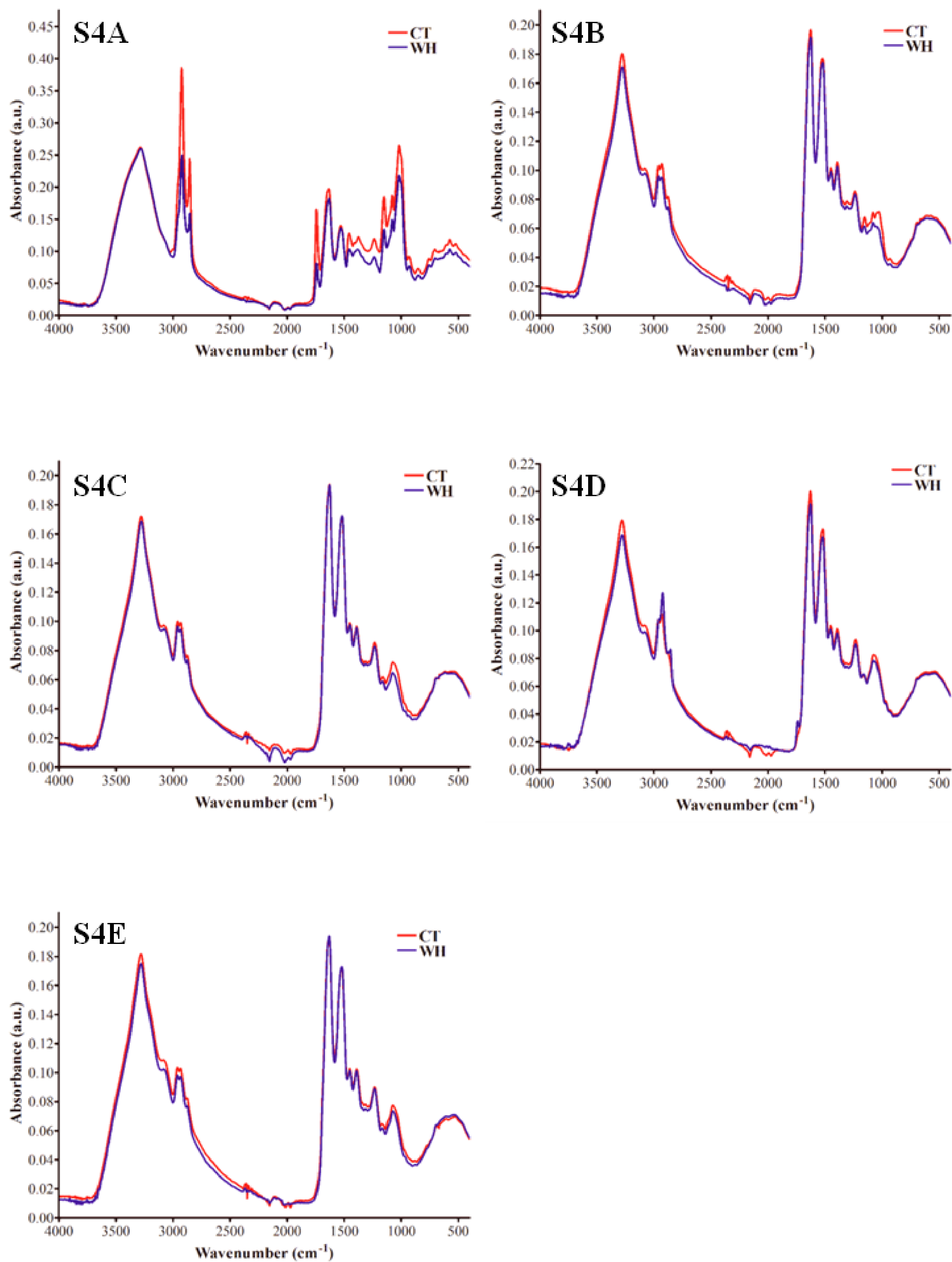


**Figure S4.** Raw mean ATR-FTIR spectra of tissue samples taken from *Rana temporaria* pro-metamorphic tadpoles. Tissues are liver **(A)**, muscle **(B)**, heart **(C)**, kidney **(D)** and skin **(E)**. Tadpoles were collected in 2013, from CT: a rural agricultural pond with no pesticide input or WH: an agricultural pond known to be impacted by pesticides ($n$=20).

# Chapter 5.

**Infrared spectroscopy detects changes in an amphibian cell line induced by fungicides: comparison of single and mixture effects.**

**Rebecca J. Strong,** Crispin J. Halsall, Kevin C. Jones, Richard F. Shore and Francis L. Martin.

**Aquatic Toxicology 178 (2016): 8-18.**

Contribution:

- I conducted all experiments for the study.
- I acquired the data and carried out all computational analysis.
- I prepared the first draft of the manuscript.

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Rebecca J. Strong Prof. Francis L. Martin

**Infrared spectroscopy detects changes in an amphibian cell line induced by fungicides: comparison of single and mixture effects**

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## Highlights

- IR spectroscopy applied to analyse the *Xenopus laevis* (A6) cell line.
- Effects of low concentrations of carbendazim or flusilazole determined.
- Alterations identified following single or binary exposures.
- A sensitive technique for examining environmentally-relevant levels of fungicides.
- A6 cells could be a useful model to identify agents that threaten amphibian health.

## Abstract

Amphibians are regarded as sensitive sentinels of environmental pollution due to their highly permeable skin and life cycle, which usually involves reproduction and development in the aquatic environment. Fungicides are widely applied agrichemicals and have been associated with developmental defects in amphibians, thus it is important to determine chronic effects of environmentally-relevant concentrations of such contaminants in target cells. Infrared (IR) spectroscopy has been employed to signature the biological effects of environmental contaminants through extracting key features in IR spectra with chemometric methods. Herein, the *Xenopus laevis* (A6) cell line was exposed to low concentrations of carbendazim (a benzimidazole fungicide) or flusilazole (a triazole fungicide) either singly or as a binary mixture. Cells were then examined using attenuated total reflection Fourier-transform IR (ATR-FTIR) spectroscopy coupled with multivariate analysis. Results indicated significant changes in the IR spectra of cells induced by both agents at all concentrations following single exposures, primarily in regions associated with protein and phospholipids. Distinct differences were apparent in the IR spectra of cells exposed to carbendazim and those exposed to flusilazole, confirming the different mechanisms of action. Exposure to binary mixtures of carbendazim and flusilazole also induced significant alterations in the IR spectra of cells, again in regions associated with phospholipids and proteins, but also in regions associated with DNA and carbohydrates, suggesting different alterations to the IR spectra of cells when the two agents are combined. Overall these findings demonstrate that IR spectroscopy is a sensitive technique for examining the effects of environmentally-relevant levels of fungicides at the cellular level. The combination of IR spectroscopy with the A6 cell line could serve as a useful model to identify agents that might threaten amphibian health in a rapid and high throughput manner.

**Keywords:** ATR-FTIR spectroscopy; carbendazim; flusilazole; *Xenopus laevis*; A6 cells

## Introduction

Large declines in amphibian populations have been reported since the 1990s (Houlahan et al., 2000; Stuart et al., 2004), with environmental pollution reported as a significant factor in these declines (Sparling et al., 2001). The life cycle of amphibians usually encompasses reproduction and early development in the aquatic environment, meaning that this group of organisms may be susceptible to run-off from agricultural sources, such as pesticide application, which is often coincident with this sensitive period of development (Hanlon and Parris, 2014; Hayes et al., 2006; Mann et al., 2009). These factors, in addition to the highly permeable skin of amphibians (Quaranta et al., 2009), mean that this group is considered to be a sentinel organism, indicative of early deterioration in environmental quality (Sparling et al., 2010).

Fungicides are widely used in agriculture in order to prevent and treat diseases in commercial crops such as wheat and soybean (Belden et al., 2010; McMullen et al., 2012). Two classes of fungicide frequently used in agricultural practice, either singly or in combination are the benzimidazole and triazole fungicides. Benzimidazole fungicides exert their toxic effect on fungal spores through inhibition of microtubule assembly, by binding to tubulin; the major component of microtubules (Berg et al., 1986; Davidse, 1986; Wolff, 2009). Triazole fungicides, in contrast, interfere with steroid biosynthesis and therefore formation of fungal cell walls through inhibition of sterol-14α-demethylase (CYP51), an enzyme present in all eukaryotes (Bossche et al., 1995; Zarn et al., 2003). As a consequence, the structure of the plasma membrane is disrupted, making it prone to further damage (Georgopapadakou, 1998; Lorito et al., 1996). Both benzimidazole and triazole fungicides have been associated with negative effects in non-target organisms, including amphibians. Such effects include endocrine disruption in adult amphibians (Poulsen et al., 2015), and developmental defects such as craniofacial abnormalities in the case of triazole fungicides (Di Renzo et al., 2011; Groppelli et al., 2005; Papis et al., 2006), or inhibition of the differentiation of neural tissues and organ dysplasia following exposure to benzimidazole fungicides (Yoon et al., 2003; Yoon et al., 2008).

