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# 1 **Hyperspectral imaging of macroinvertebrates – a pilot study for detecting metal** 2 **contamination in aquatic ecosystems**

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## 23 24 **Abstract**

25 The applicability of spectral analysis in detection of freshwater metal contamination was assessed  
26 by developing and testing a novel hyperspectral imaging (HSI) application for aquatic insect  
27 larvae (Trichoptera: Hydropsychidae). Larvae were first exposed to four different cadmium (Cd)  
28 concentrations: 0, 1, 10 and 100 µg L<sup>-1</sup> for 96 h. Individual larvae were then preserved in ethanol,  
29 inspected with microscopy for the number of anomalies in larval gills, and imaged by  
30 hyperspectral camera operating with wavebands between 500 and 850 nm. Three additional  
31 larvae from each exposure were analyzed for tissue Cd concentration. Although the larval tissue  
32 Cd concentrations correlated positively with actual water concentrations, the toxicity response of  
33 larvae i.e. frequency of gill abnormalities did not differ among the Cd concentrations. In contrast,  
34 hyperspectral imaging data indicated some concentration-response relationship of larval spectral  
35 properties to the Cd exposure, but it was too weak for reliable automatic distinction between  
36 exposed and unexposed larvae. In this pilot study a workflow for data processing for a novel  
37 application of hyperspectral imaging was developed. Based on the results of this preliminary  
38 study, the workflow in the imaging process will be optimized and its potential for detecting metal  
39 contamination of aquatic environments reassessed.

40  
41 **Keywords:** Aquatic insect larvae; cadmium toxicity; Fabry-Perot interferometer; hyperspectral  
42 imaging; metal pollution

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## 48 **Introduction**

49  
50 Metal pollution threatens aquatic ecosystems worldwide. High metal concentrations are found  
51 especially in stream ecosystems contaminated by effluents from active or abandoned mines,  
52 industrial waste waters or drainage waters from acid sulfate soils (Ljung et al. 2009; Byrne et al.  
53 2012). Metal exposure impairs physiological function and reproduction of aquatic organisms  
54 (Boening 2000; Pane et al. 2003), and causes adverse health effects in humans (Järup and  
55 Åkesson 2009).

56 Direct measurement of chemical concentrations in water is expensive, as due to typically high  
57 spatial and temporal variation, extensive sampling would be required to reliably detect also  
58 critical peak concentrations. Moreover, and perhaps more importantly, metal concentrations in  
59 the water do not directly indicate the concentrations accumulated in organisms or in food webs.  
60 Developing and improving biotesting of effluents and their effects could enable efficient  
61 detection of and rapid response to possible pollution incidents (Bae and Park 2014).

62 Hyperspectral imaging (HSI) is spectroscopy coupled with imaging. The basic principle in  
63 spectroscopy is that each substance reflects and absorbs different wavelengths of light.  
64 Consequently, a given substance often has a unique spectral signature – a fingerprint. In spectral  
65 analysis, the goal is to differentiate and recognize substances based on their spectral properties.  
66 HSI diverges from conventional reflectance spectroscopy in that it produces an image of pixel  
67 spectra. The technique enables revealing changes, which might be unseen for human eye. HSI has  
68 many applications e.g. in geology, mineralogy, agriculture and steel industry including automated  
69 detection of metal content (Antonucci et al. 2012; Gutierrez et al. 2010; Riaza et al. 2011). In this  
70 study, we attempt to apply – to our knowledge for the first time – HSI in detection of metal  
71 contamination in animals, more specifically in aquatic insects. This approach could provide a  
72 novel method for quantifying heavy metal pollution in natural water bodies through HSI and  
73 spectral data processing. If HSI proves to be a suitable method to differentiate between metal  
74 contaminated and non-contaminated larvae, it can be used to replace or support direct  
75 measurement of metal concentrations in waters affected by mines and mills and in assessment of  
76 associated ecological risk.

77 Benthic macroinvertebrates are an integral constituent of the aquatic ecosystems and essential to  
78 the system functioning (Covich et al. 2004). Furthermore, as an important food source for  
79 benthivorous fish, benthic macroinvertebrates may transfer contaminants to higher trophic levels.  
80 Larvae of caddisfly family Hydropsychidae are abundant and widespread in running waters.  
81 Metal exposure of Hydropsychidae larvae can result in morphological damages such as anal  
82 papillae and tracheal gill darkening and reduction, the incidence of which can be used for  
83 indication of metal contamination (Leslie et al. 1999; Vuori 1994, 1995; Vuori and Kukkonen,  
84 1996).

85 The main purpose of this study was to evaluate the applicability of HSI in detecting metal  
86 contamination of *Hydropsyche pellucidula* (Trichoptera: Hydropsychidae) larvae with cadmium  
87 (Cd) as a representative model compound. Cd is one of the priority hazardous substances in the  
88 US and EU legislation (CWA 2002; European Union 2013). Cadmium is mainly released locally  
89 into the environment from mining activity as a by-product from zinc (Zn), copper (Cu) and lead  
90 (Pb) ore mining (European Environment Agency 2011). Other sources of Cd include industry and  
91 agriculture. Many metals like iron (Fe), Zn and Cu are essential to biochemical function of  
92 animals, but Cd have no known metabolic function and is highly toxic for plants and fish  
93 (Gallego et al. 2012; Mebane et al. 2012). The sensitivity of insect larvae to Cd varies across  
94 different taxa (Cain et al. 2004; Buchwalter et al. 2008) and family Hydropsychidae is considered

95 quite tolerant (Mebane et al. 2012). Cd is known to accumulate to insect larvae, but the high  
96 variability in the bioconcentration factor (BCF) values, and the observed negative correlation  
97 between the exposure and the BCF indicate that BCF is not necessarily an optimal measure for  
98 Cd bioaccumulation (Poteat et al. 2012). We assume that metal exposure induces morphological  
99 changes, including color alteration, in larvae, and that these changes can be quantified by  
100 analyzing larval spectral properties, even better than by more subjective, traditional microscopic  
101 observation by humans. In addition, spectral analysis operating also in the wavebands of infrared  
102 light might reveal changes invisible to human eye.  
103 Specific issues addressed were: 1) can HSI be used to differentiate between Cd contaminated and  
104 non-contaminated larvae? and 2) can metal body burden in Hydropsyche larvae be predicted from  
105 hyperspectral data? The larvae were also screened microscopically to explore if they showed any  
106 visible morphological abnormalities in response to Cd exposure, and to compare these results  
107 with HSI data.

