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

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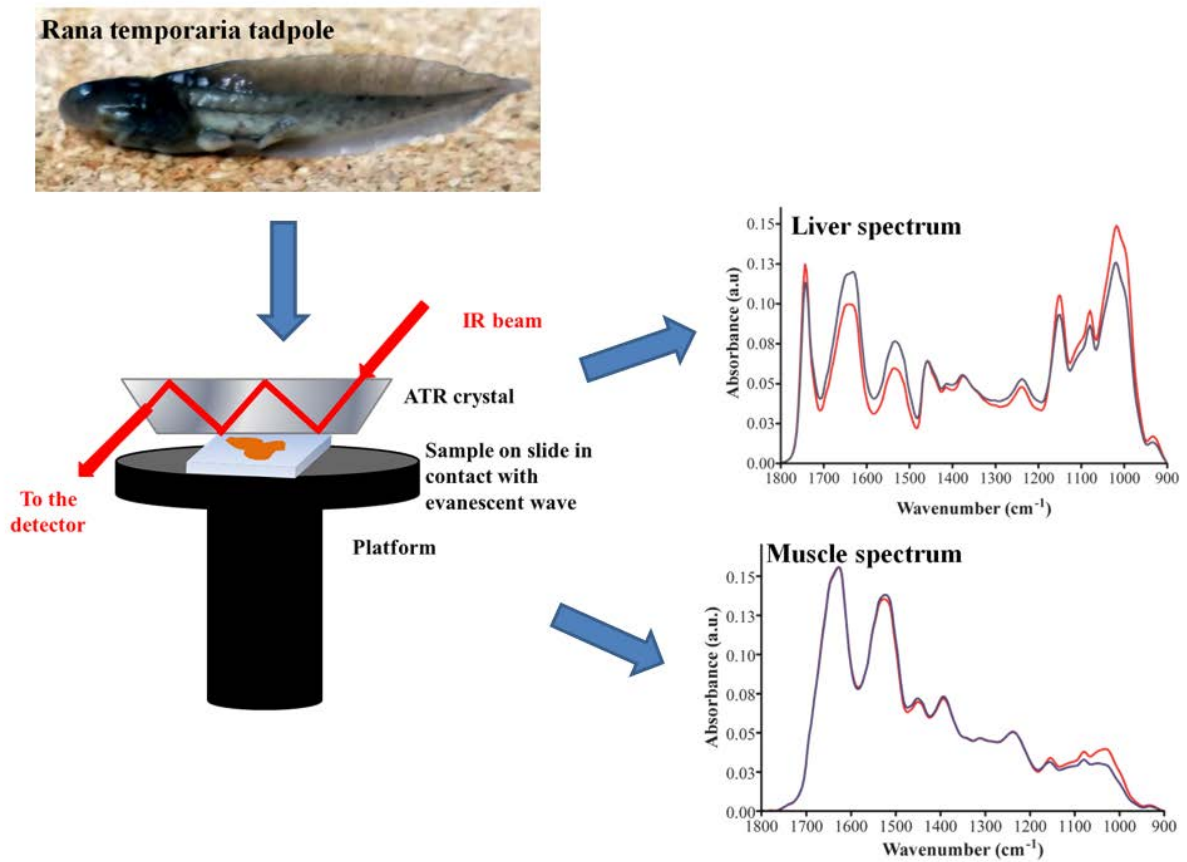
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17 **Graphical abstract**

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24 **Highlights**

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

29

30 **Abstract**

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

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

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

53 **Introduction**

54 Amphibians are facing large declines globally, with a number of hypotheses proposed to
55 explain such declines, including habitat destruction, disease, climate change, UV radiation,
56 predation and environmental contamination (Beebee and Griffiths, 2005; Blaustein et al.,
57 2003; Mann et al., 2009; Stuart et al., 2004). Whilst no one factor is likely to be the sole
58 cause of population decreases (Blaustein et al., 2011), it is known that amphibians may be
59 particularly vulnerable to environmental contamination as their reproduction and larval
60 development occurs in aquatic habitats, often adjacent to surface run-off from agricultural
61 and urban sources (Mann et al., 2009; Ralph and Petras, 1997). This coupled with the
62 permeable skin of amphibians, offering little protection against toxic contaminants (Blaustein
63 et al., 2003), means that they are regarded as indicators of environmental stress (Blaustein
64 and Wake, 1995).

65 While amphibians are considered to be most vulnerable to environmental stress during early
66 tadpole development (Bridges, 2000; Greulich and Pflugmacher, 2003), the effects of such
67 exposure may have consequences in later development (Bridges, 2000; Orton and Routledge,
68 2011; Orton and Tyler, 2014). This could include a smaller size at metamorphosis, exposing
69 the juvenile amphibian to an increased risk of predation, or delayed development and
70 metamorphosis, which could mean that the ephemeral ponds dry up before metamorphosis
71 occurs (Altwegg and Reyer, 2003; Egea-Serrano et al., 2012; Hayes et al., 2006; Venturino et
72 al., 2003). Thus, it is useful to determine the effects in the later stages of development prior to
73 metamorphic climax.

74 A technique increasingly being employed to derive detailed information from biological
75 samples is Fourier-transform infrared (FTIR) spectroscopy, which is used in three major
76 sampling modes: transmission, reflectance and attenuated total reflectance (ATR) (Kazarian

77 and Chan, 2006). FTIR spectroscopy has been widely used in several biological applications
78 including the diagnosis of disease states (Baker et al., 2014; Bellisola and Sorio, 2012; Ellis
79 and Goodacre, 2006; Kazarian and Chan, 2006; Movasaghi et al., 2008; Toyran et al., 2006),
80 imaging of tissue composition (Greve et al., 2008; Purna Sai and Babu, 2001), identifying
81 microorganisms (Mariey et al., 2001; Naumann et al., 1991) and for analysing the effects of
82 environmental contaminants at the cellular and tissue level (Abdel-Gawad et al., 2012;
83 Cakmak et al., 2006; Cakmak et al., 2003; Corte et al., 2010; Holman et al., 2000; Llabjani et
84 al., 2012; Malins et al., 2006; Obinaju et al., 2014; Palaniappan and Vijayasundaram, 2008,
85 2009; Palaniappan et al., 2011; Ukpebor et al., 2011).

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

95 FTIR spectroscopy generates large detailed datasets so is often coupled with a multivariate
96 approach such as principal component analysis (PCA) or linear discriminant analysis (LDA)
97 to extract useful information from the resulting IR absorbance spectrum (Ellis and Goodacre,
98 2006). Used in this manner, FTIR spectroscopy is able to distinguish between different
99 groups on the basis of their biochemical fingerprint and also identifies which wavenumbers,
100 and therefore which chemical bonds are altered between samples (Trevisan et al., 2012).
101 Additionally, use of derivative spectra may allow more detailed examination of overlapping

102 peaks in the spectrum, thus allowing the quantification of particular biochemical constituents
103 (Rieppo et al., 2012).

104 The aim of this study was to determine whether tadpoles of the Common frog, *Rana*
105 *temporaria*, at a pro-metamorphic stage in development. i.e. following the emergence and
106 development of hindlimbs [Gosner stage 38-40 (Gosner, 1960)] collected from ponds with
107 varying water quality could be distinguished on the basis of their ATR-FTIR spectral
108 fingerprint. Detection of underlying differences may suggest the possible application of IR
109 spectroscopy as an environmental monitoring tool. Liver and muscle samples were taken
110 from individual tadpoles and analysed with ATR-FTIR spectroscopy; previous studies in
111 amphibians using other techniques have demonstrated changes in metabolic constituents such
112 as lipid, protein and glycogen following exposure to environmental contaminants in these
113 tissues (Dornelles and Oliveira, 2014; Gendron et al., 1997; Gurushankara et al., 2007;
114 Melvin et al., 2013). Other tissues less routinely used in assessing amphibian health (heart,
115 kidney and skin) were also analysed in this study, thus providing spectral fingerprints of
116 several different tissues of an amphibian species. Although applied to fish in several studies
117 (Cakmak et al., 2006; Henczova et al., 2008; Malins et al., 2006; Malins et al., 2004; Obinaju
118 et al., 2014), this is the first time to our knowledge that IR spectroscopy has been used to
119 characterise amphibian tissue.