Investigating the effects of environmental pollutants at the cellular level is of importance in ecotoxicological research because the key interaction between chemical contaminants and organisms initially occurs within cells (Fent, 2001). In addition, the

use of an *in vitro* cell culture model reduces the number of vertebrates used in environmental risk assessment, thus reducing ethical concerns (Scholz et al., 2013). Infrared spectroscopy is being increasingly applied in cell-based assays in order to determine molecular modifications caused by chemical stressors, based on changes in the IR absorbance spectra (Holman et al., 2000a). Exposure of a sample to IR radiation will cause the functional groups within the sample to absorb the IR radiation and vibrate in several ways, including stretching, bending and deformation. These absorptions and vibrations can then be directly correlated to biochemical molecules, with peaks in the spectrum corresponding to the chemical structure of a particular entity e.g. lipid ~ 1740 $cm^{-1}$, DNA ~ 1080 $cm^{-1}$, Amide I and II ~ 1650 and 1550 $cm^{-1}$ respectively, thus providing a 'biomolecular fingerprint' in the form of an IR spectrum (Ellis and Goodacre, 2006; Kelly et al., 2011; Martin et al., 2010). . Previous studies have found a high concordance between traditional toxicological endpoints and those measured by FTIR spectroscopy. For example in HEPG2 cells exposed to TCDD, there was a positive correlation between CYP1A1 expression and IR absorption of the phosphate band (Holman et al., 2000b). MCF-7 cells exposed to 17$\beta$-estradiol showed comparable EC-50 values when assessed with either the E-screen assay or FTIR spectroscopy, with the results from FTIR spectroscopy obtained in a much shorter time; a key advantage of this technique (Johnson et al., 2014). As IR spectroscopy is able to analyse lipids, carbohydrates, proteins and nucleic acids concurrently, it is valuable technique for metabolic fingerprinting (Ellis and Goodacre, 2006). The resulting fingerprint is highly complex and information rich, comprising hundreds of features (wavenumbers), therefore multivariate techniques such as principal component analysis (PCA) or linear discriminant analysis (LDA) are often applied in order to reduce the complexity of the data sets into a small number of factors (scores). The application of chemometric methods like PCA and LDA allows the extraction of key features from the IR spectrum in the form of loadings and cluster vectors, which denote which regions of the IR spectrum are responsible for segregation between control and treated cells when viewed alongside the scores plots (Baker et al., 2014; Martin et al., 2010; Trevisan et al., 2012). The combination of IR spectroscopy and multivariate techniques for feature extraction has previously been applied in human, algal and bacterial cell types in order to distinguish between treated and control cells and generate potential biomarkers based upon the loadings and

cluster vectors generated (Heys et al., 2014; Johnson et al., 2014; Llabjani et al., 2010, 2011; Mecozzi et al., 2007; Riding et al., 2012a; Ukpebor et al., 2011) .

In this study, ATR-FTIR spectroscopy coupled with multivariate feature-extraction techniques was employed in order to detect the effects of two commonly used fungicides: carbendazim, a benzimidazole fungicide, and flusilazole, a triazole-derived fungicide at low, environmentally relevant concentrations (Chatupote and Panapitukkul, 2005; Palma et al., 2004) ranging from 0.05-5 nM in A6 cells, a continuous epithelial cell line derived from the kidney of the African clawed frog, *Xenopus laevis*. A6 cells are a well characterised cell line, having previously been used in toxicity studies (Gorrochategui et al., 2016) measuring responses such as heat shock proteins (HSPs), intracellular calcium and cell cycle progression after exposure to a variety of environmental contaminants (Bjerregaard, 2007; Bjerregaard et al., 2001; Darasch et al., 1988; Faurskov and Bjerregaard, 1997; Faurskov and Bjerregaard, 2000; Faurskov and Bjerregaard, 2002; Heikkila et al., 1987; Khamis and Heikkila, 2013; Music et al., 2014; Thit et al., 2013; Woolfson and Heikkila, 2009; Yu et al., 2007). Additionally, as amphibians are exposed to multiple chemical stressors in the environment (Hua and Relyea, 2014; Relyea, 2009), cells were also exposed to mixtures of carbendazim and flusilazole.. The aims of the study were as follows: 1) To determine if ATR-FTIR spectroscopy coupled with multivariate feature-extraction techniques could detect changes induced to cellular biomolecules by carbendazim and flusilazole across a concentration range in the A6 cell line; 2) To determine differences in the mechanism of action of each agent through direct comparison of the features extracted from their IR spectra; and 3) To determine the combined effects of carbendazim and flusilazole on cells in binary mixtures in comparison to single agent effects through comparison of the features extracted from their IR spectra.

## Materials and Methods

### Cell Culture

*Xenopus laevis* A6 kidney epithelial cells were purchased from Sigma Aldrich (Dorset, UK) and grown at 22°C in T75 tissue culture flasks in 70% (diluted with distilled water to adjust to amphibian osmolarity) Leibovitz's (L15) media supplemented with 10% v/v fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 U/mL/100 µg/mL). Cells were routinely subcultured every 7 days by partial digestion in 0.25% trypsin-EDTA and prior to incorporation into experiments. Media was replaced every 72 hours. Flasks that had reached 80-90% confluency were used for experiments. All cell culture consumables were purchased from Gibco Life Technologies (Paisley, UK) unless otherwise stated.

### Test Agents

Flusilazole (product no. 45753) and carbendazim (product no. 45368) were purchased as PESTANAL® analytical standards from Sigma Aldrich (Poole, Dorset) and made up to 10 µM stocks solutions in dimethylsulfoxide (DMSO) (also from Sigma Aldrich). Serial dilutions of the stock solutions were made to give the appropriate concentrations in the treatment flasks. Test agent/vehicle control solutions did not exceed 1% v/v in the treatment flasks.

### Cell Treatments

Routinely cultured A6 cells were trypsin-disaggregated, resuspended in complete media and seeded in T25 flasks at a density of 5 x $10^4$ cells/ml. Cells attached for 24 hours before treatment with the test agents as either single concentrations or binary mixtures for a further 24 hours. This treatment time is optimal for IR spectroscopy studies as it allows the recording of distinct spectral variations, whilst avoiding large amounts of damage from apoptosis and necrosis to the cells (Derenne et al., 2012). For the single concentrations, cells were treated with 5 nM, 1 nM, 0.5 nM, 0.1 nM and 0.05 nM of carbendazim or flusilazole, plus a vehicle control (DMSO). For the binary mixtures, cells were treated with 5 nM and 0.05 nM of flusilazole and carbendazim in the following combinations: 0.05 nM carbendazim, 0.05 nM flusilazole; 5 nM carbendazim, 5 nM flusilazole; 0.05 nM carbendazim, 5 nM flusilazole; 5 nM

carbendazim, 0.05 nM flusilazole. Cells were treated with single concentrations of carbendazim or flusilazole (5 nM and 0.05 nM) together with an equivalent volume of DMSO in order to account for any effects of volume when comparing with the mixtures. For each treatment, nine independent replicates were carried out.

Following treatments, cells were again disaggregated and the cell suspensions immediately fixed in 70% ethanol and stored at 4°C until use. Fixed cell suspensions were transferred onto 1 cm x 1cm Low-E reflective glass slides (Kevley Technologies, Chesterland, OH, USA), dried overnight and stored in a desiccator until analysis.

**ATR-FTIR Spectroscopy**

Five spectra per slide were acquired using a Tensor 27 FTIR spectrometer with Helios ATR attachment (Bruker Optics Ltd, Coventry, UK) containing a diamond crystal (≈250 μm×250 μm sampling area). Spectra were acquired at 8 $cm^{-1}$ resolution with 2x zero-filling, giving a data-spacing of 4 $cm^{-1}$ over the range 400-4000 $cm^{-1}$. Distilled water was used to clean the crystal in between analysis of each sample. A new background reading was taken prior to the analysis of each sample in order to account for changes in atmospheric conditions.

**Data Processing and Analysis**

**Single treatments**

Spectra were cut at the biochemical cell fingerprint region (1800-900 $cm^{-1}$), baseline corrected using Savitzky-Golay 2$^{nd}$ order differentiation (2$^{nd}$ order polynomial and 9 filter coefficients), and vector normalised. Data were mean-centred before the application of principal component analysis-linear discriminant analysis (PCA-LDA) with leave-one-out cross validation; this method uses a small portion of the dataset to train the model in order to prevent LDA overfitting. PCA reduces the spectra (227 wavenumbers) into a smaller number of principal components for input into LDA. In this case 14 PCs were picked for flusilazole and 15 PCs for carbendazim as this represented ~95% of the variance in the data and represented where the variance began to plateau, thus preventing noise being inputted into further analysis with LDA. LDA maximises the differences between classes and minimises the heterogeneity

within classes. The data can then be viewed as scores, to determine how the different treatments separate from the control class (Trevisan et al., 2012). The wavenumbers responsible for the separation of the scores were determined using the cluster vector (CV) approach. Cluster vectors generate 'pseudo-spectra'; which have a direct relation to the original absorbance spectra and are used to reveal biochemical alterations specific to each data class relative to the control, which is set at the origin (Trevisan et al., 2012). A peak detecting algorithm with a data-spacing of 20 $cm^{-1}$ was then employed to select the seven most prominent wavenumbers in the CVs that contributed to the segregation between control and treated cells.

**Comparison of cells treated with carbendazim *vs.* cells treated with flusilazole**

In addition, a direct comparison was made of the spectral signature of cells treated with carbendazim in comparison to those treated with flusilazole. As no significant differences were found between the individual concentrations of flusilazole and carbendazim (see results) direct comparisons between these two agents were assessed by compiling spectra of all concentrations of the data. Two approaches were taken to compare cells treated with carbendazim to those with flusilazole: cross validated PCA-LDA and forward feature selection (FFS). For both approaches, difference spectra were first calculated (following pre-processing), where the mean spectra of the vehicle control was subtracted from the mean spectra of the treated cells for each test agent giving the actual metabolic modifications caused by each test agent (Derenne et al., 2012). Cross-validated PCA-LDA was used as before, with 18 PCs incorporated into the LDA model, generating scores and a CV plot (flusilazole-treated cells at the origin) as before, with a peak detection algorithm employed as detailed previously. FFS incorporates sub-sets of wavenumbers into a dataset, ranking them based on how they contribute to the correct classification of each labelled data set. It is a useful technique for comparison with PCA-LDA, as the biomarkers generated from this approach may be more discriminatory than those generated by PCA-LDA (Trevisan et al., 2014). This approach generates a feature selection histogram (FSH) that provides a count of the frequency (number of hits) each wavenumber was selected (Trevisan et al., 2012). The FSH was produced using a Gaussian-fit classifier with random sub-sampling, repeated 100 times to randomise training and test data (90% training, 10%

data). Five variables were used to improve the stability of biomarker identification (Trevisan et al., 2014).

### Comparison of binary mixtures with single treatments

Cells exposed to the combination treatments of carbendazim and flusilazole were also pre-processed as before and analysed with PCA-LDA (9-10 PCs), generating scores and loadings plots in order to pinpoint biochemical alterations induced by each binary mixture for comparison with single-agent effects.

