



HAL
open science

A new method to measure allyl isothiocyanate (AITC) concentrations in mustard: comparison of AITC and commercial mustard solutions as earthworm extractants

Céline Pélosi, François Chiron, Florence Dubs, Mickael Hedde, Jean-François Ponge, Sandrine Salmon, Daniel Cluzeau, Sylvie Nelieu

► **To cite this version:**

Céline Pélosi, François Chiron, Florence Dubs, Mickael Hedde, Jean-François Ponge, et al.. A new method to measure allyl isothiocyanate (AITC) concentrations in mustard: comparison of AITC and commercial mustard solutions as earthworm extractants. *Applied Soil Ecology*, 2014, 80, pp.1-5. <10.1016/j.apsoil.2014.03.005>. <hal-00981416>

HAL Id: hal-00981416

<https://hal.science/hal-00981416v1>

Submitted on 23 Apr 2014

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons CC0 1.0 - Universal - International License

1 A new method to measure allyl isothiocyanate (AITC) concentrations in mustard -
2 Comparison of AITC and commercial mustard solutions as earthworm extractants

3

4 C. Pelosi^{a,*}, F. Chiron^b, F. Dubs^c, M. Hedde^a, J.-F. Ponge^d, S. Salmon^d, D. Cluzeau^e, S.
5 Nélieu^a

6

7 ^a INRA, UR251 Pessac, 78026 Versailles cedex, France

8 ^b Université Paris Sud, UMR 8079 Ecologie Systématique Evolution, 91405 Orsay, France

9 ^c IRD, UMR BIOEMCO, Centre France Nord, 93143 Bondy Cedex, France

10 ^d Muséum National d'Histoire Naturelle, CNRS UMR 7179, 4 Avenue du Petit-Château,
11 91800 Brunoy, France

12 ^e Université de Rennes 1, CNRS UMR6553 EcoBio, Station Biologique, 35380 Paimpont,
13 France

14

15 * Corresponding author: UR 251 PESSAC INRA, Bâtiment 6, RD 10, 78026 Versailles
16 cedex, France. Tel: (+33)1.30.83.36.07; Fax: (+33)1.30.83.32.59. E-mail address:

17 celine.pelosi@versailles.inra.fr

18

19

20

21

22

23

24

25

26 **Highlights**

- 27 - A new method is proposed to measure allyl isothiocyanate concentration in mustard
- 28 - AITC concentration in mustard varied five-fold, depending on its use by date
- 29 - AITC concentration was about 4 times higher in the AITC solution than in the mustard one
- 30 - AITC and commercial mustard solutions had the same earthworm extracting efficiency

31 **Abstract**

32 Earthworms are target organisms both for scientists studying the biological component
33 of soils and for farmers concerned with monitoring the quality of their soils. Different
34 expellants are used to extract earthworms from the soil but differences in chemical properties
35 and efficiency between commercial mustard and allyl isothiocyanate (AITC) solutions remain
36 unknown. The objectives of this study were to compare (i) the concentration of irritating
37 product (allyl isothiocyanate AITC) in two expellant solutions (diluted mustard or AITC
38 solution) and (ii) their efficiency in extracting earthworms from the soil.

39 AITC concentration was analyzed according to a new method, based on AITC solvent
40 extraction and HPLC quantification, in one commercial mustard brand to assess its variability
41 within and between batches of jars. According to mustard spiking with AITC standard
42 solution, extraction recovery was estimated as $98 \pm 2\%$. Earthworm field data were collected
43 in spring 2012 in 22 cultivated fields located in east Île-de-France, comparing pure AITC to
44 commercial mustard solutions. Species diversity, abundance and biomass of earthworms per
45 plot were measured.

46 We showed that AITC concentration in commercial mustard varied according to the
47 use by date but not according to the batch. We thus recommend using the freshest mustard
48 available from the same batch. Moreover, AITC solution was found to be about four times
49 more concentrated in AITC than the commercial mustard solution. Despite this result, no
50 significant differences were found in the efficiency of commercial mustard or AITC solutions
51 to bring earthworms to the soil surface in terms of abundance, biomass or diversity. We thus
52 discuss the advantage and drawbacks of using both expellants in the field.

53

54 **Keywords:** Earthworm sampling; Efficiency; Chemical extraction; Expellant.