120

121 **Materials and Methods**

122 **Pond Selection**

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

125 1. Whinton Hill (WH), Plumpton, Cumbria is a farm consisting primarily of arable land,
126 which is routinely sprayed with herbicides and fungicides. The pond surveyed was the
127 shallow pond of a pair of deep and shallow ponds (32 m long × 8 m wide x 0.5 m
128 deep), located in a boggy field, and fed by a field drain from approximately 30 ha
129 ($3 \times 10^5 \text{ m}^2$) of farmland.

130 2. Crake Trees (CT), Crosby Ravensworth is a farm used as beef grazing land and
131 marginal arable land, which has been accepted onto Natural England's Higher Level
132 Environmental Stewardship Scheme and uses minimal quantities of pesticides, with
133 buffer zones to prevent pesticide run-off into water courses. The pond surveyed was
134 the second pond of a pair of shallow ponds (each 17 m long × 6 m wide x 0.5 m
135 deep), located in a field corner, and fed by surface runoff from approximately 20 ha
136 ($2 \times 10^5 \text{ m}^2$) of farmland.

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

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

146 **Water sampling**

147 Samples of surface water (15-20 cm depth) were collected over the amphibian breeding
148 season (March-August) in 2012. Water samples for organics analysis were only available
149 from PF for March and April, and March, April and June for nutrient analysis. Water samples

150 were collected in methanol-rinsed amber bottles for organics analysis and acid-washed
151 bottles for nutrient analysis and then stored at 4°C until analysis.

152 **Chemical analysis**

153 The concentrations of trace metals (Al, Fe, Mg, Ca, K and Na) were determined in filtered
154 acidified water samples (1% HNO₃) using ICP-OES (Perkin Elmer DV 7300) while
155 concentrations of major anions (Cl, NO₃-N, SO₄-S) as well as phosphate, ammonium and
156 total organic N (TON) were determined using colorimetric methods performed by the Centre
157 for Ecology and Hydrology (Lancaster) in a quality-assured, previously published method
158 (Neal et al., 2000). For trace organic chemical analysis, 800 mL of sample water (adjusted to
159 pH 9.5 with borate buffer) underwent liquid-liquid (1:1) extraction using dichloromethane
160 (DCM) on a laboratory shaker (Gerhardt Shaker LS-500) followed by separation and
161 evaporation of the DCM on a rotary evaporator (rotavapor Büchi R-210). The concentrated
162 DCM extracts (700 µL) underwent initial qualitative screening using GC-MS (Agilent 6890N
163 GC and Agilent 5973 single quad MS) operated by ChemStation software (D.02.00.275) with
164 subsequent mass spectral identification using Mass Hunter software and comparison to the
165 NIST spectral library. The following chemicals were detected: aniline, metazachlor,
166 acetochlor, dimetachlor, triethylphosphate, tributylphosphate, tris(2-chloroethyl)phosphate,
167 tris(1-chloro-2-propyl)phosphate and flusilazole. These compounds were quantitatively
168 analysed using authentic standards using a 7-point calibration, with standards ranging from 0-
169 2000 ng/L for each analyte. Internal standards comprising of ¹³C-labelled aniline, acetochlor
170 and metalochlor were added to sample extracts and calibration standards prior to analysis.
171 Limits of quantification (LOQ) ranged from 5-10 ng/L (aniline 200 ng/L) with recoveries
172 based on spiked water samples ranging from 80-120%. Water samples were also analysed
173 for more polar, water-soluble compounds. For this analysis, 10 mL of a water sample was

174 filtered (using a 0.2 µm RC syringe filter), spiked with internal standards and analysis
175 performed on a Waters Acquity Binary Ultra Performance Liquid Chromatograph (UPLC)
176 (Waters Corporation, Milford, USA) coupled to a Waters Premier XE triple quadrupole mass
177 spectrometer (LC-MS/MS) operated by MassLynx software V 4.1. The MS was operated in
178 electrospray positive (ESI+) ionisation mode with multiple reaction monitoring (MRM). A
179 250 µL aliquot was injected via an autosampler, with analyte separation performed under a
180 MeOH/H₂O (with 5 mmol/L ammonium acetate added to both phases) mobile gradient eluted
181 through an Acquity BEH C₁₈ column (1.7 µm, 2.1 mm × 50 mm) fitted with a VanGuard
182 Acquity precolumn. The following compounds, including pesticides and pharmaceuticals,
183 were qualified/quantified: chlorotoluron, isproturon, caffeine, tebuconazole, prochloraz,
184 carbendazim, gabapentin, acetaminophen, benzotriazole, benzotriazole-methyl, ketoprofen,
185 dimethyl-chlorotoluron, metconazole, spiroxamine, boscalid, and erythromycin. Samples
186 were analysed separately for glyphosate and its degradation by-product,
187 aminomethylphosphonic acid (AMPA), using LC-MS/MS. For the analysis of glyphosate and
188 AMPA, 8 mL of a water sample was acidified to pH 1 (addition of 160 µL of 6 M HCl) and
189 subject to derivatisation using 9-fluorenylmethyl chloroformate using a previously published
190 method (Ibáñez et al., 2006). Analytes were separated using the same LC-MS/MS instrument
191 and method above. Internal standards comprised of 1,2-¹³C₂ ¹⁵N Glyphosate and ¹³C ¹⁵N
192 AMPA with a 7-point calibration with standards ranging from 0 to 2000 ng/L. Ionisation was
193 through ESI+ (precursor ions) and MRM (product ions). LOQs were 10 ng/L for both
194 glyphosate and AMPA with recoveries ranging from 70-130% (water spiked with internal
195 standards).

196

197 **Tadpole collection**

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

218 **ATR-FTIR spectroscopy**

219 Spectra of each sample were obtained using a Tensor 27 FTIR spectrometer with Helios ATR
220 attachment (Bruker Optics Ltd, Coventry, UK) containing a diamond crystal ($\approx 250 \mu\text{m} \times 250$

221 μm sampling area). Spectra were acquired at 8 cm^{-1} resolution with $2\times$ zero-filling, giving a
222 data-spacing of 4 cm^{-1} over the range $400\text{-}4000\text{ cm}^{-1}$. Ten-25 spectra were acquired from
223 each sample; these were averaged in order to give a representative spectrum per
224 organ/tadpole. Distilled water was used to clean the crystal in between analysis of each
225 sample. A new background reading was taken prior to the analysis of each sample in order to
226 account for changes in atmospheric conditions.

227 **Spectral pre-processing** Spectra were cut at the biochemical cell fingerprint region (1800-
228 900 cm^{-1}), baseline corrected using Savitzky-Golay (SG) 2^{nd} order differentiation (2^{nd} order
229 polynomial and 9 filter coefficients), and vector normalised. Processing the data with second
230 derivative spectroscopy allows overlapping peaks in the absorbance spectrum to be resolved,
231 thus allowing more detailed analysis of particular peaks. By taking second derivatives,
232 constant and linear components of baseline errors are also removed (Rieppo et al., 2012). For
233 broad spectra the derivative intensity decreases with increasing derivative order, whereas for
234 sharp spectra, the reverse is true. Therefore the underlying shape of the spectrum determines
235 the intensity of the derivative spectrum, with flat peaks decreasing in intensity with each
236 derivative order, and sharp peaks increasing in intensity, thus allowing small sharp peaks
237 overlapped by broad flat peaks to be exposed (Kus et al.).

238 SG derivation is applied by fitting a simple polynomial to a small section of given size to the
239 spectrum and calculates the derivative of the polynomial in the centre point of this section
240 (Rinnan et al., 2009). In this study, a 2^{nd} order polynomial and nine smoothing points were
241 employed in the SG algorithm. This resulted in the loss of 4 wavenumbers from each end of
242 the spectrum as a symmetric window smoothing is used requiring the number of data points
243 on each side of the centre point to be the same, and the number of wavenumbers lost equals
244 the number of smoothing points minus one (Rinnan et al., 2009). The polynomial order and
245 number of smoothing points was selected based upon a compromise between noise removal

246 and signal distortion as no method exists which is able to eliminate all noise without losing
247 important information. A small number of smoothing points and a high polynomial degree
248 can give a noisy spectrum, whereas a large number of smoothing points and a low
249 polynomial degree can distort the spectrum (Vivó-Truyols and Schoenmakers, 2006). .