All spectral pre-processing and data analysis was implemented using the IRootLab toolbox https://code.google.com/p/irootlab/ (Martin et al., 2010; Trevisan et al., 2013) in Matlab (r2012a) (The MathWorks, Inc., USA), unless otherwise stated.

### Statistical Analysis

Scores generated from PCA-LDA were averaged to give one score per replicate (9 per treatment) and either two-sample *t*-tests or one-way ANOVA followed by Tukey's post-hoc tests was applied to calculate differences between the scores generated from PCA-LDA analysis. Analysis was carried out in GraphPad Prism 6 software (GraphPad Software Inc, CA, USA).

## Results

### Single Treatments

A representative second derivative spectrum of A6 cells in the 1800-900 $cm^{-1}$ region is shown in Figure 1, with the characteristic frequency values and spectral assignments of these bands given in Table 1.

Multivariate analysis with PCA-LDA was carried out on cells treated with increasing concentrations of the fungicides carbendazim and flusilazole over the concentration range 0.05-5 nM. One-dimensional PCA-LDA scores plots and corresponding cluster vector plots representing the major biochemical alterations for cells treated with carbendazim are shown in Figures 2A and 2B respectively. Scores and cluster vector

plots for cells treated with flusilazole are shown in Figures 3A and 3B respectively. It is clear from the scores plots in Figures 2A and 3A that cells treated with all concentrations of both carbendazim and flusilazole segregate away from the vehicle control along LD1; this was confirmed by one-way ANOVA followed by Tukey's post-hoc comparison tests, which revealed that the scores generated from PCA-LDA analysis were significantly different from those of the vehicle control (Carbendazim: One-way ANOVA: $F_{5,48}$ = 8.63, $P$ <0.0001; Tukey's post-hoc tests: $P$ <0.01 for all concentrations; Flusilazole: $F_{5,48}$ = 7.12, $P$ <0.0001; Tukey's post-hoc tests: $P$ <0.01 for all concentrations). However, no significant differences were found between individual concentrations for either test agent (Tukey's multiple comparison test, $P$ >0.05).

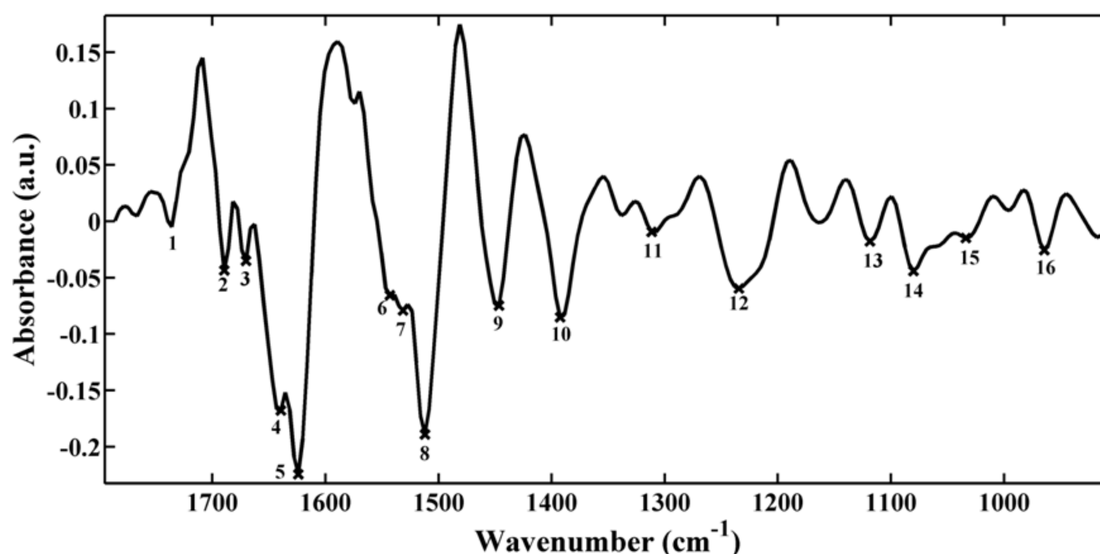


**Figure 1.** Typical mean second derivative spectrum of untreated *Xenopus laevis* kidney epithelial (A6) cells in the 1800-900 $cm^{-1}$ region following analysis with ATR-FTIR spectroscopy. Corresponding wavenumber assignments are shown in Table 1.

**Table 1.** Band assignments of major absorptions in the second derivative IR spectra of untreated *Xenopus laevis* kidney epithelial (A6) cells in the 1800-900 $cm^{-1}$ cell fingerprint region based on the literature.

| Peak Number | Wavenumber ($cm^{-1}$) | Proposed Spectral Assignment |
|---|---|---|
| 1 | 1736 | C=O stretching of esters/phospholipids |
| 2 | 1690 | Peak of nucleic acids due to base carbonyl stretching and ring breathing mode |
| 3 | 1670 | Amide I (anti-parallel $\beta$-sheet) |
| 4 | 1639 | Amide I |
| 5 | 1624 | Amide I ($\beta$-sheet) |
| 6 | 1543 | Amide II (N-H bending, C-N stretching) |
| 7 | 1531 | Amide II |
| 8 | 1512 | CH bending from phenyl rings |
| 9 | 1447 | Asymmetric $CH_3$ bending of the methyl groups of proteins |
| 10 | 1393 | Symmetric $CH_3$ bending of the methyl groups of proteins |
| 11 | 1312 | Amide III |
| 12 | 1234 | Asymmetric phosphate stretching overlapped with amide III of proteins |
| 13 | 1119 | C-O stretching mode/deformations of carbohydrates |
| 14 | 1080 | Symmetric phosphate stretching |
| 15 | 1034 | Glycogen |
| 16 | 964 | C-O deoxyribose |

Sources: (Movasaghi et al., 2008; Naumann, 2001).

The cluster vector plot for carbendazim shown in Fig. 2B revealed that the main wavenumbers associated with the segregation were very similar for each concentration. All concentrations tested revealed alterations in regions associated with C=O stretching and $CH_2$ bending of lipids (1744 and 1454 $cm^{-1}$ respectively), as well as significant contributions from the Amide II protein region. Alterations induced by particular concentrations included those associated with the anti-parallel $\beta$-sheet conformation of Amide I and the base region (5 nM and 0.5 nM respectively, Table 2).

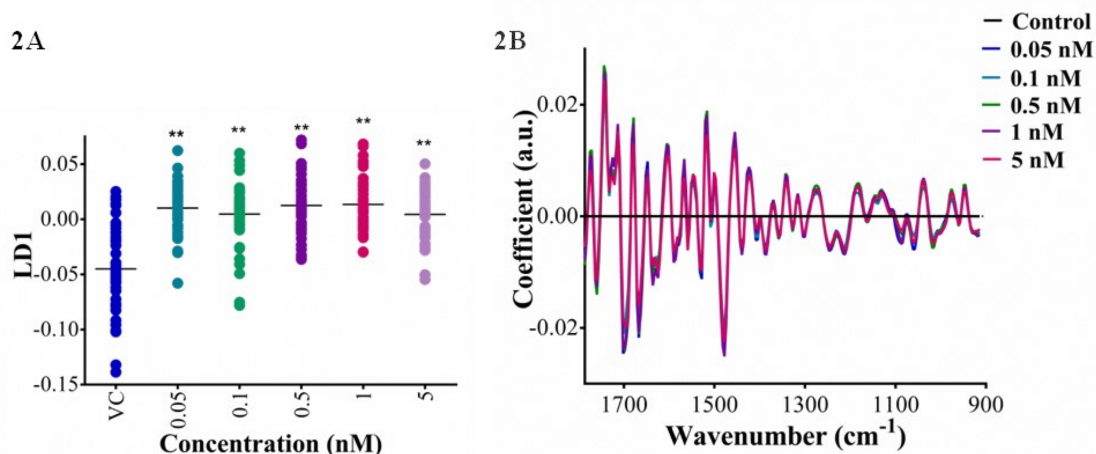


**Figure 2.** Cross-validated one-dimensional PCA-LDA scores plot **(A)** and corresponding cluster vectors plot **(B)** of A6 cells treated with increasing concentrations of carbendazim following analysis with ATR-FTIR spectroscopy. The vehicle control is set at the origin of the cluster vectors plot. Asterisks indicate a significant difference from the DMSO vehicle control (VC) at the $P < 0.01$ level as determined by One-Way ANOVA followed by Tukey's post-hoc test carried out on averages of each experimental replicate ($n = 9$). Wavenumber assignments from cluster vector plots are shown in Table 2.

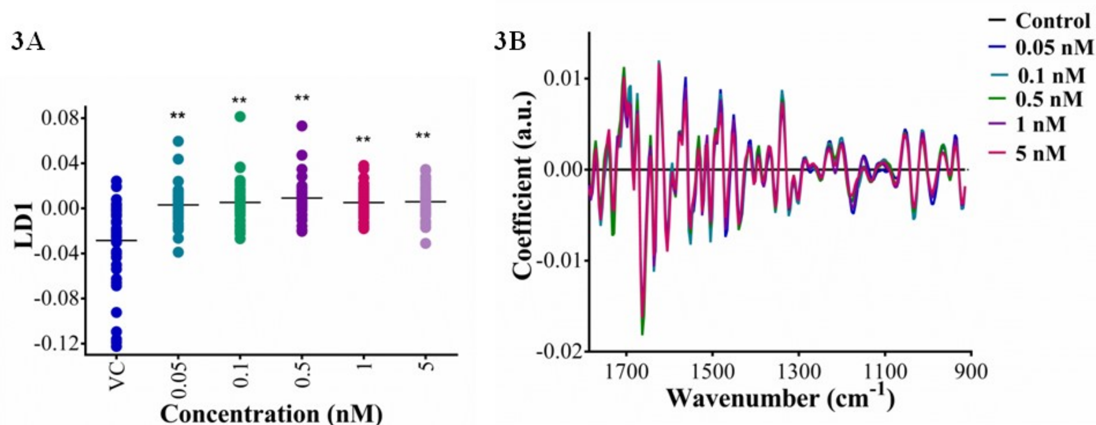


**Figure 3.** Cross-validated one-dimensional PCA-LDA scores plot **(A)** and corresponding cluster vectors plot **(B)** of A6 cells treated with increasing concentrations of flusilazole following analysis with ATR-FTIR spectroscopy. The vehicle control is set at the origin of the cluster vectors plot. Asterisks indicate a significant difference from the DMSO vehicle control (VC) at the $P < 0.01$ level as determined by One-Way ANOVA followed by Tukey's post-hoc test carried out on averages of each experimental replicate ($n = 9$). Wavenumber assignments from cluster vector plots are shown in Table 2.