55 **1. Introduction**

56 Earthworms exert important agro-ecological functions (e.g. they influence organic
57 matter dynamics and soil structure; Edwards and Bohlen, 1996; Sims and Gerard, 1999) and
58 are recognized as good biological indicators of soil quality and impacts of cultural practices
59 (Oberholzer et al., 2012; Paoletti, 1999). These organisms are interesting, both for scientists
60 studying the biological component of cultivated soils and for farmers concerned with
61 monitoring the quality of their soils and assessing the effects of different cultural practices.
62 The common interest of all these people is to move towards sustainable agriculture, producing
63 enough yields while limiting environmental damage.

64 In order to assess human impacts on soil biodiversity and soil invertebrate biomass,
65 earthworms have been sampled by scientists for several decades (Evans and Guild, 1948) and
66 more and more by the general public using standardized and simplified protocols ([http://acer-](http://acer-acre.ca/wp-content/uploads/2011/09/EMAN-MONITORING-BIODIVERSITY-IN-CANADIAN-FORESTS.pdf)
67 [acre.ca/wp-content/uploads/2011/09/EMAN-MONITORING-BIODIVERSITY-IN-](http://acer-acre.ca/wp-content/uploads/2011/09/EMAN-MONITORING-BIODIVERSITY-IN-CANADIAN-FORESTS.pdf)
68 [CANADIAN-FORESTS.pdf;](http://ecobiosoil.univ-rennes1.fr/OPVT_accueil.php) [http://ecobiosoil.univ-rennes1.fr/OPVT_accueil.php;](http://ecobiosoil.univ-rennes1.fr/OPVT_accueil.php)
69 [http://observatoire-agricole-](http://observatoire-agricole-biodiversite.fr/sites/oab.mnhn.fr/files/upload/attached/oab_guide_utilisateur_2013_protocole-vers-de-terre.pdf)
70 [biodiversite.fr/sites/oab.mnhn.fr/files/upload/attached/oab_guide_utilisateur_2013_protocole-](http://observatoire-agricole-biodiversite.fr/sites/oab.mnhn.fr/files/upload/attached/oab_guide_utilisateur_2013_protocole-vers-de-terre.pdf)
71 [vers-de-terre.pdf;](http://www.opalexplorenature.org/soilsurvey) <http://www.opalexplorenature.org/soilsurvey>).

72 Different ‘scientific methods’ are used (Valckx et al., 2011). A common one consists
73 of combining the application of a chemical expellant, which brings earthworms to the soil
74 surface with hand-sorting of the underlying soil (Bartlett et al., 2010). Various chemicals can
75 be used, like formalin (ISO 23611-1:2006; Raw, 1959), mustard powder (Högger, 1993;
76 Muramoto and Werner, 2002) or different brands of commercial mustard (Lawrence and
77 Bowers, 2002; Pelosi et al., 2009). More recently, researchers have demonstrated the
78 effectiveness of allyl isothiocyanate (AITC), an irritating molecule contained in commercial
79 mustard, for sampling earthworms in cultivated areas (Pelosi et al., 2009; Zaborski, 2003).

80 Moreover, according to Čoja et al. (2008), while formalin may harm soil organisms, AITC
81 has no undesirable side-effects on soil organisms.

82 In France, the participatory method of earthworm sampling consists of the application
83 of commercial hot mustard Amora[®] ‘Fine et Forte’ solution on the soil ([http://observatoire-
86 vers-de-terre.pdf](http://observatoire-agricole-
84 biodiversite.fr/sites/oab.mnhn.fr/files/upload/attached/oab_guide_utilisateur_2013_protocole-
85 vers-de-terre.pdf)). However, due to commercial confidentiality, the concentration of AITC in
87 this mustard is unknown, as for all other mustard brands used for earthworm sampling. To
88 date, no study has compared the chemical properties of commercial mustard and AITC. The
89 objectives of the present work are to compare (i) the irritating properties of two chemicals
90 used for sampling earthworms and (ii) the efficiency of the two expellant solutions in terms of
91 diversity, abundance and biomass of earthworms.

92

93 **2. Materials and Methods**

94 *2.1. Chemical analysis of commercial hot mustard*

95 *2.1.1 Commercial hot mustard and chemicals*

96 AITC concentration was analyzed in the commercial mustard Amora[®] ‘Fine et Forte’.
97 To assess variability in AITC concentration, ten 150 g jars from different batches and ten 150
98 g jars from the same batch were compared. AITC (synthetic grade, estimated 97.3%) was
99 purchased from VWR and acetonitrile (HPLC-plus grade) from Carlo-Erba. LC-grade water
100 (resistivity > 18.2 MOhm cm) was produced by a Maxima system (USF Elga, High
101 Wycombe, UK).