250 **Multivariate analysis**

251 **PCA**

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

262 After the data were mean-centred, PCA was employed to reduce the 227 absorbance values
263 into 10 principal components (PCs), which represented > 95% of the variance in the datasets.
264 The most statistically significant PCs were retained, as these represented the best separation
265 in the data (see table S1 in SI for *P* values of scores for each PC for each tissue) (Malins et
266 al., 2006; Malins et al., 2004). Loadings from the most significant PCs were used to identify
267 wavenumbers accounting for the separation between ponds. A peak detecting algorithm was

268 employed to determine the five largest loadings values (constrained by a minimum of 20 cm⁻¹
269 spacing between values).

270 **LDA**

271 In addition to PCA, linear discriminant analysis (LDA) was also employed to improve the
272 discrimination between the spectra of tissues between ponds. LDA is a supervised technique
273 (the class groupings are known *a priori*) which maximises the differences between classes,
274 while minimising within-class heterogeneity (Martínez and Kak, 2001). For small data sets,
275 like the ones in this study, LDA alone can over-fit the data, resulting in good data separation
276 by chance, as the number of variables (wavenumbers) are much larger than the number of
277 samples, therefore a data reduction technique is necessary to overcome this (Gromski et al.,
278 2015). In this case, PCA was used prior to LDA to reduce the variables to a smaller number
279 of PCs, which still represented ~95% of the variance in the data (see SI table S2 for the
280 number of PCs selected for each data set). PCA also removes colinearity between variables
281 (Gromski et al., 2015)

282 Data were standardised prior to the application of PCA-LDA and leave-one-out cross
283 validation, where a small portion of the data set is used to train the model was used, again to
284 prevent over-fitting and so as to prevent bias in the output (Trevisan et al., 2012). The output
285 from PCA-LDA again generates scores and loading plots, however this technique generates
286 $n-1$ linear discriminants (LDs), which optimally separate n classes; in the case of this study a
287 one-dimensional scores plot and one loading is generated per data set. To aid with the
288 interpretation of the scores plots, a linear discriminant classifier (LDC) was also employed,
289 which uses the same principle as LDA but fits a Gaussian classifier to separate the data and
290 provides a % classification rate for each data set (Trevisan et al., 2012). Data were
291 standardised and cross-validated as before.

292 **Comparison of absorbance values**

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

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

302 .

303 **Statistical analysis**

304 Body condition indices (BCI) were calculated for each tadpole as follows: $(\text{body mass}/\text{SVL}^3)$
305 $\times 100$ (Melvin et al., 2013). Hepatosomatic indices (HSI) were also calculated for tadpoles
306 collected from CT and WH in 2013 as follows $(\text{liver mass}/\text{body mass}) \times 100$.

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

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

317 **Results and discussion**

318 **Water quality analysis**

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

330 Results from the analysis of water samples for trace organic chemicals are shown in Table 2.
331 Screening of the water samples collected from CT, PF and WH revealed large differences in
332 the organic contaminants detected. CT and PF appeared to be the least contaminated sites;
333 xenobiotics detected in water samples from these sites included caffeine, several OP flame
334 retardants and the pharmaceutical drugs acetaminophen and gabapentin, commonly found in
335 surface waters (Mompelat et al., 2009). Both sites also had detectable levels of

336 aminomethylphosphonic acid (AMPA), the degradation product of glyphosate.
337 Aminomethylphosphonic acid may also form following the degradation of other phosphonate
338 compounds including detergents, so is not necessarily indicative of glyphosate residue (Botta
339 et al., 2009; Van Stempvoort et al., 2014). However, as glyphosate was also detected at PF
340 and AMPA levels were higher here than at CT, this suggests that glyphosate was the likely
341 source. Interestingly, relatively high levels of benzotriazole and benzotriazole-methyl were
342 detected at CT. These compounds are commonly used as corrosion inhibitors so may have
343 leached from farm machinery etc and they were frequently detected in a recent European-
344 wide survey of river water (Loos et al, 2009). Water samples collected from PF also showed
345 detectable levels of naphthalene, which has previously been associated with detrimental
346 effects in aquatic species, although at much higher concentrations than those found in this
347 study (Farré et al., 2008; Pillard et al., 2001).

348 Water samples collected from WH demonstrated relatively high levels of aniline, a
349 compound generated during the degradation of several herbicides and pesticides (Xiao et al.,
350 2007) early in the season. In contrast to CT, the other agricultural site, several pesticides,
351 particularly fungicides were detected at WH during April and June: these included
352 carbendazim, flusilazole, tebuconazole, boscalid, dimethachlor, chlorotoluron, metconazole
353 and glyphosate. Carbendazim and flusilazole displayed the highest concentrations in April,
354 with much lower levels in June and August. Glyphosate and boscalid showed the highest
355 concentrations in June, coinciding with tadpole metamorphosis, but with much lower levels
356 by August. Like CT, WH showed detectable levels of the corrosion inhibitors benzotriazole
357 and benzotriazole-methyl.

358 All three sites showed detectable levels of OP flame retardants, the particular type varying
359 between each site: TEP present at PF and WH but absent from CT; TBP only present at CT.

360 TCPP and TCEP were detected at all three sites, with TCPP generally detected at the highest
361 levels, particularly at WH, where it reached a maximum level of 1600 ng/L, which is similar
362 to that found in other studies, where it is the dominant OP flame retardant (van der Veen and
363 de Boer, 2012). These compounds are frequently detected in surface waters due to their lack
364 of biodegradability in wastewater treatment (Regnery and Püttmann, 2010) (Fries and
365 Püttmann, 2003). As PF receives treated wastewater as well as run-off from landfill, this may
366 explain the higher levels found here.

367 **Body measurements**

368 As shown in Figure 1, tadpoles from PF (2012) were significantly larger than those from CT
369 (2012) on all measures of body size (fig. 1A-D); tadpoles from PF also had a significantly
370 higher BCI (fig. 1E), as determined by two sample t-tests (SVL: $t_8 = 4.02$, $P = 0.004$; HW: t_8
371 $= 2.83$, $P = 0.022$; tail length: $t_8 = 4.67$, $P = 0.002$; body mass: $t_8 = 5.28$, $P = 0.0007$; BCI: t_8
372 $= 3.08$, $P = 0.015$). This finding is somewhat unexpected considering that CT is regarded as
373 the pond with better water quality. However, there were many factors not measured in this
374 study that could account for the differences. Such factors include selection pressures such as
375 predation/presence of competing species, population density, food availability, abiotic factors
376 (pH, temperature and dissolved oxygen), and changes in pond depth.

377 In contrast, tadpoles collected from CT (2013) only differed from those collected from WH
378 (2013) on measures of tail length and body mass (fig. 2A-E); tadpoles from CT were
379 significantly larger on these two measures (Two sample t-test: SVL: $t_{18} = 1.41$, $P = 0.17$; HW:
380 $t_{18} = 0.57$, $P = 0.57$; tail length: $t_{18} = 2.40$, $P = 0.027$; body mass: $t_{18} = 2.22$, $P = 0.04$; BCI: t_{18}
381 $= 1.16$, $P = 0.26$). Additional measurements were made for tadpoles from CT (2013) and those
382 from WH (2013) of liver mass and HSI (fig. 2F and 2G), with the finding that tadpoles from
383 WH had significantly larger values of liver mass and HSI than those from CT (Two sample t-
384 test: liver mass: $t_{18} = 2.31$, $P = 0.033$; LSI: $t_{18} = 4.23$, $P = 0.0005$). Again, the differences in

385 body mass and tail length could simply be due to uncontrolled factors such as food
386 availability and pond size (Vences et al., 2002). However, the greater liver mass and HSI of
387 tadpoles from WH in comparison to those from CT is indicative of liver inflammation or
388 growth abnormalities (Olivares et al., 2010). Larger livers may be reflective of biochemical
389 changes that occur as an organism attempts to maintain homeostasis and have been associated
390 with exposure to environmental contaminants in aquatic species, including amphibians
391 (Edwards et al., 2006; Kim et al., 2013; Lowe-Jinde and Niimi, 1984; Melvin et al., 2013;
392 Tetreault et al., 2003). Therefore the larger HSI seen in tadpoles from WH, coupled with their
393 smaller mass and tail length is a clear indicator of environmental stress most likely
394 attributable to poor water quality and marked by environmental contamination through
395 agricultural run-off at this site.