Cells treated with flusilazole showed some similarities to those treated with carbendazim in the cluster vector plot; however regions associated with the Amide I and II region of proteins were more dominant (see Fig. 3B). Wavenumbers associated with the Amide I and II regions of proteins (1663 and 1562 $cm^{-1}$) were identified as common to all concentrations tested; however, there was a greater variability in the other segregating wavenumbers between concentrations in comparison to cells treated with carbendazim, with some concentrations also associated with C=O stretching of lipids (0.5 nM) and fatty acids (0.05, 0.5, 1, and 5 nM, Table 2).

**Table 2.** Distinguishing wavenumbers and proposed assignments generated from the cluster vector plots of A6 cells analysed with ATR-FTIR spectroscopy after treatment with increasing concentrations of either carbendazim or flusilazole.

| Treatment | Wavenumber ($cm^{-1}$) | Tentative Assignment | Concentration (nM) |
|---|---|---|---|
| **Carbendazim** | 1744 | C=O stretching of phospholipids, triglycerides, cholesterol esters | All |
| | 1701 | Fatty acid esters | 0.05, 0.1, 1 |
| | 1697 | Base region | 0.5 |
| | 1693 | Anti-parallel $\beta$-sheet of Amide I | 5 |
| | 1666 | C=O stretching of pyrimidine bases | All |
| | 1605 | Amide I | 0.05, 0.5, 1 |
| | 1601 | C=N cytosine, N-H adenine | 0.1, 5 |
| | 1516 | Amide II | All |
| | 1477 | Proteins | All |
| | 1454 | $CH_2$ bending of lipids, with some contribution from proteins | All |
| **Flusilazole** | 1732 | C=O stretching (phospholipids)/fatty acid esters | 0.5 |
| | 1705 | Fatty acid esters | 0.05, 0.5, 1, 5 |
| | 1690 | Peak of nucleic acids due to base carbonyl stretching and ring breathing mode | 0.1 |
| | 1663 | Amide I | All |
| | 1636 | $B$-sheet structure of amide I | 0.05, 0.5, 1 |
| | 1624 | Amide I ($\beta$-sheet) | 0.1, 5 |
| | 1605 | Asymmetric stretch of polysaccharides/pectins | 0.05, 0.5, 1 |
| | 1562 | Amide II region | All |
| | 1504 | In-plane CH bending from phenyl rings | 0.05, 0.1 |
| | 1481 | Amide II | 0.05, 0.1, 1, 5 |
| | 1439 | Protein | 5 |
| | 1339 | In-plane C-O stretching vibration combined with the ring stretch of phenyl | 0.1, 0.5, 1, 5 |

Sources: (Movasaghi et al., 2008; Naumann, 2001).

## Comparison of cells treated with carbendazim *vs.* cells treated with flusilazole

As no significant differences were found between the individual concentrations of flusilazole and carbendazim, direct comparisons between these two agents were assessed by compiling spectra of all concentrations of the data. After subtraction of the vehicle control spectra, spectra of flusilazole and carbendazim-exposed cells were compared using cross-validated PCA-LDA followed by two sample *t*-test to assess the significance of the resulting scores, with the finding that cells treated with flusilazole were significantly different to those treated with carbendazim (see Fig. 4A). The cluster vector plot shown in Fig. 4B denotes where these differences were most apparent, with the seven most segregating wavenumbers in regions associated with C=C stretching of lipids and fatty acids (1755 $cm^{-1}$), nucleic acids (1690 $cm^{-1}$), proteins (1477, 1562, 1601 $cm^{-1}$) and glycogen (1018 $cm^{-1}$), as shown in Table 3.

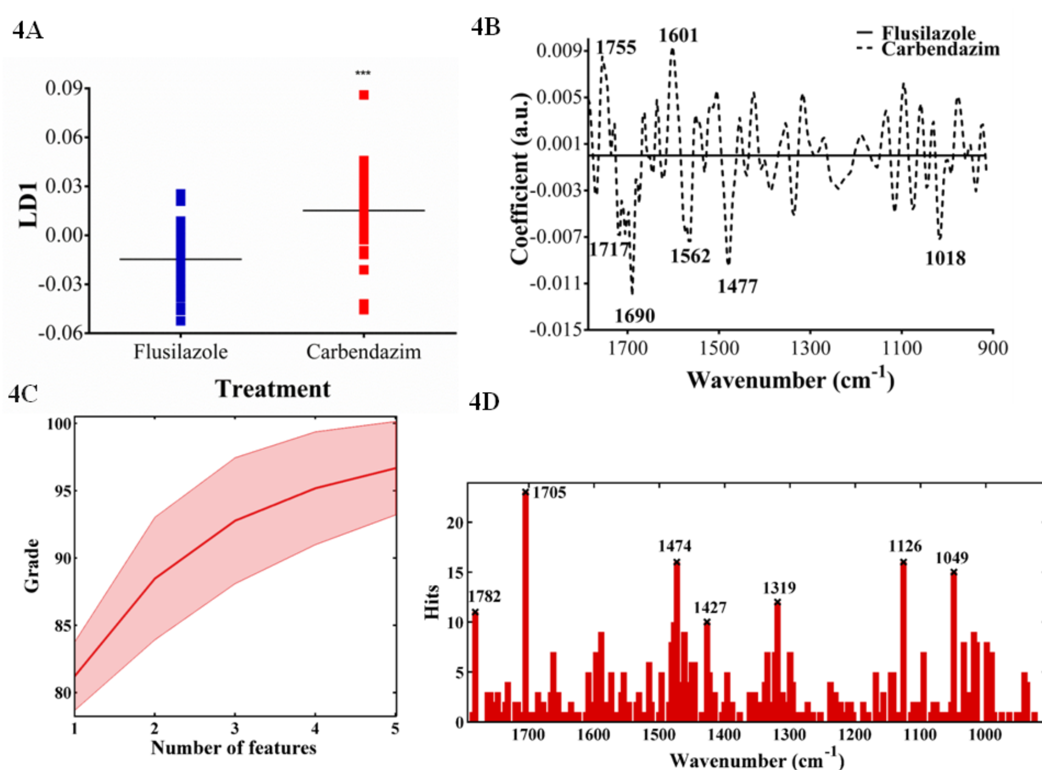


**Figure 4.** Cross-validated one dimensional PCA-LDA scores plot **(A)** and corresponding cluster vectors plot **(B)** directly comparing cells treated with either carbendazim or flusilazole following analysis with ATR-FTIR spectroscopy. Flusilazole is set at the origin for direct comparison with carbendazim in the cluster vectors plot. For a comparison with PCA-LDA, figures **C** and **D** show the classification of cells treated with either flusilazole or carbendazim into their respective categories and the feature histogram generated following forward feature selection respectively. Wavenumber assignments are shown in Table 3.

As well as PCA-LDA, FFS was also used as a technique to identify discriminating wavenumbers. The high classification rate (~97%) generated by FFS is shown in Fig. 4C, and the FSH with key discriminating wavenumbers marked is shown in Fig. 4D. Wavenumbers responsible for the segregation of cells treated with flusilazole from those treated with carbendazim were also associated with lipid/fatty acid regions (1782, 1705, 1474 $cm^{-1}$), with some protein contribution (Amide III) and again significant contribution from carbohydrates/glycogen (1126, 1049 $cm^{-1}$; see Table 3).

**Table 3.** Distinguishing wavenumbers and proposed assignments generated from cluster vectors and feature histograms following a direct comparison of A6 cells treated with either carbendazim or flusilazole after analysis with ATR-FTIR spectroscopy. Vehicle control spectra were first subtracted from treated spectra to directly compare the difference in alterations induced by each agent. All concentrations were combined as there were no significant differences between concentrations for each agent.

| Analysis Type | Wavenumber ($cm^{-1}$) | Tentative Assignment |
|---|---|---|
| **Cluster Vectors** | 1755 | Stretching C=C, phospholipids fatty acids |
| | 1717 | C=O stretching of carbonic acid |
| | 1690 | Peak of nucleic acids due to base carbonyl stretching and ring breathing mode[1] |
| | 1601 | C=N cytosine, N-H adenine |
| | 1562 | Amide II |
| | 1477 | Proteins |
| | 1018 | Glycogen |
| **Feature Histograms** | 1782 | Fatty acid esters |
| | 1705 | Fatty acid esters |
| | 1474 | Protein/$CH_2$ bending of lipids |
| | 1427 | $CH_2$ bending |
| | 1319 | Amide III |
| | 1126 | C-O stretching, disaccharides/sucrose |
| | 1049 | Glycogen/carbohydrates |

Sources: (Movasaghi et al., 2008; Naumann, 2001).

**Comparison of binary mixtures with single treatments**

Cells were also treated with a mixture of carbendazim and flusilazole in the following combinations 0.05 nM carbendazim + 0.05 nM flusilazole; 0.05 nM carbendazim + 5 nM flusilazole; 5 nM carbendazim + 0.05 nM flusilazole; and 5 nM carbendazim + 5 nM flusilazole to determine if this generated any different alterations detectable by ATR-FTIR spectroscopy. All treatment combinations segregated significantly away from the control (see supplementary information Figs. S1A-D). Cluster vectors of cells treated with 0.05 nM and 5 nM of either carbendazim or flusilazole are shown in Figs. 5A and 5B respectively for comparison with mixtures of these agents in different combinations (shown in Figs. 5C-F, with wavenumber assignments in Table 4). Comparison of the single agent cluster vectors and the binary mixture cluster vectors reveals some similarities and some differences. With the binary mixtures, again there was a dominance of lipid and protein alterations, as seen with the single treatments. However the alterations in the phospholipid region (~1750-1730 $cm^{-1}$) induced by the binary mixtures indicated a more pronounced effect in comparison to the single agent treatments, which was consistently seen for all treatment combinations.