102

103 *2.1.2 Extraction procedure*

104 To our knowledge, only one method has been published to measure AITC in mustard
105 (Hils et al., 2001). This method uses water vapor distillation into an ammonia-holding
106 receiver, the allyl thiourea from the AITC then being measured by spectrophotometry. It thus
107 requires a specific distillation apparatus and allows quantification of a transformation product,
108 but not AITC itself. We thus chose to develop a new method, based on AITC solvent
109 extraction and HPLC quantification. According to the optimized method, 2.5 g of mustard
110 were weighed in a 50 mL polypropylene tube (Falcon BD) and immediately suspended in 5
111 mL LC-grade water. Then, 7.5 mL of acetonitrile were added and the mixture was agitated for
112 10 min on an orbital shaker at 300 rpm (KS501 digital, Ika), sonicated for 30 min at a
113 temperature below 30 °C and centrifuged (Allegra X-15R, Beckman Coulter) for 10 min at
114 1300 g and 20 °C. The supernatant was removed with a Pasteur pipette and collected in a 50
115 mL glass vial with a screw cap with PTFE coated seal and protected from light. The sample
116 was extracted three times consecutively with water and acetonitrile as described above and all
117 extracts were collected in the same vial. The final volume (35-37 mL) was estimated by
118 weighing, the extract density being estimated at 0.871. A 2 mL aliquot was filtered with 0.45
119 µm hydrophilic PTFE filter (4 mm, Millex-LH, Millipore) prior to HPLC-UV analysis,
120 collecting filtrate from the fifteenth droplet into the HPLC-injection vial. According to
121 mustard spiking with AITC standard solution, extraction recovery was estimated as $98 \pm 2\%$
122 (approximately 77%, 17% and 4% at each successive extraction cycle).

123

124 *2.1.3 HPLC-UV analysis*

125 HPLC-UV analyses were performed on a Dionex system, including an ASI100T
126 autosampler, a P580 pump, a STH585 column oven and a UVD380S UV-photodiode array
127 detector. Separation was done on a 125 x 2 mm 3 µm Nucleodur C18 HTec reversed-phase
128 column (Macherey-Nagel[®], Düren, Germany) at 20 °C, using a gradient of water and

129 acetonitrile at 0.3 mL min⁻¹ flow rate (75/25 to 25/75 v/v in 20 min). Samples were kept at 15
130 °C and protected from light in the autosampler before being injected. The injection volume
131 was 5 µL. Quantifications were carried out at 242 nm using external calibration. The limit of
132 quantification was estimated at ca. 14 µg.g⁻¹ and the linear range was above 2800 µg.g⁻¹
133 (AITC/mustard). All analyzed mustard jars were extracted and analyzed in triplicate, with a
134 standard deviation of less than 6%.

135

136 2.2. *Earthworm sampling*

137 2.2.1. *Sites and crop systems*

138 Field data were collected in spring 2012 in 22 cultivated fields located in Seine-et-
139 Marne, east of Île-de-France. Among them, 11 fields were under conventional farming and 11
140 under organic farming. Soils were clay loamy (70% silt, 25% clay and 5% sand on average) at
141 near-neutral pH (Appendix A).

142 The climate was temperate oceanic, with a mean annual rainfall of 640 mm and a
143 mean annual temperature of 10.4 °C.

144 Conventional plowing at 25-30 cm depth was carried out in all fields, at a frequency
145 ranging from every year to once every three years. The last plowing was done in 2010 or
146 2011, depending on the field. All fields were cultivated with winter wheat at the time of
147 sampling. Samplings with AITC and commercial mustard were done on the same fields and
148 on the same days.

149

150 2.2.2. *Samplings with AITC*

151 We use the procedure proposed by Pelosi et al., 2009 for AITC application. First,
152 AITC was diluted with isopropanol (propan-2-ol, RPE grade, Carlo-Erba) to obtain a 5 g.L⁻¹
153 solution (Pelosi et al., 2009; Zaborski, 2003). This solution was then diluted with water to

154 reach a concentration of 0.1 g.L⁻¹. After removing vegetation present at the ground surface,
155 two applications of 3.2 L of the prepared AITC solution were applied to the soil at 10-min
156 interval within a 40 x 40 cm metal frame. Taking into account the sample area, a jug with a
157 spout has been used for the application of the AITC solution since a watering can with a rose
158 would not have been appropriate. Emerging individuals were collected for 20 min and
159 preserved in 4% formalin solution. Three replicates, spaced approximately 7-10 meters apart,
160 were made in each field, at least 10 meters from the field edge (Table 1).