396 *Rana temporaria* tadpoles, like other species, are able to show developmental plasticity,
397 where developmental rate is adjusted according to environmental conditions, producing
398 smaller metamorphs under conditions of low food availability and high population density.
399 Food availability and quality may also affect metamorphic performance and body size, with
400 higher protein diets associated with a larger size at metamorphosis, (Audo et al., 1995;
401 Beebee and Richard, 2000; Kupferberg, 1997; Álvarez and Nicieza, 2002) although this can
402 vary with species (Casta et al., 2006). Therefore there is uncertainty regarding the effect of
403 these uncontrolled factors and their interactions on body size parameters and a future study
404 would aim to control such factors. Tadpoles collected in this study were at stages 38-40; a
405 stage of development regarded as pro-metamorphic and defined as when the hindlimbs
406 emerge and differentiate (Chambers et al., 2011; Gosner, 1960). Whilst slight differences in
407 developmental stage can impact on size, the stages between 30-40 are considered to be one of
408 stability in the development of key traits (Gosner, 1960). The body size of tadpoles peaks in
409 late pro-metamorphosis before forelimb emergence (stage 42) and declines during

410 metamorphic climax (Álvarez and Nicieza, 2002). Therefore as the tadpoles collected in this
411 study were at a late stage in pro-metamorphosis, but before metamorphic climax, the
412 differences in size are unlikely to be due to this factor.

413 **ATR-FTIR spectroscopy**

414 Figures 3A-F show the 2-dimensional scores plots and corresponding loadings following
415 PCA for tissues which separated significantly in tadpoles collected from CT (2012) and PF
416 (2012) (tentative assignments in table S3 in SI); 1-dimensional scores plots are shown in
417 figures S1A-E in the SI, with corresponding statistical and classifier analysis shown in tables
418 S4 and S5 respectively with tentative assignments in table S6. Figures 4A-E show the mean
419 spectra for each tissue type following second derivative analysis.. Figures 5A-E show the 2-
420 dimensional scores plots following PCA for tissues analysed from tadpoles collected from
421 CT (2013) and WH (2013); corresponding loadings are shown in figure 6A-D (tentative
422 assignments in table S3 in SI), with 1-dimensional scores plots following analysis with PCA-
423 LDA shown in fig S2A-E in the SI; corresponding statistical and classifier analysis are shown
424 in tables S4 and S5 respectively with tentative assignments in table S6. Figures 7A-E show
425 the mean spectra for each tissue type following second derivative analysis. Table 3 shows a
426 list of all the major second derivative peaks from each tissue and their corresponding
427 tentative assignments. Raw spectra are shown in figures S3 and S4 in the SI.

428 **Liver samples**

429 Results from ATR-FTIR spectroscopy demonstrated that the liver was the tissue which best-
430 distinguished tadpoles collected from CT or PF in 2012, and also tadpoles collected from CT
431 or WH in 2013. This is perhaps expected, as the liver is the organ responsible for metabolism
432 of xenobiotics in vertebrates, including amphibians; therefore any changes induced by
433 environmental contamination may be detected here (Fenoglio et al., 2011). In addition, the

434 liver is an energy store in tadpoles, and lipids, protein and glycogen are utilised for the
435 completion of metamorphosis (Sheridan and Kao, 1998); thus changes in the levels of these
436 constituents may be reflective of the energy status and thus condition of the tadpole (Melvin
437 et al., 2013). However, other factors such as food availability and composition and predation
438 may also impact the stress status of amphibians in synergy with chemical-insult (Relyea and
439 Mills, 2001). This must be taken into account in the interpretation of the results and is a
440 limitation of this study.

441 Comparison of liver samples from tadpoles collected from CT (2012) and those from PF
442 (2012) demonstrated significant separation along PC1 (fig. 3A), following PCA which was
443 associated with alterations in C-O ribose (991 cm^{-1}), carbohydrate (1153 cm^{-1}), Amide II
444 (1516 cm^{-1}), C=N cytosine (1601 cm^{-1}) and Amide I β -sheets (1624 cm^{-1}) as seen in the
445 loadings plot in figure 3B and table S3 (SI). Further analysis with PCA-LDA led to improved
446 separation in the scores plot (SI fig. S1A), with a correct classification rate of 99 and 90% for
447 CT and PF respectively (see tables S4 and S5 in SI). Loadings were in regions associated
448 with carbohydrates and proteins as before, as well as some lipid contribution (table S6 in SI).
449 Analysis of the second derivative peak heights also showed significant differences between
450 tadpole livers from CT (2012) and those from PF (2012) in regions associated with protein
451 (Amide I and II), with the finding that peak heights in these regions were larger in tadpole
452 livers from PF (2012) in comparison to those from CT (2012) (table 3, fig. 4A).

453 Results from PCA demonstrated that tadpole livers from CT (2013) segregated from tadpole
454 livers from WH (2013) along PCs 1 and 4 (fig. 5A); the major loadings accounting for this
455 separation were in regions assigned as C-O ribose, ($988\text{-}991\text{ cm}^{-1}$), glycogen (1022 cm^{-1}),
456 symmetric phosphate stretching (1080 cm^{-1}), Amide I ($1616, 1624, 1639$ and 1697 cm^{-1}) and
457 stretching of triglycerides (1744 cm^{-1}), as shown in fig. 6A and table S3 (SI). Supervised
458 analysis with PCA-LDA showed an improvement in the separation of the data in the scores

459 plot with a high classification accuracy of 98 and 100% for CT and WH respectively, as
460 shown in fig. S2A and table S4 in the SI. Loadings associated with this separation were again
461 in regions assigned as carbohydrates, proteins and lipids (table S6 in SI) Analysis of the
462 second derivative peak heights showed larger peak heights in regions associated with proteins
463 (both Amide I and II) and symmetric phosphate stretching vibrations in tadpole livers from
464 WH (2013) in comparison to CT (2013); however, in regions associated with lipids, peak
465 heights were larger in tadpole livers from CT (2013) (table 3 and fig. 7A).

466 Lipid levels are generally low in pre-metamorphic tadpoles, rising during pro-
467 metamorphosis, as lipids are the main energy source metabolised during metamorphic climax
468 (Sheridan and Kao, 1998). As the tadpoles in this study were at the pro-metamorphic stage of
469 development (emergence of hindlimbs), it was expected that a clear lipid peak would be
470 present in the liver (figs. 4A and 7A, see SI figs. S3A and S4A). Previous studies have
471 demonstrated changes in lipid levels in the livers of tadpoles and adult amphibians exposed to
472 pesticides, with some reporting a decrease (Dornelles and Oliveira, 2014; Gurushankara et
473 al., 2007), while others report an increase (Melvin et al., 2013) or no change (Zaya et al.,
474 2011). Although no differences in hepatic lipid levels were detected between tadpoles
475 collected in 2012 from CT and PF, tadpoles collected in 2013 from WH had significantly
476 lower levels of hepatic lipid than those from CT in the same year. This coupled with the
477 finding that tadpoles from WH had significantly larger livers than those from CT is
478 suggestive of exposure to an environmental stressor, which may have resulted in the tadpoles
479 using the lipid stored in the liver as an energy source to overcome the noxious stimuli and
480 maintain homeostasis.

481 Glycogen levels may be altered in amphibian livers exposed to environmental stress due to
482 contaminant exposure or hypoxia (Gendron et al., 1997; Loumbourdis and Kyriakopoulou-
483 Sklavounou, 1991); levels may decrease as the organism utilises this energy source in order

484 to overcome the stressful situation. Results from PCA demonstrated separation between
485 tadpole livers from CT (2012) and those from PF (2012) along PC1, with one of the largest
486 identified loadings associated with carbohydrates including glycogen (1153 cm^{-1}). Separation
487 along PC4 between tadpoles from CT (2013) and those from WH (2013) also had some
488 contribution from glycogen (1022 cm^{-1}).

489 Protein levels were also found to be altered in tadpole livers collected from CT (2012) in
490 comparison to those from PF (2012), and between tadpole livers from CT (2013) and those
491 from WH (2013), with the finding that tadpoles from CT had lower protein levels than those
492 from the other two sites. This is unexpected as CT is considered to have the best water
493 quality based on the analysis conducted in this study. Reduced protein levels have previously
494 been associated with pesticide exposure/hypoxia in amphibian livers (Dornelles and Oliveira,
495 2014). However, increased protein levels have also been associated with pesticide exposure
496 in the livers of fish, with the suggestion that higher protein synthesis is initiated to
497 compensate for protein loss, leading to a higher protein turnover (Oruç and Üner, 1999).