108

## 109 **Methods and materials**

110

### 111 *Test organism and exposure set up*

112 Fifth instar larvae of *Hydropsyche pellucidula* (Trichoptera: Hydropsychidae) were collected  
113 from unpolluted River Siikakoski, Finland in November 2012. R. Siikakoski is an appropriate  
114 reference site with a demonstrated high ecological and good chemical status (Finnish  
115 Environment Institutes Water Quality Database Hertta). Median water pH in R. Siikakoski was  
116 7.0 (range 6.6–7.3) and total hardness (Ca + Mg) 0.2 mmol L<sup>-1</sup> during 2000 – 2013. Cd  
117 concentration in the R. Siikakoski measured during 2003 – 2004 ranged between 0.05 and 0.25  
118 µg L<sup>-1</sup>. Larvae were transported in aerated river water to the laboratory, where the animals were  
119 acclimatized slowly to the test temperature (19 ± 1°C) and test water, kept with photoperiod of  
120 light:dark 16:8 h, and fed with aquarium fish flake food Tetramin® ad libitum. Only viable  
121 animals after 14 d acclimation period were chosen for the exposures. Animals were not fed  
122 during the exposure, or one day prior to the experiment to reduce the amount of fecal material  
123 and possible metal sorption to it during the experiment.

124 The test design included one control and three different exposure concentrations each in three  
125 replicates. One replicate consisted of 11 larvae in one test container, a 2 L borosilicate glass  
126 beaker, and total number of exposed individuals was hence 132. Test concentrations of Cd were  
127 0.0 µg L<sup>-1</sup> (control), 1.0 µg L<sup>-1</sup>, 10.0 µg L<sup>-1</sup> and 100.0 µg L<sup>-1</sup>. Volume of experimental water was  
128 0.15 L per larvae. Glass beakers and all vessels and pipets were washed with acid (10% HCl),  
129 and then rinsed and soaked in MilliQ water before the exposure. During the exposure, glass  
130 beakers were continuously aerated to maintain rapid water circulation and oxygen saturation, and  
131 were covered with Parafilm® to prevent water evaporation. In each test vial, artificial plastic  
132 substrate was provided for larvae for net spinning. Exposure time was 96 h.

133

### 134 *Experimental water and chemicals*

135 Artificial freshwater prepared according to the standard ISO 6341 (1996) (CaCl<sub>2</sub>·2 H<sub>2</sub>O, MgSO<sub>4</sub>·7  
136 H<sub>2</sub>O, NaHCO<sub>3</sub>, KCl) with total hardness (Ca + Mg) of 0.5 mmol L<sup>-1</sup> was used in all the  
137 exposures, prepared into a MilliQ-water, and buffered prior to exposures with 0.5 M phosphate  
138 buffer (Na<sub>2</sub>HPO<sub>4</sub>·H<sub>2</sub>O, Na<sub>2</sub>H<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O) to pH 7 ± 0.1. After buffering, water was aerated  
139 overnight and pH adjusted with 1 M HCl, if needed. Stock solutions of 10 000 mg L<sup>-1</sup>, 1000 mg  
140 L<sup>-1</sup> and 100 mg L<sup>-1</sup> were prepared into a MilliQ-water from anhydrous cadmium chloride, CdCl<sub>2</sub>

141 (Alfa Aesar GmbH & Co.KG), and test concentrations were prepared by dilution of the stock  
142 solution to 5000 ml of test water, which was partitioned as 1650 ml into each glass beaker.  
143 Based on the results of a preliminary exposure with similar test setup, Cd concentration of  
144 experimental water decreases rapidly due to animal uptake. In order to maintain actual Cd  
145 concentrations  $\geq 90\%$  of the nominal, experimental water was completely changed at 3 h, 9 h, 24  
146 h, 48 h and 72 h of test duration by decanting. Water samples (50 ml) for Cd analysis from each  
147 test concentration and control ( $n = 4$ ) were taken two times right after test solutions had been  
148 prepared to see pipetting accuracy in spiking, and just before each water renewal, preserved with  
149 supra pur nitric acid (65%  $\text{HNO}_3$ ) and stored in the refrigerator prior to analyses. Water samples  
150 before water renewals at 3 h, 9 h and 24 h were taken only from the first replicate from the  
151 control and each exposure concentration, but at 48 h and 96 h water samples were taken from all  
152 replicates. pH, temperature ( $^{\circ}\text{C}$ ) and oxygen saturation (%) were measured daily from each  
153 replicate.

#### 154 155 *Measured end points*

156 Larval mortality was checked daily. After the exposure, one larva per replicate ( $n = 3$  per Cd  
157 concentration) was rinsed quickly to remove Cd adsorbed on *H. pellucidula* cuticula with pH-  
158 buffered artificial freshwater, weighed and freeze-dried for tissue Cd concentration analysis. All  
159 other ten larvae were preserved in 70% ethanol (purity 91.2%) for examination of gill damages  
160 and for HSI. Gill examination or HSI imaging results were not acquired from larvae which were  
161 prepared into Cd tissue analysis. In the present study, we measured larval wet weight, but  
162 according to another study conducted in our laboratory, the mean wet weight to dry weight ratio  
163 for *H. pellucidula* larvae ( $n = 74$ ) is 3.61 ( $\pm 0.50$ ) (Ruuth 2017), which may be used as an  
164 estimate of conversion factor for tissue concentrations. Hydropsychid gill abnormality index  
165 (HY-index, HYI) (Vuori and Kukkonen 2002) was calculated as  $\text{HYI} = \sum \text{NAG} / n$ , where NAG is  
166 the number of abnormal gills, and  $n$  is the number of individuals. HYI values can vary from 0 to  
167 19 as *H. pellucidula* has 9 pairs of abdominal gill tufts with one extra gill on the 2nd abdominal  
168 segment. Normal, undamaged gills are whitish and branching as shown in the picture in Online  
169 Resources 1 (ESM 1). A gill tuft was considered damaged, if it was totally reduced or darkened  
170 with its basal or distal parts, or if the gill tuft had dark spots on  $> 50\%$  of its branches (Ratia et al.  
171 2012). Hyperspectral images were taken from ethanol-preserved larvae ( $n = 120$ ) from their  
172 dorsal, ventral, left and right sides. Larvae were individually immersed in tap water and placed  
173 with insect needles on a Petri dish just before imaging. Petri dishes were placed on a grey  
174 background and larvae imaged in a random order. Normality of the HYI distribution was tested  
175 using Shapiro-Wilk-test and equality of variances by Levene test. 1-way ANOVA was used to  
176 test if the HYI values differed across exposure concentrations. Spearman correlation was used to  
177 study larval Cd concentration in relation to actual water Cd concentrations. IBM SPSS Statistics  
178 20 software was used in statistical analyses.