Other parts of the spectrum were also highlighted as contributing towards the segregation in cells treated with binary mixtures, including those associated with DNA (1057, 1080 and 964 $cm^{-1}$). The peak at 1169 $cm^{-1}$ (asymmetric stretching of CO-O-C in carbohydrates) was highlighted as a wavenumber consistently associated with treatment with binary mixtures in 3 out of 4 treatment combinations (Figs. 5C, D and F). From the cluster vector plot of cells treated with single concentrations of flusilazole (Fig. 5B), there is also a small peak at 1169 $cm^{-1}$, which becomes more prominent and is highlighted by the peak detector when the agents are combined in a binary mixture, suggesting that when in combination there is a greater effect in this region.

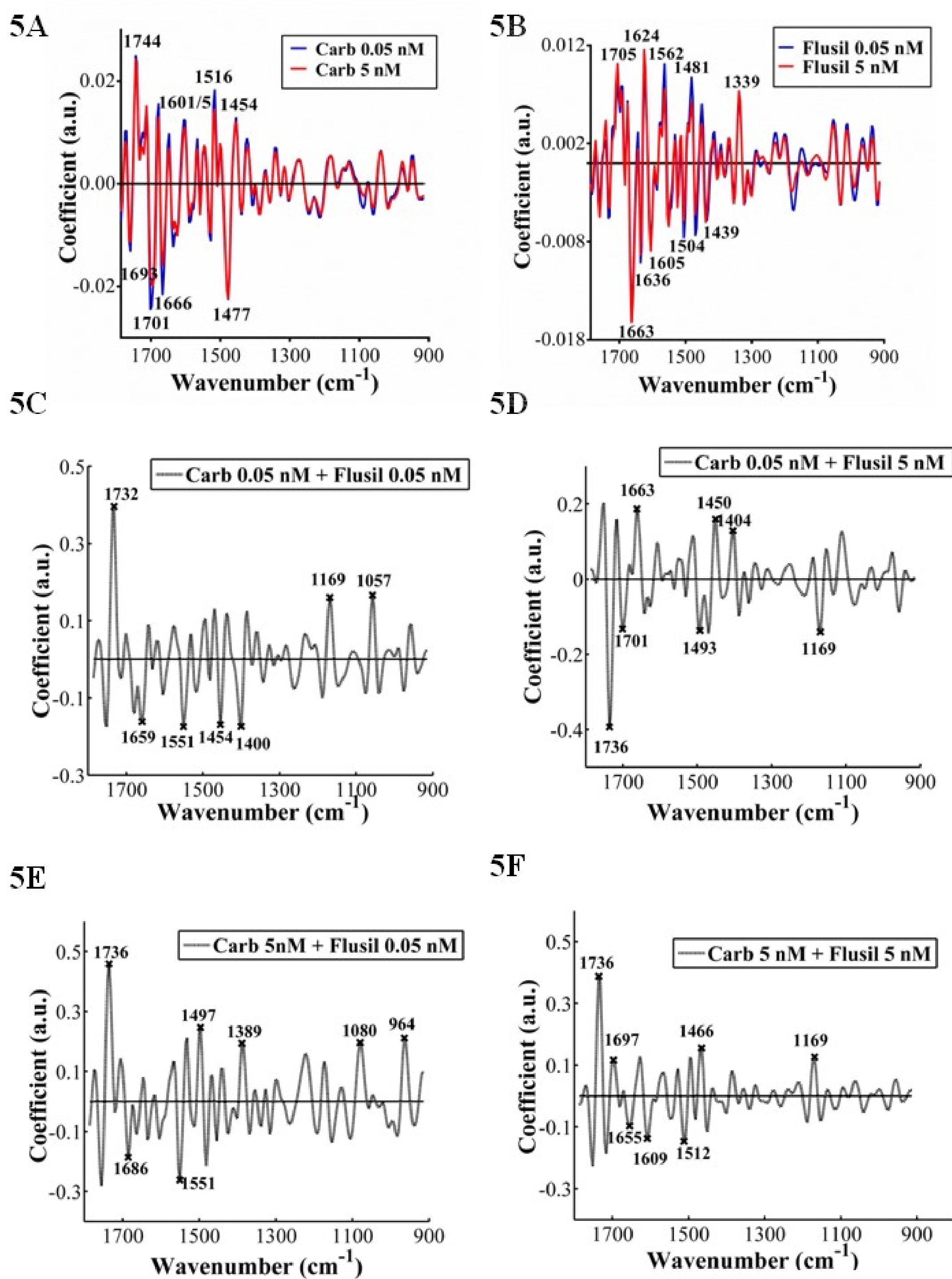


**Figure 5.** Cluster vector plots generated from single treatments of A6 cells with carbendazim **(A)** and flusilazole **(B)** for comparison with loadings plots generated following treatment of A6 cells with binary mixtures of a combination of carbendazim and flusilazole at different concentrations **(C-F)**. Wavenumber assignments are shown in Table 4.

**Table 4.** Distinguishing wavenumbers and proposed assignments generated from loadings plots following treatment of A6 cells with binary mixtures of carbendazim and flusilazole after analysis with ATR-FTIR spectroscopy.

| Treatment | Wavenumber ($cm^{-1}$) | Tentative Assignment |
|---|---|---|
| **0.05 nM Carbendazim + 0.05 nM Flusilazole** | 1732 | Fatty acid esters/C=O stretching of phospholipids |
| | 1659 | Amide I |
| | 1551 | Amide II |
| | 1454 | $CH_2$ bending of lipids, with some contribution from proteins |
| | 1400 | Symmetric stretching of methyl groups in proteins/$COO^-$ vibration of fatty acids |
| | 1169 | Asymmetric stretching of CO-O-C in carbohydrates |
| | 1057 | C-O-C stretching of nucleic acids and phospholipids |
| **0.05 nM Carbendazim + 5 nM Flusilazole** | 1736 | C=O stretching of phospholipids |
| | 1701 | Fatty acid esters |
| | 1663 | Amide I |
| | 1493 | In-plane CH bending vibration |
| | 1450 | Methylene deformation |
| | 1404 | $CH_3$ asymmetric deformation |
| | 1169 | Asymmetric stretching of CO-O-C in carbohydrates |
| **5 nM Carbendazim + 0.05 nM Flusilazole** | 1736 | C=O stretching of phospholipids |
| | 1686 | Amide I (disordered structure) |
| | 1551 | Amide II |
| | 1497 | C=C deformation, C-H |
| | 1389 | Stretching C-O, C-H deformation |
| | 1080 | Symmetric phosphate stretching |
| | 964 | C-O deoxyribose, C-C |
| **5 nM Carbendazim + 5 nM Flusilazole** | 1736 | C=O stretching of phospholipids |
| | 1697 | Base region |
| | 1655 | Amide I of proteins (α-helix) |
| | 1609 | Adenine vibration in DNA |
| | 1512 | CH bending from phenyl rings |
| | 1466 | $CH_2$ scissoring mode of the acyl chain of lipid |
| | 1169 | Asymmetric stretching of CO-O-C in carbohydrates |

Sources: (Movasaghi et al., 2008; Naumann, 2001).

## Discussion

This study determined the application of ATR-FTIR spectroscopy coupled with multivariate feature extraction techniques in assessing the effects of environmentally-relevant concentrations of two differently acting agricultural fungicides in an amphibian cell line. The results confirmed distinct differences in cellular constituents treated with flusilazole or carbendazim, as may be expected from agents with different molecular targets (Derenne et al., 2011; Derenne et al., 2012). Cells treated with all concentrations of either flusilazole or carbendazim segregated significantly away from the vehicle control, demonstrating that ATR-FTIR spectroscopy is a sensitive technique capable of detecting cellular alterations even at very low concentrations, as has been recorded with other test agents (Johnson et al., 2014; Llabjani et al., 2011; Ukpebor et al., 2011). This result is significant as the concentration range used for each test agent was similar to that found in the aquatic environment (Chatupote and Panapitukkul, 2005; Palma et al., 2004) including areas in which amphibians are typically present (Strong et al., 2016). These results suggest that the combination of IR spectroscopy and chemometric analysis with the A6 cell line could serve as a useful model in identifying agents that might threaten amphibian health, however extrapolation from the cellular to the whole organism and population level needs to be interpreted with caution, as there are differences in how chemical interact with whole organisms in comparison to individual cells (Schirmer, 2006).

IR spectroscopy, as well as being able to detect differences between control and treated cell populations in a rapid and high-throughput manner, provides detailed information about how particular agents affect cellular biochemistry through interpretation of the generated IR spectra (Jamin et al., 1998; Movasaghi et al., 2008). The use of chemometric methods such as PCA-LDA allows key features of the IR spectrum to be extracted in the form of loadings and cluster vectors; the largest values corresponding to the most important wavenumbers responsible for segregation between control and treated cells, and can thus be considered biomarkers (Llabjani et al., 2010; Martin et al., 2010; Trevisan et al., 2012). Cell exposed to carbendazim revealed alterations in regions associated with C=O stretching and $CH_2$ bending of lipids, with some protein contributions, indicative of a significant effect on cell membranes including the phospholipid bilayer. Lipids and Amide proteins are

principally associated with the outer cell membrane, and the large spectral alterations seen in these regions are suggestive of disruption to membrane structure and integrity, indicative of lipid peroxidation (Gasper et al., 2009; Riding et al., 2012a). Lipid peroxidation is the initial step in damage to cell membranes caused by pesticides and other contaminants following the generation of reactive oxygen species (Costa et al., 2008), and may be detectable using FTIR due to changes in the band assigned as C=O stretching of lipids at ~ 1740 $cm^{-1}$ (Lamba et al., 1994; Riding et al., 2012a; Riding et al., 2012b), as seen here. The generation of reaction oxygen species by environmental contaminants and subsequent oxidative damage may negatively affect tadpole reproduction and development and is related to amphibian population declines (Costa et al., 2008), therefore there may be population-level consequences of these low-dose exposures. Carbendazim has previously been associated with lipid peroxidation in milk fish (Palanikumar et al., 2014) and in rats (Rajeswary et al., 2007), although only developmental and genotoxic effects have been measured thus far in amphibians (Yoon et al., 2008; Zoll-Moreux and Ferrier, 1999), despite the wide usage and detection of carbendazim in surface waters at low concentrations (Palma et al., 2004).