498 **Muscle samples**

499 No significant differences were detected between tadpole muscle samples from CT (2012)
500 and those from PF (2012) following analysis with either PCA or PCA-LDA (figs. 3C, 4B and
501 S1B in SI). In contrast, the comparison of muscle tissue from tadpoles collected from CT
502 (2013) and WH (2013) with PCA demonstrated separation along PC1 (fig. 5B) in regions
503 associated with the OCH_3 band of polysaccharides (972 cm^{-1}) glycogen (1022 cm^{-1}), C-O
504 stretching of the phosphodiester and ribose (1065 cm^{-1}), carbohydrates (1154 cm^{-1}) and
505 Amide II (1501 cm^{-1}), as shown in the loadings plot in figure 6B and table S3 (see SI).
506 Analysis with PCA-LDA led to some improvement in the separation of the data in the scores
507 plot, with a reasonable classification accuracy of 71 and 80% for tadpoles from CT and WH
508 respectively (fig. S2B and tables S4 and S5 in SI). Loadings confirmed separation based upon

509 changes in the phosphodiester and protein regions, with additional contributions from lipids
510 (table S6 in SI). Second derivative peak heights show greater absorbance in muscle samples
511 from CT (2013) in regions associated with glycogen, carbohydrates, symmetric phosphate
512 and Amide I and II; in regions associated with asymmetric phosphate, and Amide III, peaks
513 heights were larger in tadpole muscle samples from WH (2013) (fig. 7B, table 3).
514 Lower levels of both glycogen and protein have previously been found in muscle samples
515 from tadpoles exposed to pesticides (Dornelles and Oliveira, 2014). Reduced glycogen levels
516 in muscle tissues have also been associated with pesticide-induced stress in several species of
517 fish, where glycogenolysis and glycolysis occur in order to provide more energy so that the
518 organism can overcome stressful stimuli (Ferrando and Andreu-Moliner, 1991; Gluth and
519 Hanke, 1985; Oruç and Üner, 1999).

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

521 Differences between the other tissues analysed: heart, kidney and skin were small in
522 comparison to the differences in liver and muscle tissue. Whilst analysis with PCA showed
523 no significant differences between hearts from tadpoles collected from CT (2012) and those
524 from PF (2012) (fig. 3D), the use of PCA-LDA led to an improvement in data separation, as
525 shown in fig. S1C, and tables S4 and S5 in the SI. The largest loadings values accounting for
526 the separation were in regions associated with symmetric phosphate stretching vibrations
527 (1088 cm^{-1}) as well as carbohydrates (1138 cm^{-1}) and collagen (1196 cm^{-1}), as shown in table
528 S6 (SI). Analysis of the second derivative peak heights revealed a significant difference at the
529 peak associated with asymmetric phosphate stretching; where it was larger at CT (2012) than
530 PF (2012) as shown in figure 4C. Tadpole hearts collected from CT (2013) and WH (2013)
531 demonstrated some separation along PC3 following PCA (fig. 5C), but this was not
532 statistically significant ($P=0.06$); however a significant improvement in data separation was
533 seen when PCA-LDA was employed, with a classification accuracy of 76 and 71% for

534 tadpoles from CT and WH respectively (fig S2C and table S4 and S5 in SI) Loadings values
535 confirmed the separation in regions assigned as collagen and protein (amide I) as shown in
536 table S6 (SI). Analysis of the second derivative peak heights demonstrated significant
537 differences between CT (2013) and WH (2013) in the region associated with CH₃ bending of
538 lipids, where peak height was smaller at CT than WH, and in the Amide I region, where the
539 peak height was larger at CT in comparison to WH (fig 7C, table 3). Previous work in fish
540 has also shown differences in heart tissue in fish collected from polluted rivers, in regions
541 associated with Amide I and lipids, as measured with ATR-FTIR spectroscopy (Obinaju et
542 al., 2014). Whilst no previous spectroscopic measurements of tadpole hearts have been
543 published, it is known that cardiac output in tadpoles may be altered in response to stressful
544 situations induced by xenobiotics (Costa et al., 2008). Future work could attempt to correlate
545 differences in cardiac output with the spectral signature.

546 No significant differences were found in the spectral signature of tadpole kidney samples
547 collected from CT (2012) or PF (2012) when analysed with either PCA or PCA-LDA (figs.
548 3E and 4D, fig. S1D in SI). However, PCA revealed significant separation between kidney
549 samples from tadpoles from CT (2013) and those from WH (2013) as shown in fig. 5D.
550 Loadings from PCA revealed that these differences were attributable to protein (Amide I and
551 II) and lipid alterations (fig. 6C, see SI table S2). Analysis with PCA-LDA actually led to
552 poorer data separation, as shown in fig S2D and tables S4 and S4 in the SI which may occur
553 when working with small data sets, as in this study (Martínez and Kak, 2001). Second
554 derivative peak analysis also confirmed alterations associated with Amide I/stretching of fatty
555 acids at 1670 cm⁻¹, with kidney samples from CT having a larger peak height than those from
556 WH (fig 7D, table 3). The kidney, like the liver is susceptible to the effects of several
557 toxicants, with a previous study in amphibians finding differences in the structure and
558 histochemistry of kidney samples from adult frogs collected from polluted compared to

559 unpolluted sites (Fenoglio et al., 2011). Alterations in the kidneys of fish from polluted sites
560 have previously been detected using ATR-FTIR spectroscopy; these were also in regions
561 associated with Amide I and II of proteins, as in this study; however, no alterations in lipids
562 were detected, in contrast to that found in this study (Obinaju et al., 2014).

563 There were no differences detected in the tadpole skin samples from CT (2012) in
564 comparison to those from PF (2012) when the data were analysed with PCA, PCA-LDA or
565 using the peak absorbances (fig. 3F and 4E, table 3, fig. S1E; tables S4 and S5 in SI). In
566 contrast, skin samples taken from tadpoles from CT (2013) and WH (2013) showed some
567 separation along PC3 following PCA (fig. 5E); this was mainly in regions associated with
568 Amide I (1616, 1640 cm^{-1}), with some contribution from lipids (1497, 1694 cm^{-1}), as shown
569 in the loadings plot in figure 6D and table S3 (see SI). The use of PCA-LDA led to improved
570 data separation as shown in the scores plot in fig S2E and tables S4 and S5, associated with
571 amide I proteins as before, with some contributions from collagen and C-O stretching of
572 carbohydrates (table S6 in SI). Analysis of second derivative peak heights showed no
573 significant differences between skin samples from CT (2013) and WH (2013) (fig. 7E, table
574 3). That some separation was apparent between skin samples is of note, given that the skin is
575 the first organ that environmental contaminants come into contact with in amphibian species.
576 The skin of amphibians is permeable to water, where it plays a vital role in respiration and
577 osmoregulation; therefore the skin provides a significant exposure route to chemicals in
578 addition to that from ingestion and has previously been proposed as a bioindicator of
579 deleterious environmental conditions, with structural changes detected following exposure to
580 environmental contaminants (Bernabò et al., 2013; Fenoglio et al., 2009; Fenoglio et al.,
581 2006; Haslam et al., 2014). The skin of larval amphibians may also be more susceptible to
582 chemical insult than that of adults due to the lack of specialised cells and many of the
583 detoxifying enzymes, which are present in adults (Fenoglio et al., 2009).

584 **Conclusions**

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

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

605 This study is the first to characterise a range of tissues from an amphibian species with ATR-
606 FTIR spectroscopy. Additionally, this study demonstrates the possible use of this technique
607 as a rapid and cost-effective environmental monitoring tool. This technology could be of

608 great promise as an early warning for assessing the health of amphibian populations exposed
609 to varying or diminished water quality.

610

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617

618 **Figure headings**

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

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

632 **Figure 3.** Two-dimensional scores plots and significant loadings following principal
633 components analysis (PCA) of ATR-FTIR spectra obtained from several different tissues
634 taken from *Rana temporaria* pro-metamorphic tadpoles. Tissues are liver **(A: scores, B:**
635 **loadings)**, muscle **(B)**, heart **(C)**, kidney **(D)** and skin **(E)**. Tadpoles were collected in 2012
636 from CT: a rural agricultural pond with no pesticide input or PF: an urban pond impacted by
637 wastewater and landfill run-off ($n = 10$). Two sample *t*-tests were employed to detect
638 differences in the PC scores between ponds within each year. Asterisks indicate a *P* value of
639 < 0.05 (*) or < 0.01 (**). Values in parentheses show the contribution of each principal
640 component to the overall variance.

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

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

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

662 **Figure 7.** Second derivative mean spectra of tissues taken from *Rana temporaria* pro-
663 metamorphic tadpoles. Spectra were cut at the biochemical fingerprint region (1800-900 cm⁻¹),
664 processed with Savitzky-Golay second-order differentiation and vector-normalised.