#### 179 180 *Cd analyses*

181 Weighed and freeze-dried *H. pellucidula* samples were pre-treated for elemental analysis using  
182 an ultrasound-assisted digestion method. The samples were digested using 2 mL of aqua regia as  
183 a digestion solution. The digestion solution was added, after which sample vessel was closed and  
184 placed into a 35 kHz, model Sonorex RK 512 CH ultrasonic water bath supplied by Bandelin.  
185 The ultrasound-assisted digestion procedure was carried out in a temperature of about  $60^{\circ}\text{C}$ . The  
186 optimized sonication procedure lasted 15 minutes, divided into five equal steps. The sample

187 vessel was shaken by hand between each step to ensure effective mixing of the sample and the  
188 digestion solution. After cooling, the sample solution was transferred into a volumetric flask and  
189 diluted to a final volume of 10 ml with water. All the ICP-OES measurements (larval tissue and  
190 water samples of 10 and 100  $\mu\text{g L}^{-1}$ ) were performed with a Perkin-Elmer (Norwalk, CT, USA)  
191 model Optima 8300 inductively coupled plasma optical emission spectrometry. A cyclonic spray  
192 chamber and GemCone Low-flow nebulizer were used throughout. The determination of Cd  
193 concentrations was performed using parameters of the instrument (nebulizer flow 0.6  $\text{L min}^{-1}$ ,  
194 auxiliary gas flow 0.2  $\text{L min}^{-1}$ , plasma gas flow 8  $\text{L min}^{-1}$  and plasma power of 1450 W). Two  
195 wavelengths with the axial plasma viewing used in the determination were 228.802 nm and  
196 214.440 nm. A Perkin Elmer Model AAnalyst 800 atomic absorption spectrometer with an AS-  
197 800 autosampler was used for GFAAS measurements in order to perform accurate analysis of Cd  
198 at low level of concentrations (water samples of 0 and 1  $\mu\text{g L}^{-1}$ ). All the measurements were  
199 based on integrated absorbance and were performed using a Zeeman-effect background  
200 correction system. The determination of Cd was performed at 228.8 nm using a Perkin Elmer  
201 hollow cathode lamp supplied by Perkin Elmer. Pyrolytic graphite-coated THGA tubes with end  
202 caps and an integrated L'vov-type platform (Perkin Elmer) were used. Argon (AGA, Espoo,  
203 Finland) was used as a protective gas throughout. 20  $\mu\text{L}$  of sample solution and 5  $\mu\text{L}$  of matrix  
204 modifier solution (a mixture of 0.1%  $\text{NH}_4\text{H}_2\text{PO}_4$  + 0.06%  $\text{Mg}(\text{NO}_3)_2$ ) were injected into the  
205 furnace. All the ICP-OES measurements were carried out using a four-point calibration. The  
206 determination of Cd was performed by taking the most sensitive emission lines to attain the  
207 sensitivity required. The detection limits of the determination of water samples using  
208 wavelengths 228.802 nm and 214.440 nm resulted in 0.69 and 0.76  $\mu\text{g L}^{-1}$ , respectively. The  
209 detection limit for the *H. pellucidula* tissue samples using same wavelengths resulted in 0.15 and  
210 0.16  $\text{mg kg}^{-1}$ . All the GFAAS measurements were done using a six-point calibration. The  
211 characteristic masses for each analysis were calculated by the measurement of 2.5  $\mu\text{g L}^{-1}$  of Cd  
212 standard solution. The characteristic masses calculated were within 10% of the recommended  
213 values. The detection limit of the determination of Cd in water samples by GFAAS resulted in  
214 concentration of 0.23  $\mu\text{g L}^{-1}$ . The accuracy of the method was tested by the analysis of  
215 SRM1643f (Trace elements in water) and DOLT-4 (Dogfish Liver) certified reference materials.  
216 The determination of Cd resulted in recovery rates from 95.6% to 99.5% for both of the CRMs  
217 analyzed.

### 218 219 *Hyperspectral imaging*

220 The main instrument used was a compact and lightweight hyperspectral imager developed by  
221 VTT Technical Research Centre of Finland. Spectral separation in this device is based on the  
222 piezo-actuated Fabry-Perot interferometer (FPI). The component is hermetically sealed into a  
223 metal can filled with nitrogen. Both the parallelism and the distance between the mirrors of the  
224 Fabry-Perot interferometer need to be controlled with great degree of accuracy. This is achieved  
225 with three closed loop control systems positioned at the edges of the mirror plates. Each channel  
226 has a piezoelectric actuator with associated capacitive measuring element, which is used to  
227 determine the mirror separation. Each channel is controlled with nanometer accuracy to obtain  
228 the desired parallelism and air gap between the mirrors. Detailed information about camera is  
229 given in Table 1.

230  
231  
232  
233

234 **Table 1** Specifications of VTT's Fabry-Perot hyperspectral imager.