With flusilazole, the effects elicited in cells were mainly in the regions associated with Amide I and II proteins, with some lipid contribution. This again may be related to the cellular membranes as the functional properties of the plasma membrane are determined principally by the orientation of proteins within the membranes, which are readily detected by IR spectroscopy (Gasper et al., 2009; Kong and Yu, 2007; Naumann, 2001). Flusilazole, like other triazole fungicides acts by interrupting the formation of fungal cell walls through inhibition of sterol-14α-demethylase (CYP51), which is highly conserved across taxa including animals, where it is utilised in the pathway to cholesterol formation; thus effects on cell membranes may not be restricted to the target species (Bossche et al., 1995; Zarn et al., 2003). The hazards posed by triazoles to wildlife is because their effects may not be limited to CYP51, and there is emerging evidence that they may accumulate in the tissues of amphibians, (Hansen et al., 2014; Poulsen et al., 2015; Smalling et al., 2013), although this may vary depending on the levels found in the environment (Smalling et al., 2015). At low levels of exposure, triazole fungicides have been associated with endocrine disruption, disrupting steroidogenesis in adult male frogs at concentrations as low as 1 μg/L (Poulsen et al., 2015), as well as developmental defects in tadpoles and embryos

(Bernabò et al., 2016; Di Renzo et al., 2011; Groppelli et al., 2005; Papis et al., 2006). The changes in IR spectra elicited by flusilazole at similarly low concentrations in this study suggest that triazole fungicides are capable of generating responses in non-target organisms at environmentally-relevant concentrations. The possible endocrine disrupting effects of triazole fungicides have the potential to elicit population-level effects in amphibians by affecting sex ratios and disrupting normal reproductive behaviour (Kloas and Lutz, 2006).

High concentrations of agrichemicals are not typically measured in environmental compartments and instead much lower concentrations, often in complex mixtures tend to be detected, therefore amphibians are likely to be exposed to multiple agents in the environment (Khamis and Heikkila, 2013; Relyea, 2009; Smalling et al., 2015). Thus, in order to represent a more environmentally-realistic scenario, cells were also exposed to binary mixtures of flusilazole and carbendazim. Results from the cluster vector plots demonstrated a dominance of lipid and protein alterations, as seen with the single treatments; however the alterations in the phospholipid region (~1730-40 $cm^{-1}$) induced by the binary mixtures indicated a more pronounced effect in comparison to the single agent treatments, which was consistently seen for all treatment combinations, suggestive of further effects on the lipid bilayer when flusilazole and carbendazim were combined (Gasper et al., 2009). Other parts of the spectrum were also highlighted as contributing towards the segregation in cells treated with binary mixtures, including those associated with DNA (1057, 1080 and 964 $cm^{-1}$). Although carbendazim is not a direct-acting DNA damaging agent, it is a mitotic spindle poison and aneuploidogen which may secondarily affect DNA synthesis through the blocking of nuclear division (Davidse, 1986; McCarroll et al., 2002) and has been previously associated with genotoxicity in *X.laevis* tadpoles, although at higher concentrations than those used here (Zoll-Moreux and Ferrier, 1999). Instability of cell membranes may make cells more susceptible to further damage (Georgopapadakou, 1998; Lorito et al., 1996). As the phospholipid region of the spectrum showed consistent alterations following exposure to all mixtures, this suggests lipid peroxidation (Lamba et al., 1994; Riding et al., 2012a; Riding et al., 2012b). If this was indeed the case, the effects induced in other areas of the spectrum associated with DNA and carbohydrates could be caused indirectly by the products generated following the oxidation of lipids within the cell membrane (Burcham, 1998;

Riding et al., 2012b); if the two agents are acting together and destabilising cell membranes the effects seen on other parts on the spectrum may be more pronounced.

## Conclusions

- Amphibians are at risk of exposure to pesticides in the environment and there is emerging evidence that certain fungicides may accumulate in tissues, causing deleterious effects at low levels of exposure.

- This study presents the use of ATR-FTIR spectroscopy coupled with multivariate analysis to detect changes induced by environmentally-relevant concentrations of two mechanistically distinct fungicides in an amphibian cell line, both singly and in binary mixtures.

- Results suggested effects on cell membranes, as determined by alterations in the lipid and protein regions of the IR spectrum likely to be as a result of lipid peroxidation.

- Binary mixtures of flusilazole and carbendazim demonstrated consistent effects on areas of the spectrum associated with lipids, with alterations to other cell constituents including DNA also noted, suggestive of destabilisation of cell membranes, thus allowing further damage to subcellular moieties.

- Future work could aim to determine the effects of other common water constituents, such as nitrate/phosphate and metal ions in cell culture with these agents. Amphibians are known to be exposed to multiple stressors in the environment and creation of a more environmentally realistic scenario is key to understanding the effects at a cellular level.

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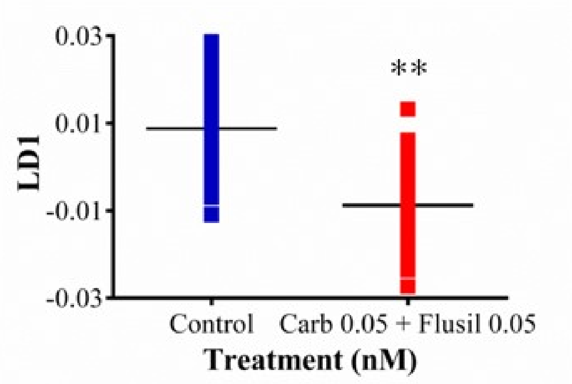
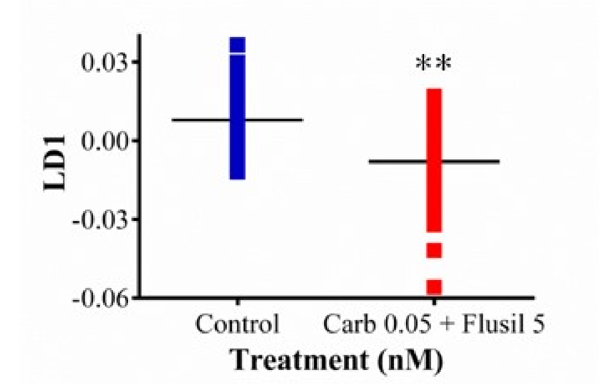
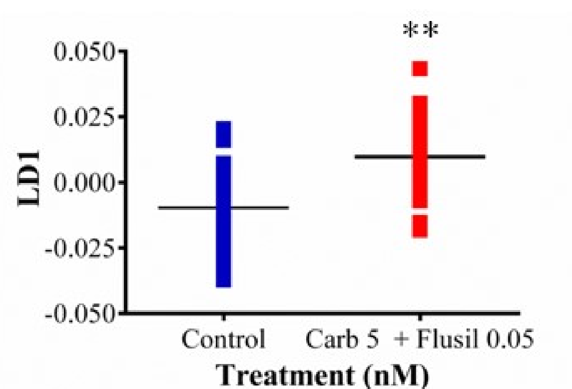
**Appendix A: Supplementary Information**



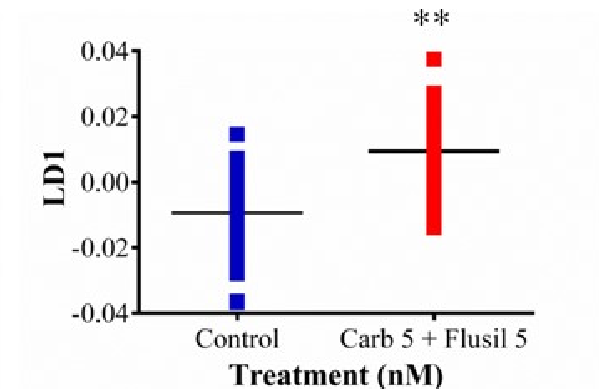




**Figure S1.** Cross-validated one-dimensional PCA-LDA scores plots of A6 cells treated with binary mixtures of flusilazole and carbendazim following analysis with ATR-FTIR spectroscopy. Asterisks indicate a significant difference from the vehicle control (DMSO) at the $P$ <0.01 level as determined by two-sample *t*-tests.

# Chapter 6. General Discussion

Amphibians as a group are facing threats to their health and survival, with environmental pollution regarded as a significant factor in population declines (Davidson, 2004; Mann et al., 2009). The common frog is a widely distributed species in the UK and Europe and as such may be a useful sentinel organism. Although numbers are not generally declining, local extinctions may occur due to habitat destruction, pollution sources and disease (Beebee, 2014). While there has been previous research conducted in the UK using native amphibians, the number of studies remain low and many were conducted decades ago (Cooke, 1972, 1973, 1977, 1981; Oldham et al., 1997; Orton and Routledge, 2011; Orton and Tyler, 2014). Therefore there are a lack of recent data regarding the impact of current-use pesticides on native amphibian populations (Beebee, 2014).

There are a variety of approaches for assessing the impacts of environmental pollution on amphibian health both in the field and in the laboratory, including for example, measurement of enzymes, endocrine disruption, growth, mortality and genotoxicity (Mann et al., 2009; Venturino et al., 2003). Although these approaches are highly useful, some of them may be time-consuming, expensive and involve the use of reagents. Another technology being utilised in environmental toxicology is IR spectroscopy, which has already been implemented both in the laboratory and in the field in other taxa at risk of harm from environmental pollutants including algae, fish and birds (Cakmak et al., 2006; Cakmak et al., 2003; Llabjani et al., 2012; Malins et al., 2006; Malins et al., 2004; Mecozzi et al., 2007). However, no studies to date have examined its use in assessing the health of amphibian populations at risk from the effects of environmental pollutants from different sources. IR spectroscopy identifies and quantifies cellular constituents and characterises differences in macromolecular composition, thus it is a useful technique for identifying biomarkers of environmental quality (Mecozzi et al., 2007). Measurements of carbohydrates, proteins, lipids and nucleic acids can be taken simultaneously, thus providing an integrated metabolic fingerprint of the sample (Ellis and Goodacre, 2006). The application of IR spectroscopy in ecotoxicology offers an approach that can rapidly and cost-effectively detect changes in the biochemical composition of a sample as a result of exposure to chemical agents at sub-lethal levels of exposure (Cakmak et al., 2006). Alterations in

the spectral fingerprint of the samples can thus be used as biomarkers, which can identify the biological effects of environmental contamination in sentinel species (Llabjani et al., 2012; Malins et al., 2006; Malins et al., 2004; Obinaju et al., 2014; Obinaju et al., 2015).