665 Tissues are liver (**A**), muscle (**B**), heart (**C**), kidney (**D**) and skin (**E**). Tadpoles were collected
666 in 2013, from CT: a rural agricultural pond with no pesticide input or WH: an agricultural
667 pond known to be impacted by pesticides ($n = 20$). Peaks are labelled with the corresponding
668 wavenumbers. Two sample t -tests were employed to detect differences in the second
669 derivative peak height at each labelled peak between ponds within each year. Asterisks
670 indicate a P value of < 0.05 (*) or < 0.01 (**).

671

672

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 runoff. 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 March	CT April	CT June	CT August	PF March	PF April	PF June	WH March	WH April	WH June	WH August
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
NH₄-N	0.028	0.412	1.47	0.014	0.06	0.128	1.28	0.303	0.282	5.50	0.033
NO₃-N	< 0.001	0.219	0.012	1.62	0.427	1.18	0.016	0.01	2.49	0.017	0.912
PO₄-P	0.029	0.033	0.121	0.15	0.068	0.304	0.17	0.006	0.639	0.584	0.089
SO₄-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
Metconazole	<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

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	$\nu(\text{CO})$, $\nu(\text{CC})$ 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	$\delta(\text{CH})$, 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 β -sheets ⁵	NS	NS

	1647	Amide I ¹	NS	CT > WH *
	1670	Amide I (anti-parallel β -sheet) $\nu(\text{C}=\text{C})$ 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	ν C-O and δ 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 β -sheets ⁵	NS	NS
	1643	Amide I, C=O stretching vibrations ¹	NS	NS
	1670	Amide I (anti-parallel β -sheet) ν (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 CH ₃ 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 β -sheets ⁵	NS	NS
	1647	Amide I ¹	NS	NS
	1670	Amide I (anti-parallel β -sheet) ν (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 ν (CC), lipid cis ⁹	NS	NS
	1080	ν (CC), lipid trans ⁹	NS	NS
	1119	ν (CC), lipid trans ⁹	NS	NS
	1165	ν (CC), δ (COH) ⁹	NS	NS
	1231	Amide III protein ^{1, 10}	NS	NS
	1312	Amide II protein ¹	NS	NS
	1393	δ [C(CH ₃) ₂] symmetric ^{1,9}	NS	NS
	1447	δ [C(CH ₃) ₂] symmetric ¹	NS	NS
	1512	Amide II ¹	NS	NS
	1543	Amide II ¹	NS	NS

1624	$\nu(\text{C=O})$, amide I, β ⁹	NS	NS
1643	Collagen ¹⁰	NS	NS
	$\nu(\text{C=O})$, amide I, α ⁹		
1690	Amide I ¹	NS	NS
1744	Lipid ¹	NS	NS

ν : 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.

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Figure 1

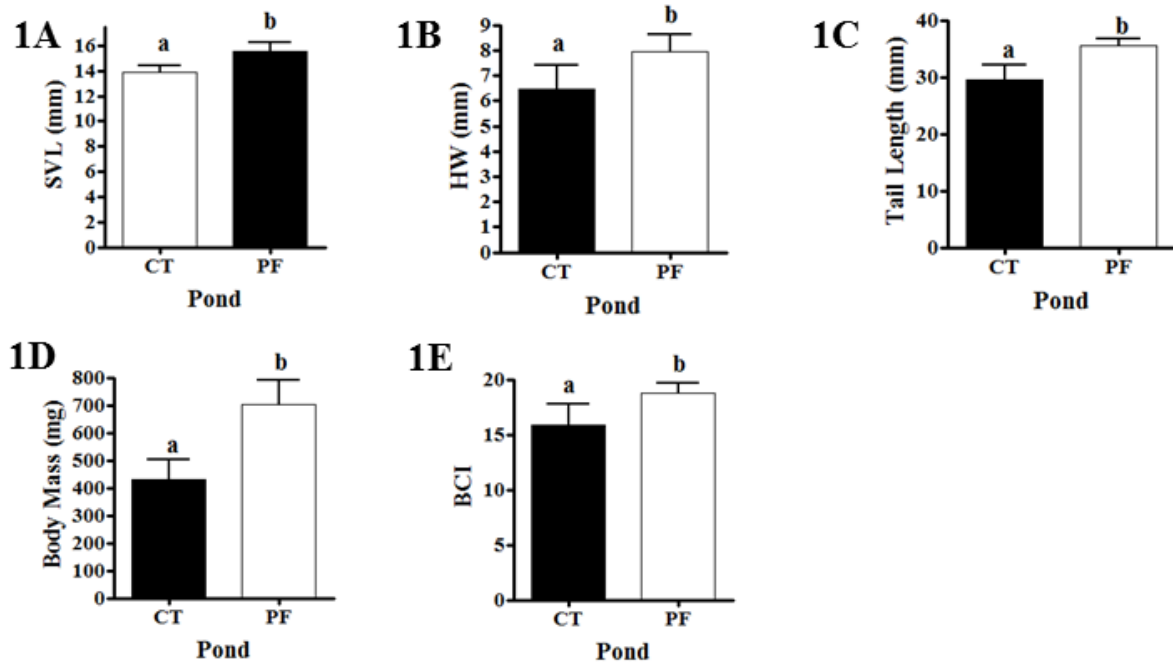


Figure 2

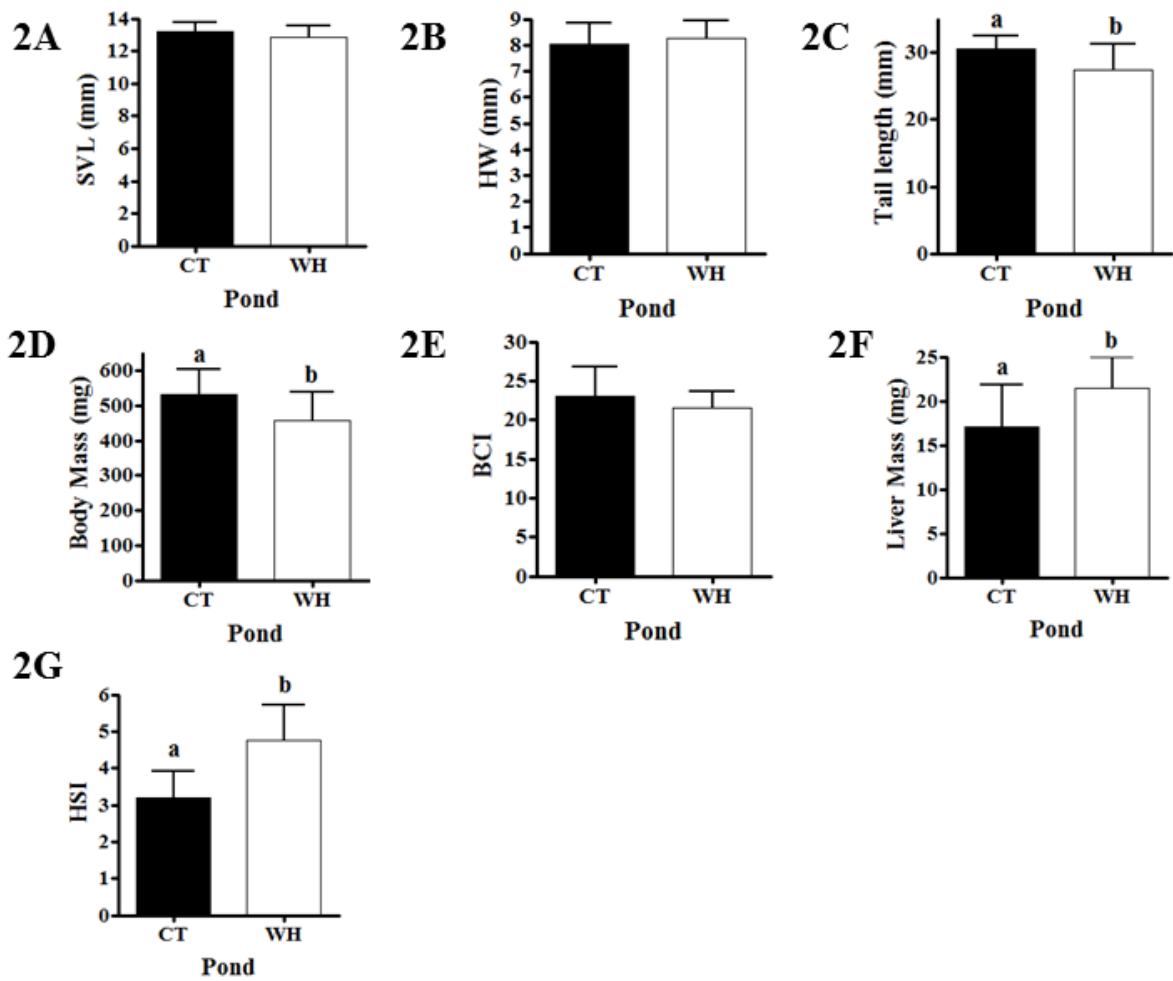
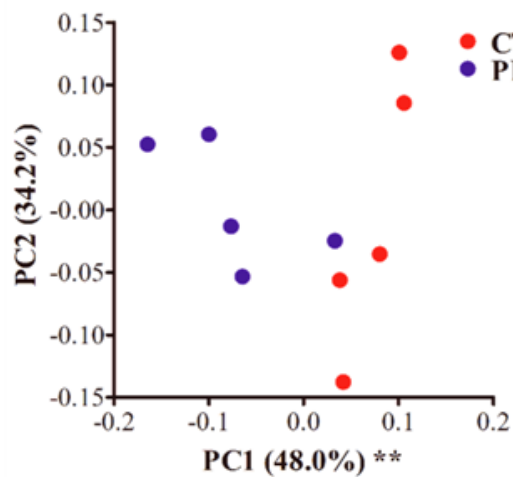
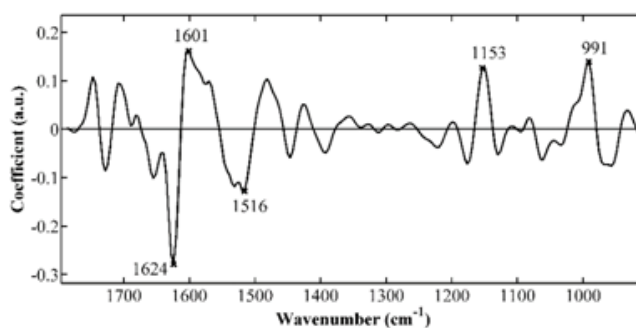