Parameter	
Horizontal and vertical FOV (deg.)	> 36 > 26
Nominal focal length (mm)	9.3 ± 3 (Custom lenses)
Wavelength range (nm)	500–885
Spectral resolution at FWHM (nm)	9–40
Adjustable spectral resolution step	< 1
f-number	< 6.7
Maximum spectral image size (pixels)	2592 × 1944
Spectral image size with default binning (pixels)	320 × 240
Camera dimensions (mm)	62 × 66 × 219
Weight (g)	< 450

235 In imaging setup HSI was mounted to a stand using a macro objective for sufficient  
 236 magnification. Illumination was provided from both sides using one 200 W broadband halogen  
 237 light on each side of the specimen holder. This lighting setup was selected to provide even light  
 238 distribution over the subject without significant shadows. Imaging system setup is illustrated in  
 239 Online Resources 2 (ESM 2). Incandescent light sources were utilized to provide a broadband  
 240 illumination spectrum without significant spikes or other abnormalities. Each larva was prepared  
 241 and mounted with non-reflective matte black metal pins prior to imaging using the same exposure  
 242 and integration period settings, with the same sequence of spectral bands for all specimens. In our  
 243 experiment, we used default spatial pixel binning, which improved noise performance of imaged  
 244 spectra. Water level in the specimen holder was maintained at a level sufficient to fully immerse  
 245 each specimen to prevent any reflective border effects from parts of the specimen disturbing the  
 246 surface of the water. This also ensured similar additional spectral absorption caused by the water  
 247 covering the specimen. Further standardizing the depth of this immersion is of importance for  
 248 achieving as comparable imaging conditions as possible. Petri dish where specimens were set  
 249 was on diffuse grey surface (approx. 30% reflectance). Used wavelengths and full width at half  
 250 maximum (FWHM) of each band are listed (Table 2).  
 251  
 252

**Table 2** Used wavelengths and full width at half maximum (FWHM) of each band

Wavelength	496.2	496.4	499.2	503.2	507.2	510.8	514.6	518.9	523.7	528.4
	532.4	533.5	535.9	538.5	539.6	543.2	547.3	552	557.1	561.8
	566.2	571.5	579.1	582.8	586.1	589.8	593.8	597.7	601.9	606.3
	610.9	612.2	618	625.9	634.1	639.8	646	653.6	659.9	660.8
	666.6	666.9	674.5	682.3	688.9	695.3	701.8	709.1	716.4	723
	729.5	735.6	744	750.5	756.7	762.5	769.8	777.7	786.7	793.4
	800.2	806.2	812.3	818.3	824.9	832.7	838.6	846.5	852.5	859.8
FWHM	32.1	30.3	29.3	45.6	30.2	29.2	30.8	25.2	30.7	20.9
	30.9	31.7	31.2	30.3	30.3	31	30.6	30.7	19.4	29.9
	21.9	30.3	21.4	30.7	21	30.7	21.8	29.4	22.8	29
	23.1	29.4	22	31	20.4	31.9	19.8	31.6	19.8	30.3
	20.2	29.6	21.4	28.5	22.2	28.1	22.4	28.6	21.8	29
	23.3	28.7	24.9	28.3	23	28.6	20.2	27.6	19.2	28.4
	19.7	28.5	19.6	19.5	19.8	20.4	11.2	20	12.9	15.3

253  
 254 A hyperspectral image is a stack of grayscale images (Figure 1). Each of these images represents  
 255 intensities at a given wavelength of light. A given pixel in each image is from the same target  
 256 location. Thus, if a vector of pixels is taken through this stack, it forms a spectrum from a given  
 257 point in the imaged object. After all specimens were imaged with the system, all pixels from all

258 spectral cubes were transformed to one large data matrix so that each row corresponded to a  
 259 certain spectrum from a certain image. From this matrix unique spectral fingerprints were looked  
 260 for. These fingerprint spectra are called as endmembers. The occurrence of each endmember can  
 261 then be computed through inversion. Our assumption was that based on the statistical features for  
 262 each specimen we could create supervised classification for specimen's contamination. In this  
 263 case specimens would be classified to four groups based on the exposure concentration.

264  
 265 Our data processing pipeline was:

- 266 • Data normalization to reflectance. From each image baseline spectrum  $s_0$  was selected  
 267 manually. Raw imaging data was converted to reflectance  $x = s_{i,j}/s_0$  for all pixels in image,  
 268 where  $s_{i,j}$  is spectrum of a single pixel.
- 269 • Spectral unmixing to delineate specimen from image. Vertex component analysis (VCA) was  
 270 used to detect spectrum of specimen from image. With detected spectrums inversion was  
 271 calculated using filter vector algorithm (FVA) to create a mask for specimen.
- 272 • All spectra from specimen were gathered and endmembers were selected from Figure 2 scatter  
 273 plot. Endmembers are considered as vertices of convex hull of a data set.
- 274 • Inversion for selected endmembers was calculated using FVA and abundance maps were  
 275 formed.
- 276 • For abundance images statistical features were calculated. Features were calculated based on the  
 277 abundance maps and textures of abundance maps using local binary pattern algorithm. Used  
 278 statistical features were scale and rotation invariant (average, median, mode, variance, standard  
 279 deviation, entropy, difference entropy, difference variance, difference standard deviation).
- 280 • Manifold learning approach was utilized to classify specimens to different groups. For the  
 281 manifold learning feature space's dimension was reduced using diffusion maps. For specimens in  
 282 embedded space k-nearest neighbour (KNN) based classification was used. For cross-validation  
 283 leave-one-out method was used with KNN.

284 We will here explain in details how to find the unique spectral fingerprints from the data matrix.  
 285 We utilized a spectral unmixing method. An assumption behind spectral unmixing is that the  
 286 spectrum arriving at a given pixel is a mixture of reflections that have been given off from  
 287 different materials present in the scene being imaged. To effectively separate these materials, a  
 288 model is required to describe this process, making it possible to devise a reverse operation. One  
 289 common assumption is a so- called linear mixing model, which assumes the detected spectrum  
 290 for each pixel to consist of a linear combination of substance- originated constituent spectra,  
 291 termed as endmembers.