The aim of this thesis was to explore the application of ATR-FTIR spectroscopy coupled with multivariate analysis as a novel method to assess amphibian health. This was achieved by sampling several aquatic life stages of the common frog in the field exposed to pollutants from both agricultural and urban sources, and through the use of a continuous cell line derived from *X.laevis* exposed to specific pollutants associated with agriculture. Below are highlighted key findings from each experimental chapter that contribute to the overall rationale for employing IR spectroscopy as a sensitive technique in determining the effects of environmental pollutants from both agricultural and urban sources in amphibians; thus raising the potential for its wider use in environmental monitoring.

## 6.1. Initial assessment of biospectroscopy in amphibian environmental monitoring.

Chapter two was a pilot study which aimed to assess if it was possible to distinguish between embryos and early-stage tadpoles of *R.temporaria* collected from ponds with differing water quality in using ATR-FTIR spectroscopy. *R.temporaria* is a habitat generalist and as such is able to tolerate wide-ranging environmental conditions, exploiting habitats both in agricultural and urban settings (Carrier and Beebee, 2003; Hamer and McDonnell, 2008). However, this also means that as a consequence, such species may be exposed to sub-lethal levels of pollutants associated with these environments during the sensitive stages of breeding and development, potentially having a deleterious impact on their future health and survival (Ruiz et al., 2010; Smalling et al., 2015). Therefore ATR-FTIR spectroscopy was used to distinguish between embryos and early-stage tadpoles of *R.temporaria* collected from ponds impacted by both urban and agricultural water contaminants in comparison to a relatively pristine pond, and the resulting outputs from multivariate analysis used to ascertain which areas of the spectrum were responsible for the differences between samples.

Results revealed smaller differences between embryos collected from the different ponds in comparison to tadpoles, which is in agreement with previous studies where the jelly coat surrounding the embryos has been proposed as offering protection against particular water contaminants (Berrill et al., 1994; Brodeur et al., 2009; Wagner et al., 2015). Tadpoles however, showed distinct differences in the spectral fingerprint generated, depending on which pond they were collected from, which was despite no significant differences in body size measurements being recorded. The areas of the spectrum attributed to the differences between ponds were primarily in regions associated with glycogen and carbohydrates, with tadpoles collected from the ponds impacted by pesticides and other water contaminants showing reduced absorbance in this region, indicative of a reduction in glycogen levels. Reduced glycogen levels have previously been noted in amphibians exposed to a range of water contaminants and may be indicative of a stress response, as energy is diverted away from processes such as growth and development and instead towards detoxification as the organism attempts to restore homeostasis (Dornelles and Oliveira, 2014; Gendron et al., 1997; Melvin et al., 2013).

This study demonstrated that it was possible to distinguish between tadpoles, and to a lesser extent, embryos from ponds with differing water quality using ATR-FTIR spectroscopy. However, as the results were from a single year of sampling (2012), this may not be representative of the effects of long term contaminant exposure. Therefore this initial study paved the way for a longer-term monitoring study of the same ponds over a three year period, which was the basis for Chapter 3.

## 6.2. Monitoring amphibian populations over time with biospectroscopy

Ideally in any environmental monitoring study, the same areas should be studied over time in order to build up a clearer picture of risks from environmental contamination to populations, and long-term and multi-generational studies which monitor amphibian populations over different developmental stages are needed, as transgenerational effects may become apparent (Bergeron et al., 2010; Sparling et al., 2010). Therefore, building on the data collected in Chapter 2, embryos and early-stage tadpoles of *R.temporaria* were collected in 2013 and 2014 from the same study areas, giving a three year data collection period, which is presented in Chapter 3. The overall

aim was to determine whether ATR-FTIR spectroscopy coupled with multivariate data analysis and classification techniques was still capable of distinguishing between embryos and tadpoles collected from ponds with different water quality, despite expected inter-annual differences from factors which could not be controlled for in a field study of this nature (e.g. spawning date, temperature, rainfall etc). If biospectroscopy alongside multivariate analysis is to be implemented as a tool in environmental monitoring, it must be specific enough to distinguish between embryos/tadpoles from ponds based upon water quality. This is analogous to the application of biospectroscopy in medical research, where although there is case-matching of patients on factors such as age, all sources of inter-individual variation cannot be removed, and thus the combination of spectroscopy plus data analysis techniques must be able to distinguish individuals on the basis of disease state rather than another factor (Wood et al., 1998).

Tadpoles could be distinguished based upon pond of origin with a high degree of accuracy (a classification rate of up to 94%), despite annual differences. Taken together over the whole study period, tadpoles did not vary markedly in measures of body size between ponds, and therefore this is unlikely to account for the differences seen in the spectra of tadpoles. However, there also were significant differences in the spectra of tadpoles between years within each pond, but this annual variation was generally confined to different areas of the spectrum compared to those observed between ponds, and may be related to body size differences, which also showed annual differences. Embryos in contrast showed much poorer classification and seemed to be more affected by annual variations than differences between ponds, possibly as a result of temperature variation which is known to affect egg development markedly (Neveu, 2009). Therefore, results from this study suggest that tadpoles are a more appropriate life stage to use in an environmental monitoring study and where possible should be matched on the basis of their body size and developmental stage so as to control any factors that may affect biochemical parameters, as in studies where biospectroscopy has been applied in disease diagnosis (Theophilou et al., 2015).

## 6.3. Using biospectroscopy to distinguish between tissues of pro-metamorphic common frog tadpoles

In this chapter (Chapter 4), late-stage pro-metamorphic tadpoles of the common frog were collected from the same ponds previously sampled in 2012 and 2013, and individual tissues: liver, muscle, heart, kidney and skin excised for comparison between ponds with ATR-FTIR spectroscopy. This study was implemented because although earlier life stages of tadpole are considered to be more sensitive to environmental pollution, sub lethal effects may manifest themselves later in development (Bridges, 2000; Orton and Routledge, 2011) and some studies also point to later developmental stages being more sensitive to particular contaminants (Howe et al., 1998).

This study found that the liver consistently distinguished between tadpoles collected from ponds with differing water quality; liver size was also found to be increased in tadpoles from the pond with pesticide exposure. This is in agreement with other studies and is likely to be due to the fact that the liver is responsible for the metabolism of xenobiotics in amphibians as in other verebrates, thus differences due to environmental contamination may manifest here (Fenoglio et al., 2005; Melvin et al., 2013). There were also significant differences in other tissues, mainly between tadpoles collected from the pond impacted by pesticides and the relatively clean pond, where all tissues showed some differences between ponds, suggesting that the effects detectable by IR spectroscopy were not limited to the liver. Perhaps most interestingly, the skin samples distinguished between tadpoles collected from the minimally-impacted pond and the pond impacted by agricultural pesticides. The skin of amphibians is highly permeable and plays a vital role in osmoregulation and respiration and thus provides a significant exposure route to chemicals, with previous studies also noting significant structural changes in the epidermis following exposure to environmental contaminants (Bernabò et al., 2013; Fenoglio et al., 2009). As the skin is the first organ that environmental contaminants come into contact with, it may be useful as an early indicator of deteriorating environmental quality. In addition, the sensitivity of skin may allow for the development of non-destructive methods to assess responses to environmental pollution in larval amphibians. Tail-tip clipping has previously been used successfully for non-destructive DNA analysis in tadpoles (Snell and Evans, 2006), and in reptiles and salamanders for assessing exposure to

environmental contaminants (Hopkins et al., 2001; Townsend and Driscoll, 2013); it would be interesting to explore the use of IR spectroscopy to assess the effects of environmental pollution on this non-destructive measure.

While previous studies in fish have documented the effects of environmental contaminants in several tissues using spectroscopy (Cakmak et al., 2006; Obinaju et al., 2014; Palaniappan and Vijayasundaram, 2008, 2009), this study is the first of its kind to report such effects in the tissues of amphibians (Strong et al., 2016).

## 6.4. Application of biospectroscopy in elucidating the effects of low-dose fungicides in amphibian cells

Infrared spectroscopy has been shown to be a sensitive technique for assessing the effects of low, environmentally-relevant levels of chemicals in a variety of cell types (Johnson et al., 2014; Li et al., 2016; Riding et al., 2012a; Ukpebor et al., 2011). In vitro studies provide useful mechanistic information regarding the interaction of particular chemicals with cells, and biospectroscopy, as well detecting differences between treated and untreated cell populations, gives detailed information regarding the effects on cellular biochemistry through the IR spectrum produced (Jamin et al., 1998).

In this study (Chapter 5), a cell-line derived from *X.laevis* kidney epithelia was exposed to concentrations of two fungicides used in agriculture with different mechanisms of action: carbendazim, a benzimidazole fungicide, and flusilazole, a triazole fungicide. Cells were exposed to agents singly or as a binary mixture at environmentally-relevant concentrations, in order to add more environmental realism as amphibians are typically exposed to multiple agents in the environment (Khamis and Heikkila, 2013; Relyea, 2009). All concentrations induced alterations in cells, which led to cells segregating away from the control when analysed with PCA-LDA. The areas of the spectrum attributable to the separation in treated cells were in regions primarily associated with lipids and Amide proteins, which is likely due to effects on the cell membrane (Riding et al., 2012b). Mixture effects also induced effects in regions associated with DNA, potentially due to increased lipid peroxidation of the cell membrane leading to the damage of sub cellular components from the products generated through oxidation of the lipids detected at ~1740 $cm^{-1}$ in the cell membrane (Burcham, 1998). Benzimidazole fungicides are frequently detected in surface waters

and associated with lipid peroxidation in fish, and developmental defects in amphibians although there are very few studies examining their effects in non-target species (Palanikumar et al., 2014; Palma et al., 2004; Yoon et al., 2008). Likewise, despite their wide usage and emerging evidence of accumulation and detrimental effects at low concentrations in amphibians triazole fungicides are poorly studied (Poulsen et al., 2015; Smalling et al., 2013).