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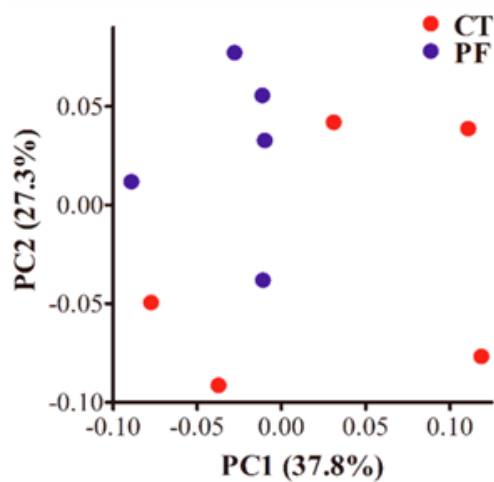
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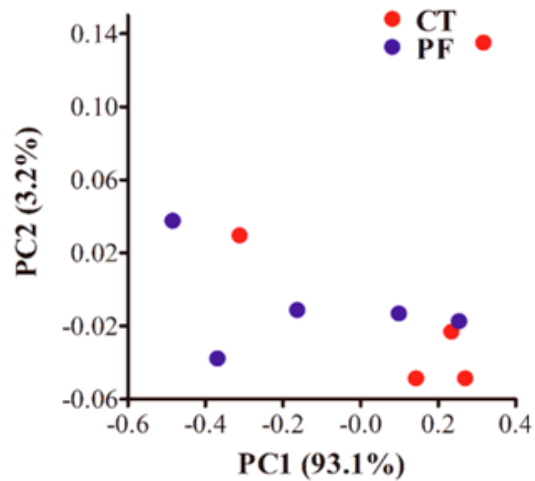
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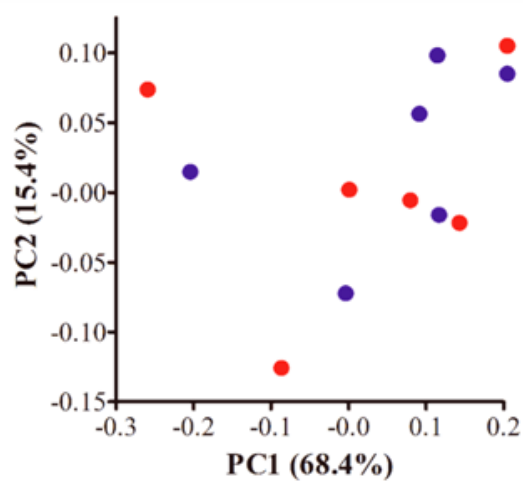
3C