292 As given in (Keshava 2003), linear mixing model (LMM) for single spectrum can be described as

293

$$294 \quad \mathbf{x}[\lambda] = \sum_{i=1}^M \mathbf{a}_i \mathbf{s}_i[\lambda] + \mathbf{w}[\lambda],$$

295  
 296 where  $\mathbf{x}$  is the detected spectrum as a function of wavebands  $\lambda$ ,  $\mathbf{a}_i$  is an abundance coefficient for  
 297 endmember  $\mathbf{s}$ ,  $M$  is the number of endmembers and  $\mathbf{w}$  is the noise term. Expanding LMM to all  
 298 observed pixel spectra, we shall have a matrix form of  $X = AS + W$ , where  $X = [\mathbf{x}_1, \mathbf{x}_2 \dots, \mathbf{x}_N]^T$   
 299 is  $N \times d$  matrix,  $A = [\mathbf{a}_1, \mathbf{a}_2, \dots, \mathbf{a}_M]$  is  $N \times M$  matrix,  $S = [\mathbf{s}_1, \mathbf{s}_2 \dots, \mathbf{s}_M]^T$  is  $M \times d$  matrix and  
 300  $W$  is  $N \times d$  noise matrix.

301

302 Goals of unmixing processes are to estimate these constituent spectra, and their relative



303 abundance for each pixel. Given these abundance coefficients, new images displaying the relative  
 304 occurrence of a given endmember within the scene can be drawn, usually termed as abundance  
 305 maps.

306 Unmixing can be done manually projecting data to a lower dimensional space. We used principal  
 307 component analysis to project specimens spectra to a lower dimensional space, where  
 308 endmembers were selected. One unsupervised method used to unmix the spectral data is Vertex  
 309 Component Analysis, as outlined in Nascimento and Bioucas Dias (2005). This method assumes  
 310 presence of pure pixels  $S$  in the input data  $X$ , and proceeds by performing iterative orthogonal  
 311 projections of the data onto subspace spanned by the previously determined endmembers. The  
 312 extremity of this projection is taken as being the new endmember signature. This process repeats  
 313 until  $M$  endmembers have been extracted.

314 As such, the assumption of pure pixels existing is a strong one, and not necessarily true in all  
 315 types of data. For purpose of discovering material differences present within the scene imaged in  
 316 contrast to finding endmember spectra directly usable for substance identification, the behavior of  
 317 selecting the most purest pixel spectra as the endmember signatures is often sufficient.

318 To obtain abundance maps, given spectral datacubes and endmember spectra, two different  
 319 methods were examined. The first one was a traditional non-negative least squares inversion,  
 320 which proved to provide good abundance maps, but also is computationally expensive. The other  
 321 method utilized was the Filter Vector Algorithm (Bowles et al. 1995) (FVA), which in turn  
 322 provided reduced computational cost.

323 The FVA forms a set of filter vectors  $F$ , which are used to estimate abundance coefficients.

324 Estimation is performed in the following way  $A = FX$ , where  $F = [RS]^{-1}R$   
 325 and  $R = S^T - (I/NS)^T$ . Here  $I$  is  $N \times N$  unit matrix.

326  
 327 Proposed workflow offers us features, which can be utilized in separation of different spectra. In  
 328 the case of *H. pellucidula* this gave us an opportunity to utilize numerical features based on  
 329 specimen's reflectance spectrum.

330 After unmixing and inversion we calculated scale and rotation invariant statistical features for  
 331 each specimen. These features were listed earlier. With this feature space we got 54 different  
 332 features for each specimen, which also means 54 dimensional space. Because of large  
 333 dimensionality diffusion maps (Coifman and Lafon 2006) were used to reduce it. Diffusion maps  
 334 is a non-linear dimensionality reduction algorithm, which detects underlying manifolds from the  
 335 data.

336  
 337 Now each specimen  $i = 1, 2, \dots, 120$  has a feature vector  $x_i^f \in \mathbb{R}^{54}$  and all these feature vectors  
 338 form a feature matrix  $X^f = [x_1^f, x_2^f, \dots, x_{120}^f]^T$ . Diffusion matrix  $W$  is formed using Gaussian  
 339 heat kernel

$$340 \quad k_{i,j} = \exp \frac{|x_i^f - x_j^f|}{\epsilon}$$

341 in every element of the  $W_{i,j \in [1,2,\dots,120]}$ . By normalizing  $W$  with symmetric matrix  $D_{i,i} = \sum_{j=1}^{120} W_{i,j}$   
 342 we will get Markovian transition matrix  $P = D^{-1}W$ . After this the conjugate matrix

343  $\hat{P} = D^{1/2}PD^{-1/2}$  is calculated to determine the eigenvalues of  $P$ . Now,  $\hat{P} = D^{-1/2}WD^{-1/2}$ ,

344 which is also known as normalized graph Laplacian and it maintains eigenvalues. Singular value  
 345 decomposition is used to find the singular values  $\varphi_i$  and singular vectors  $\psi_i$ . Now,  $\Phi_i = \varphi_i\psi_i$  are  
 346 the coordinates of the embedded data.

347

348 After dimensional reduction step, we can use classification algorithm to classify specimens to  
349 four different exposure groups. We apply simple three nearest neighbor classification to this  
350 using the embedded data  $\Phi$  and more precisely 2nd, 3rd and 4th eigenvectors and  $\lambda$ -values. To test  
351 accuracy of classification cross-validation scheme is built with a leave-one-out method. In this  
352 method, one data point is removed and the rest are used for training the classifier. Now this  
353 removed data point is classified with a trained classifier. This procedure is repeated for all the  
354 data points.

## 355 **Results and discussion**

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358 Water chemistry remained stable throughout the exposure. Average pH ( $\pm$  SD) was 7.14 (0.03),  
359 temperature 18.8 °C (0.3) and oxygen saturation 100.0% (0.6). The actual Cd concentrations in  
360 experimental water were slightly over the nominal throughout the exposure at the concentrations  
361 of 10.0 and 100.0  $\mu\text{g L}^{-1}$ . At the lowest Cd concentration (1.0  $\mu\text{g L}^{-1}$ ) actual Cd concentration  
362 was only 48–80% of the nominal concentration despite frequent water renewals.

363 Larval tissue Cd concentration ( $\mu\text{g g}^{-1}$  ww) varied somewhat within treatments (Figure 5), but  
364 showed a significant positive correlation with actual water concentrations ( $\mu\text{g L}^{-1}$ ) ( $r_s = 0.75$ ,  $p =$   
365 0.005).

366 There was no larval mortality in any of the exposure concentrations. HYI showing the average  
367 number of damaged gills per larvae in the exposed population was low and varied from 1.47 ( $\pm$   
368 0.42) in the control to 1.63 ( $\pm$  0.25) in the exposure concentration of 10.0  $\mu\text{g L}^{-1}$  with no  
369 significant differences among exposure populations ( $F = 0.077$ ,  $df = 3$ ,  $p = 0.971$ ) (Figure 3).