This study provided evidence that ATR-FTIR spectroscopy is capable of detecting alterations induced in target cell populations at low levels of exposure using commonly found, yet poorly studied environmental contaminants.

## 6.5. Conclusions and future research

Implementation of IR spectroscopy in environmental research is a growing area, which is proving to be a useful technique in assessing the effects of environmental pollutants in a variety of taxa. This technique provides quantitative information about functional groups in the analysed samples and is a highly sensitive technique capable of detecting small alterations in vibrational modes. In this thesis, ATR-FTIR spectroscopy coupled with multivariate analysis and classification techniques has been shown to be a sensitive technique for assessing the impact of differing water quality on multiple life stages of *R.temporaria*, as well as the specific effects of low-dose exposure to fungicides in vitro in a *X.laevis* cell line. A summary of the conclusions and future research needs is provided in Figure 14.

While this study provides a useful starting point for the application of spectroscopy in assessing the effects of pollutants on amphibian populations, there are limitations that must be highlighted. Infrared spectroscopy was able to detect differences in *X.laevis* cells exposed to fungicides, however there may be issues in extrapolating cell-based studies to higher levels of organisation (Schirmer, 2006); future work should look at 3D cell culture systems (Baron et al., 2012), possibly in co-culture with other cell lines. In addition, other markers of water quality such as nitrate and phosphate in concert with environmental contaminants could be explored. Although attempts were made to reduce sources of variability by sampling tadpoles and embryos of around the same developmental stage for comparison, other factors than those measured in the study may have impacted the results, which are difficult to control in a field study. These include the effect of other stressors on amphibian health such as food

availability, food content, predation, population density and abiotic factors such as temperature and pH. Tadpoles are known to show developmental plasticity, speeding up or slowing down metamorphosis depending on the environmental conditions (Alvarez and Nicieza, 2002). Future research could look at attempting to control some of these factors by the use of complementary laboratory and outdoor mesocosm studies. In addition, the results reported are from a small number of ponds; a bigger study sampling more ponds would potentially allow the correlation of different types of pollutants in the aquatic environment with the spectral measurements from tadpoles and embryos. While there are several studies in fish that have utilised IR spectroscopy in ecotoxicogical research (e.g. Malins et al., 2004, 2006, Obinaju et al., 2014) the data presented in this thesis are the first to apply this technique in amphibians, therefore ideally future work would seek to further validate the observed changes in the spectral data with that from more conventional approaches. For example, the decreases seen in the absorbance bands of glycogen and lipid in whole tadpoles (chapters 2 and 3) and tadpole tissues (chapter 4) could be correlated to results obtained from the direct quantification of these constituents and markers of oxidative stress and detoxification enzymes such as glutathione-S-transferase and superoxide dismutase, as has been measured in other studies in amphibians (Attademo et al., 2007; Dornelles and Oliveira, 2014; Dornelles and Oliveira, 2016; Melvin, 2015; Melvin et al., 2013; Melvin et al., 2016).

At the onset of the PhD it was envisaged that one or more ponds could be selected to serve as a 'control' i.e. sites that have minimal anthropogenic perturbations. However, this is unlikely to be achieved in such a small survey and further to this; other factors such as pH, temperature, food availability etc can play a role as demonstrated by the longitudinal (multi-year) study, most notably on frog embryos. While the focus of this study was on water pollution and thus employed aquatic life stages of *R.temporaria*, terrestrial amphibians could be sampled in a future study as exposure to environmental contaminants at the terrestrial stage may be an important and overlooked area of research (Brühl et al., 2013).

The wider application of studying amphibian populations with IR spectroscopy could involve sampling of a non-destructive nature, of both larval and metamorphosed amphibians. There is a growing field of biofluid analysis using IR spectroscopy in

medical diagnostics using samples such as blood, urine and sputum (Krafft et al., 2009; Lewis et al., 2010; Ollesch et al., 2013). This has the potential to be applied in amphibians (and other groups of sentinel organisms), as currently blood, urine samples and buccal and skin swabs are used as non-destructive indices in assessing responses to pollution, and in DNA, disease and hormone analysis in adult and larval amphibians (Berubé et al., 2005; Graham et al., 2013; Kindermann et al., 2012; Pidancier et al., 2003). The implementation of IR spectroscopy in a non-destructive manner could be of great benefit in the many species of amphibian vulnerable to extinction, where collection of organisms would be detrimental to the species.

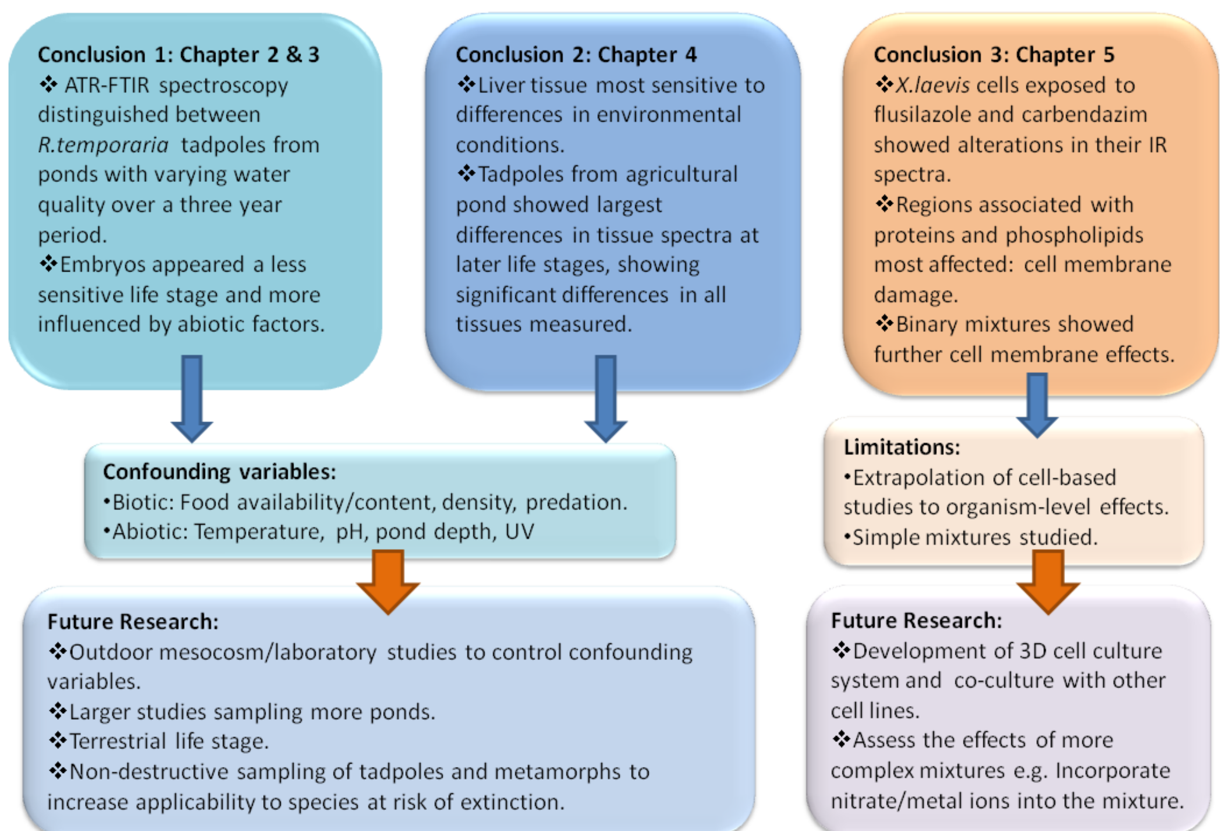



**Figure 14.** Flow diagram of conclusions, limitations and future research needs of the thesis.

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## Appendix

**List of publications from collaborative research:**

1. Li, J., **Strong, R.J,** Trevisan, J., Fogarty, S. W., Fullwood, N. J., Jones, K. C., & Martin, F. L. 2013. Dose-related alterations of carbon nanoparticles in mammalian cells detected using biospectroscopy: potential for real-world effects. Environmental science & technology 47(17), 10005-10011.

2. Heppenstall, L. D**., Strong, R. J.,** Trevisan, J., & Martin, F. L. 2013. Incorporation of deuterium oxide in MCF-7 cells to shed further mechanistic insights into benzo [a] pyrene-induced low-dose effects discriminated by ATR-FTIR spectroscopy. Analyst 138(9), 2583-2591.

3. 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.

4. Heys, K.A., Riding, M.J**., Strong, R.J.,** Shore, R.F., Pereira, M.G., Jones, K.C., Semple, K.T., Martin, F.L., 2014. Mid-infrared spectroscopic assessment of nanotoxicity in Gram-negative vs. Gram-positive bacteria. Analyst 139, 896-905.

5. Ahmadzai, A.A., Trevisan, J., Pang, W., Riding, M.J., **Strong, R.J.,** Llabjani, V., Pant, K., Carmichael, P.L., Scott, A.D. and Martin, F.L., 2015. Classification of agents using Syrian hamster embryo (SHE) cell transformation assay (CTA) with ATR-FTIR spectroscopy and multivariate analysis. Mutagenesis 30(5), 603-612.

6. 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.

**Conference Abstracts:**

1. **Strong R.J**., H., C.J., Jones K.C., Martin F.L. Environmentally relevant levels of the genotoxin, cyclophosphamide induce alterations in MCF-7 cells detectable by biospectroscopy approaches. Mutagenesis 27, 814-814.

2**. Strong, R.J.,** Halsall, C.J., Jones, K.C., Shore, R.F., Martin, F.L., 2014. Biospectroscopy as a tool to detect the effects of environmentally-relevant levels of two fungicides in an amphibian cell line. Mutagenesis 29, 539-539.