3D



3E



3F

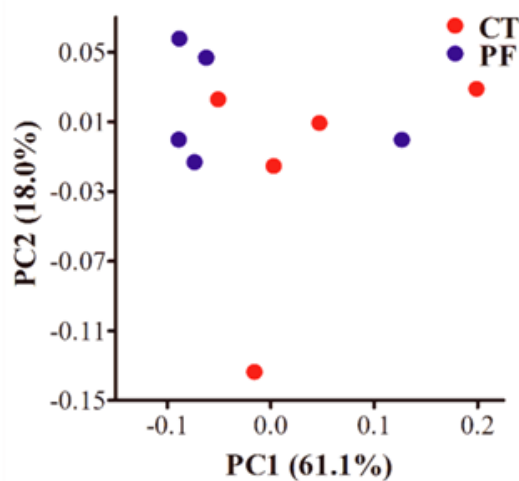


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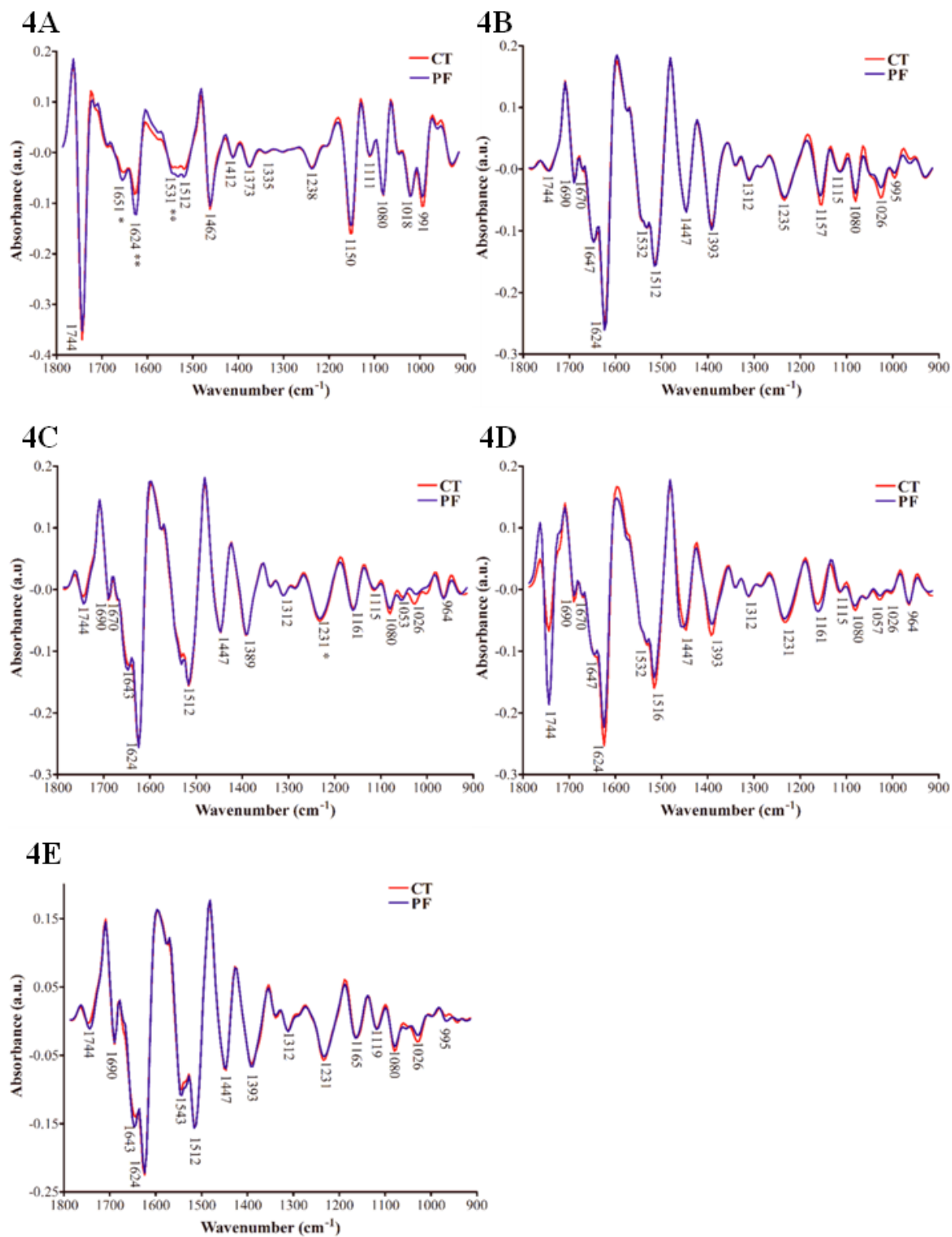
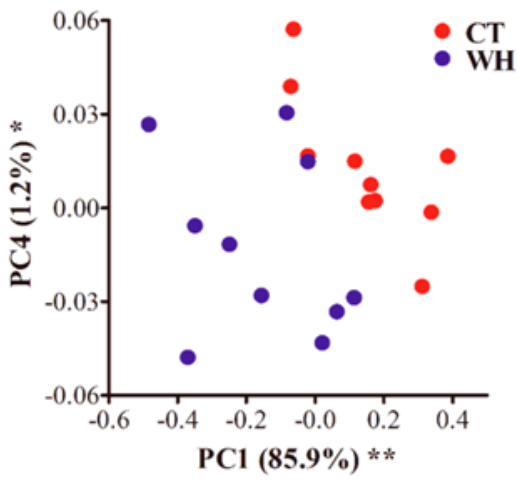
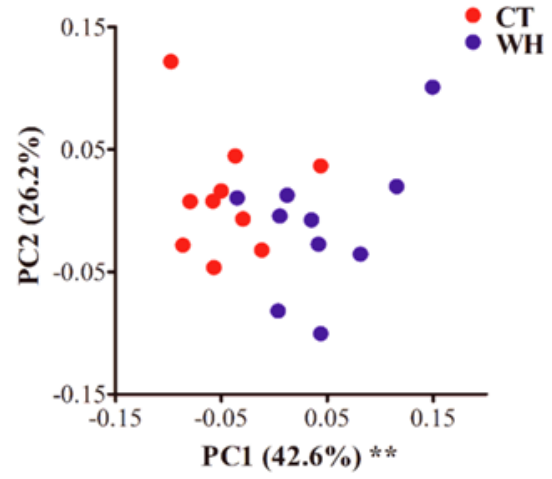


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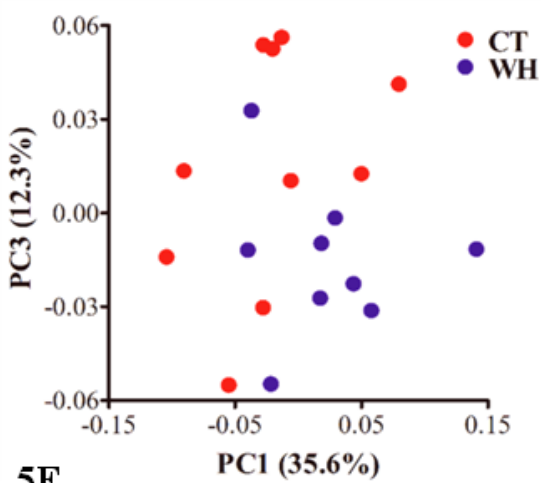
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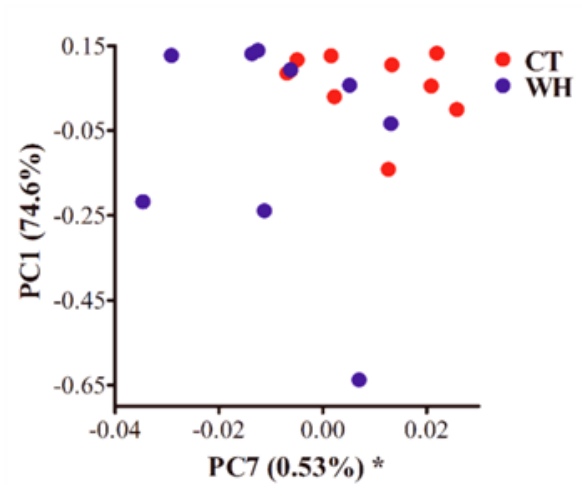
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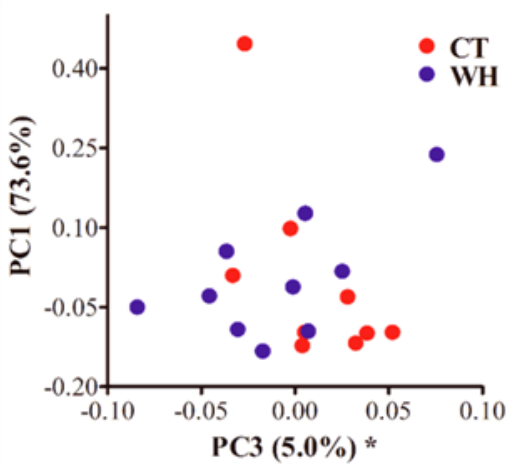


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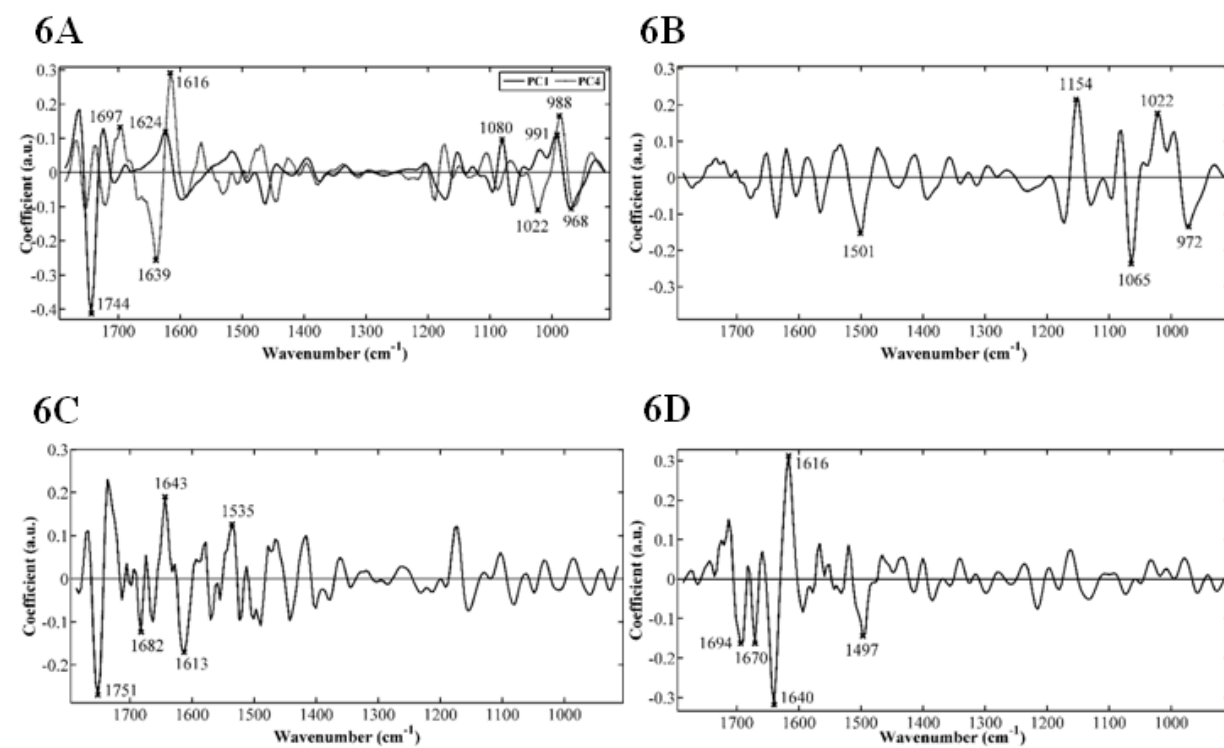


Figure 7