370 HSI data analysis indicated that larvae exposed to high Cd concentrations might have different  
371 spectral properties than control larvae. First of all, different parts of the specimen based on  
372 spectral unmixing were able to separate. In Figure 4 is one specimen presented as RGB image  
373 (A) and abundances of the found endmembers (B, C, D). It seems that B represents dark parts of  
374 the thorax and abdomen. C and D present endmembers for the segments, where tissue is soft and  
375 does not include pigment.

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395 **Table 3** Average water ( $\mu\text{g L}^{-1}$ ) and larval tissue ( $\mu\text{g g}^{-1}$  ww) Cd concentrations ( $\pm$  SD) during the  
 396 exposure. Experimental water was completely changed five times (at 3, 9, 24, 48 and 72 h), and exposure  
 397 duration was 96 h

Time	Nominal water Cd $\mu\text{g L}^{-1}$	Actual water Cd after spiking $\mu\text{g L}^{-1}$	Actual water Cd before water renewal $\mu\text{g L}^{-1}$	Larval tissue Cd $\mu\text{g g}^{-1}$ ww	Larval ww mg
3 h	0.0	>0.22	>0.22		
9 h	0.0	>0.22	>0.22		
24 h	0.0		>0.22		
48 h	0.0		>0.22		
72 h	0.0		>0.22		
96 h	0.0		>0.22	0.62 (0.41)	53.3 (4.32)
3 h	1.0	1.88	0.80		
9 h	1.0	1.26	0.85		
24 h	1.0		0.75		
48 h	1.0		0.62		
72 h	1.0		0.48 (0.11)		
96 h	1.0		0.54 (0.05)	3.98 (1.96)	50.27 (8.09)
3 h	10.0	12.00	10.83		
9 h	10.0	12.20	10.98		
24 h	10.0		9.90		
48 h	10.0		10.00		
72 h	10.0		10.35 (0.34)		
96 h	10.0		10.74 (0.13)	12.76 (6.43)	49.17 (1.83)
3 h	100.0	119.70	117.10		
9 h	100.0	123.60	116.90		
24 h	100.0		117.80		
48 h	100.0		121.90		
72 h	100.0		121.10 (2.08)		
96 h	100.0		122.90 (0.54)	8.92 (3.23)	60.17 (7.27)

398 Classifier gave 32% accuracy, which is better than guessing (25%). The most promising  
 399 individual set of features, more specifically the inverse of the variance of pixel intensities in a  
 400 specimen, correlated with the Cd exposure ( $r_s = 0.29$ ) (Figure 5). These data were captured from  
 401 the dorsal side of the insect. This result can be interpreted as abundance image's pixel intensities  
 402 having less difference between each other when Cd exposure concentration is higher. The result  
 403 indicates that soft parts of the larvae are darker in higher exposure concentrations (Figure 5).  
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405 *H. pellucidula* larvae accumulated waterborne Cd into their tissues in this study. Accumulated Cd  
406 concentration reflected the actual water Cd concentrations, but was on average highest at the  
407 second highest exposure concentration,  $10 \mu\text{g L}^{-1}$ . There is evidence of active internal Cd  
408 concentration regulation and some physiological control seem to exist for protecting insects from  
409 excess uptake (Buchwalter et al. 2008; Poteat et al. 2012). Larval body burden represents the  
410 bioavailable fraction of metals, not the entire metal exposure in the water, and therefore reflects  
411 the potential harmful ecotoxicological effects of metals on biota. In the present study, larval body  
412 burden was high compared to Cd tissue concentrations in field-collected larvae of the same  
413 family (e.g., Cain et al. 2000; Awrahman et al. 2016; Prommi and Payakka 2018).

414 All exposure concentrations  $1\text{--}100 \mu\text{g L}^{-1}$  used in this study were acutely in a sublethal range for  
415 *H. pellucidula* as there was no mortality during this 96 h test. In streams receiving mine drainage,  
416 Cd concentration may vary  $0.3\text{--}2.0 \mu\text{g L}^{-1}$  (Ramani et al. 2014), but rivers receiving waters from  
417 historical deep metal mines can contain  $< 10\text{--}2600 \mu\text{g L}^{-1}$  Cd (Byrne et al. 2012). In Finnish  
418 mine-impacted streams and lakes, Cd concentration has exceeded on a spill occasion the  
419 maximum environmental quality standard, MAC-EQS, over tenfold (European Union 2013;  
420 Kauppi et al. 2013). Water hardness generally protects against Cd toxicity since calcium (Ca)  
421 ions can compete with Cd for binding sites on cell membrane thus preventing Cd uptake  
422 (Penttinen et al. 1998). In this study, a soft water characteristic of Finnish water bodies and  
423 natural habitats of *Hydropsyche* larvae was used. According to Mebane et al. (2012), larval  
424 Arctopsychidae (closely related to Hydropsychidae) were very tolerant for Cd with 96-h EC<sub>50</sub>  $>$   
425  $458 \mu\text{g Cd L}^{-1}$  when stream water was used as an experimental water with estimated dissolved  
426 organic carbon being  $0.6 \text{ mg L}^{-1}$  and field measured pH 6.8–7.5. There are differences in Cd  
427 sensitivity between insect families reflecting their phylogeny (Buchwalter et al. 2008).

428 *Hydropsyche* spp. are considered metal tolerant with more efficient detoxification processes and  
429 accumulated Cd whole-body concentrations in metal polluted sites lower than e.g., in mayfly  
430 larvae (Cain et al. 2004). *H. californica* has shown very high Cd elimination rate compared to  
431 other aquatic invertebrate taxa (Buchwalter et al. 2008). Also animal size affects metal tolerance.  
432 Larger individuals may be more tolerant with decreased surface area:mass ratio compared to  
433 smaller individuals with higher ion uptake rates (Poteat and Buchwalter 2014) which makes  
434 earlier instar larvae more susceptible to metals. Metal exposure through dietary sources is found  
435 to be potentially more toxic than uptake of dissolved metal from water (Xie and Buchwalter  
436 2011).

437 No differences were observed in the number of gill damages in *Hydropsyche* larvae among the  
438 three different Cd concentrations and the control. Overall, the incidence of gill abnormalities was  
439 low compared to much higher incidence of gill damage (average HYI 5–15) found by Vuori and  
440 Kukkonen (2002) when exposing *H. siltalai* larvae to very high Cd concentrations (160 and 10  
441  $000 \mu\text{g L}^{-1}$ ). Although the visual inspection for gill damages did not reveal any relation to Cd  
442 concentrations, HSI succeeded to detect a weak correlation between water Cd concentrations and  
443 spectral features, showing a relationship between darkening of larval soft tissues and Cd exposure  
444 concentration. These features could not be correlated to larval Cd tissue concentration directly  
445 since the larvae that were imaged were not suitable anymore for tissue Cd analysis due to ethanol  
446 preservation. This correlation and maybe those for some other features might have become  
447 stronger if some apparent sources of error could be avoided. Spectral features of the larvae might  
448 have been measured better if the larvae were not immersed in water. In the current imaging  
449 system, this was required to avoid larvae from drying out under illumination. Because there was a  
450 range of larval sizes present, there was additional variance in the level of water surface. Water  
451 absorption can be an additional source of error, and would thus be advantageous to eliminate. As

452 the larvae are not flat, the thickness of water layer on them varies among different parts of the  
453 larva, and potentially, to some extent among specimens. However, for the wavelengths used in  
454 imaging, the absorption by water is limited and has relative small effect on the imaged spectra.  
455 Thus, this was handled as a noise factor of linear mixing model, and was thereby controlled for in  
456 the data processing. The manual preparation with needles also gives rise to a further uncertainty,  
457 as each larva is positioned and stretched out in a slightly different manner, which also affects  
458 what areas are imaged and to what extent.

459 Physiological explanation for the soft tissue darkening in the higher Cd concentrations indicated  
460 by HSI is unclear. Vuori (1994) and Vuori and Kukkonen (2002) observed heavy visual  
461 darkening of hydropsychid soft tissues at high Cd exposure levels. This phenomenon was  
462 associated with heavy darkening and reduction of tracheal gills of the larvae. HSI is known to  
463 detect color changes in organisms before the changes are visible to human eye (Little and Summy  
464 2012). By the same token, HSI indication of soft tissue darkening of hydropsychids in the present  
465 study may be an early-warning signal of color change related to metabolic disturbances. Cd is  
466 observed to alter normal cell ultrastructure in insects, which is linked to protein-based defense  
467 system activation (Braeckman et al. 1999a, b). Another explanation may be related to adsorption  
468 of Cd on body surfaces. Significant proportion of metals can be adsorbed on the body surfaces of  
469 aquatic insects, mainly as oxide coating on the cuticle surfaces (Dittman and Buchwalter 2010) or  
470 direct binding to chitin (Hare 1992). The main component of abdominal cuticle of insects is  
471 chitin. Number of studies has shown rapid biosorption of Cd to chitin (Gonzalez-Davila et al.  
472 1990; Zhou et al. 2004). The high affinity of chitin to Cd and other metals is related to its  
473 structure comparable to the polysaccharide cellulose assembled into crystalline nanofibrils  
474 (Vincent 2002). Short-term preservation of larvae in 70% ethanol has probably no significant  
475 impact on Cd concentrations. Concentrations of closely related zinc in invertebrates have also  
476 been shown to be unaffected by ethanol preservation (Braun et al. 2009).

477 In this pilot study the feasibility of a novel technique, HSI, was tested to detect metal  
478 contamination of aquatic insects, and a workflow for processing hyperspectral data for this kind  
479 of an application was developed. The actual imaging process needs further development,  
480 especially concerning stabilization of specimens for spectral imaging in a standardized manner  
481 without damaging them. Moreover, it is important to improve the study protocol so that  
482 individual level information of both tissue Cd accumulation, gill damages, and specific spectral  
483 features are acquired. The results show quite large variation in Cd accumulation, and in the  
484 spectral features of larvae, so in the future studies it is essential to get both of these parameters  
485 from each individual. Reliable estimates of Cd body burden also may require larger sample sizes  
486 than used in this study, where only three larvae per treatment were analyzed. Although a relation  
487 between metal exposure concentration and a spectral feature was discovered in the data, it proved  
488 to be too weak for performing automatic distinction between the exposed and unexposed  
489 specimens. Thus the current data set as such is insufficient for directly estimating Cd water or  
490 tissue concentrations. Prediction could be potentially performed by similar machine learning  
491 methodologies as here was done directly against the observed data. The absolute values of  
492 features for each exposure concentration occupy much the same numerical range (Figure 5),  
493 making any potential prediction results considerably more uncertain. Still, we consider these  
494 preliminary results encouraging enough to study further if HSI has a potential for detection of  
495 metal contamination in aquatic insects. To further develop and test HSI to this end, focus in the  
496 future studies should focus on set-ups that will enable a standardized manner of larvae  
497 preparation for spectral imaging, and will yield accurate dose-response data.

498

499 Conflict of Interest: The authors declare that they have no conflict of interest.

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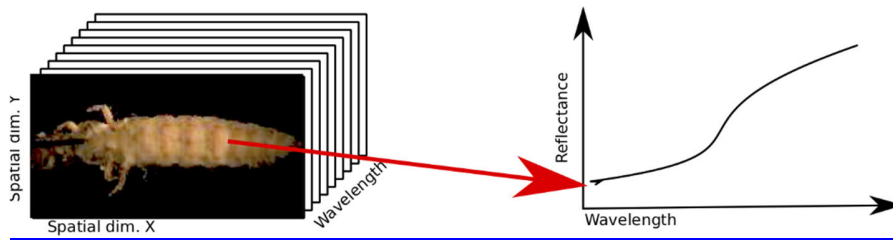
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659 **Fig. 1** Illustration of a hyperspectral datacube. Each spatial pixel forms a spectrum through the  
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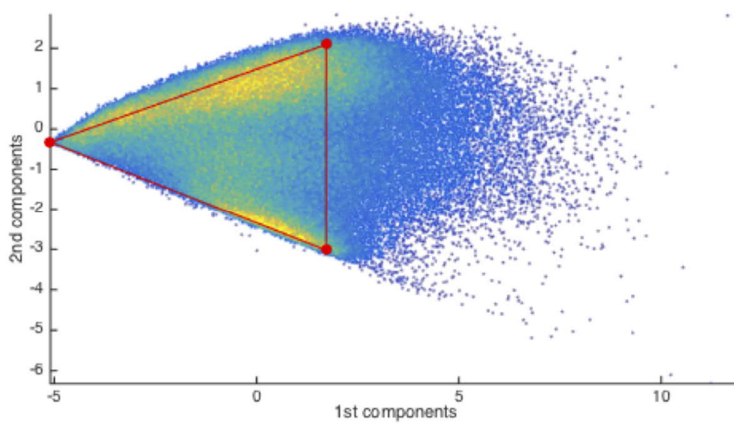
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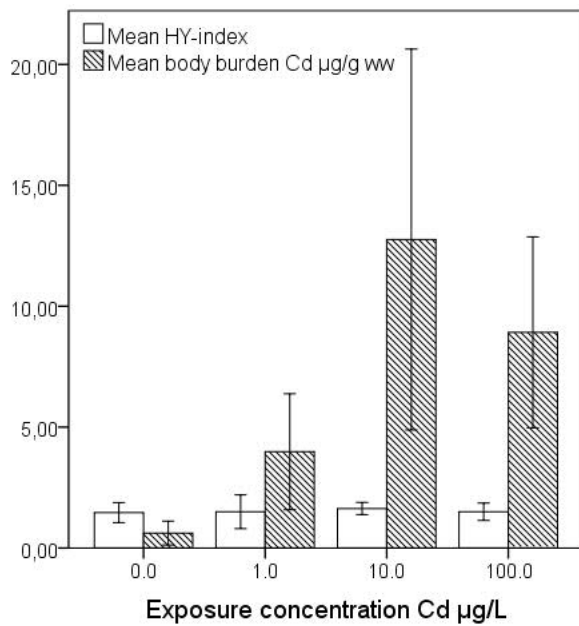
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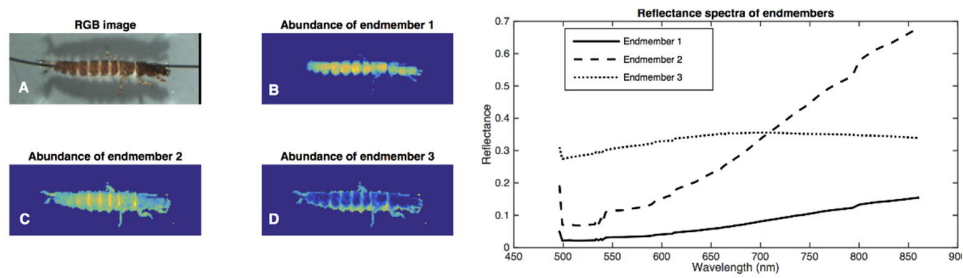


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700 **Fig. 2** Illustrative density scatter plot of all the specimen spectra projected to the lower  
701 dimensional space with principal component analysis. Projection of the data set is almost convex.  
702 Color in scatter plot indicates spatial density of projection. In more yellow (lighter) area there are  
703 more scatter points than in blue (darker) area. Majority of the data is between marked three red  
704 dots. These dots can be seen as endmembers for the data and data points inside this convex hull  
705 can be presented as linear combination of these endmembers. Data points outside of hull consist  
706 more noise  
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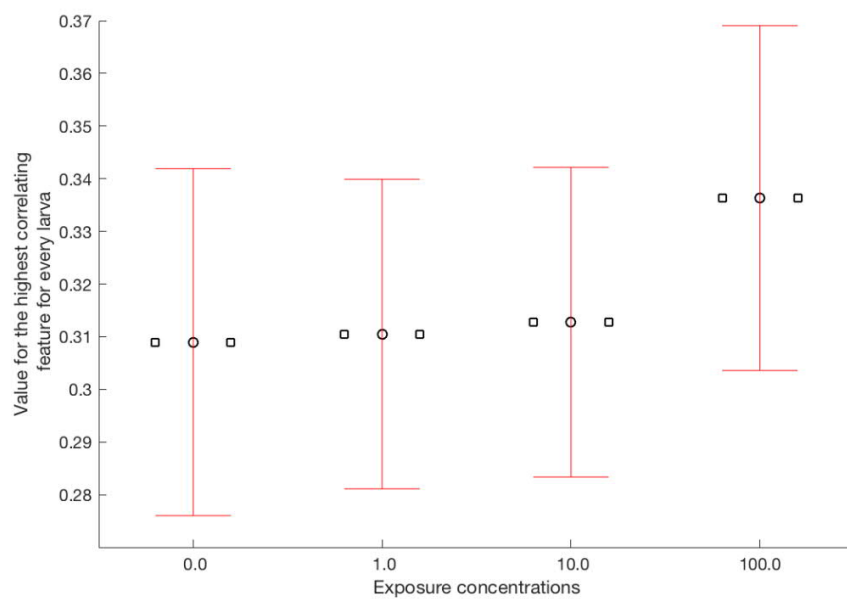


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 710 **Fig. 3** Mean HY-index ( $\pm$  SD) ( $n = 30$ ) describing the average number of damaged gill tufts of *H.*  
 711 *pellucidula* in the exposed population, and mean larval body burden Cd  $\mu\text{g g}^{-1}$  ww ( $\pm$  SD) ( $n = 3$ )  
 712 in Cd-exposure concentrations of  $0.0 \mu\text{g L}^{-1}$  (control),  $1.0 \mu\text{g L}^{-1}$ ,  $10.0 \mu\text{g L}^{-1}$ , and  $100.0 \mu\text{g L}^{-1}$   
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 739 **Fig. 4** Separated parts of the specimen based on spectral unmixing. Here is one *H. pellucidula*  
 740 larvae presented as RGB image (A) and abundances of the found endmembers (B, C, D).  
 741 Extracted endmembers on the right  
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744 **Fig. 5** Values for the highest correlating spectral feature (mean  $\pm$  SD) for every larva in Cd  
745 concentrations of 0 (control), 1, 10, and 100  $\mu\text{g L}^{-1}$