161

162 2.2.3. *Samplings with commercial mustard*

163 We use the procedure proposed by the French participatory method of earthworm
164 sampling ([http://observatoire-agricole-
165 biodiversite.fr/sites/oab.mnhn.fr/files/upload/attached/oab_guide_utilisateur_2013_protocole-
166 vers-de-terre.pdf](http://observatoire-agricole-biodiversite.fr/sites/oab.mnhn.fr/files/upload/attached/oab_guide_utilisateur_2013_protocole-vers-de-terre.pdf)). After removing vegetation present at the ground surface, two applications
167 of 10 L of a mustard solution were applied to the soil 15 min apart over 1 m². A suspension in
168 water was prepared by mixing two 150 g jars of commercial hot mustard (Amora[®] 'Fine et
169 Forte') in 10 L of water. All jars were from the same batch and had the same expiry date. A
170 watering can with a rose was used to spread the solution evenly over the sampling surface.
171 Emerging individuals were collected for 30 min and preserved in 4% formalin solution. Three
172 replicates, spaced approximately 7-10 meters apart, were made in each field, at least 10
173 meters from the field edge (Table 1).

174

175 2.2.4. *Earthworm identification*

176 All individuals (juveniles, sub-adults and adults) were counted and weighed with their
177 gut content. Sub-adults and adults were identified at species level according to the
178 identification key of Sims and Gerard (1999). Juveniles were attributed to species according

179 to morphological characteristics and to the specific form they take in formalin in comparison
180 with that of identified adults.

181

182 *2.3. Statistical Analysis*

183 Observations were first transformed to record earthworm abundance and biomass per
184 square meter. Medians of the three replicates per field were calculated and used for statistical
185 analysis. Data presented in the results section are means of these medians. AITC
186 concentration of irritating chemical as well as earthworm diversity (species richness),
187 abundance and biomass in each field recorded with the different chemicals were compared
188 with Wilcoxon Mann-Whitney non-parametric tests because conditions for parametric tests
189 were not fulfilled. We used the R software (R Development Core Team, 2005).

190

191 **3. Results**

192 *3.1. Comparison of AITC concentrations*

193 Chemical analysis of the ten jars of commercial hot mustard from the same batch
194 revealed that concentrations of AITC were relatively uniform within the same batch. The
195 mean of the 10 jars was $860 \mu\text{g}\cdot\text{g}^{-1}$ with a standard deviation of $39 \mu\text{g}\cdot\text{g}^{-1}$, corresponding to a
196 5% standard deviation. Values ranged from 811 to $922 \mu\text{g}\cdot\text{g}^{-1}$.

197 For the 10 jars from different batches, AITC concentrations differed considerably
198 between jars, with a standard deviation of 36%. Values ranged from 273 ± 11 to 1306 ± 13
199 $\mu\text{g}\cdot\text{g}^{-1}$ (mean \pm standard deviation). We noticed that the concentration of AITC in the jars was
200 higher when the indicated time before expiry of the mustard was longer (Fig. 1).

201 For the sampling with pure AITC (proposed by Zaborski, 2003 and Pelosi et al.,
202 2009), 1 mL AITC was poured into 10 L of water. Since the density of the AITC was $1 \text{ g}\cdot\text{cm}^{-3}$,
203 then 1 mL of AITC diluted in 10 L of water corresponded to a concentration of $0.1 \text{ g}\cdot\text{L}^{-1}$.

204 For the sampling with commercial mustard ([http://observatoire-agricole-](http://observatoire-agricole-biodiversite.fr/sites/oab.mnhn.fr/files/upload/attached/oab_guide_utilisateur_2013_protocole-vers-de-terre.pdf)
205 [biodiversite.fr/sites/oab.mnhn.fr/files/upload/attached/oab_guide_utilisateur_2013_protocole-](http://observatoire-agricole-biodiversite.fr/sites/oab.mnhn.fr/files/upload/attached/oab_guide_utilisateur_2013_protocole-vers-de-terre.pdf)
206 [vers-de-terre.pdf](http://observatoire-agricole-biodiversite.fr/sites/oab.mnhn.fr/files/upload/attached/oab_guide_utilisateur_2013_protocole-vers-de-terre.pdf)) involving two 150 g jars of mustard diluted in 10 L of water, the average
207 concentration of AITC was approximately 0.025 g.L^{-1} in the solution that was spread on the
208 soil. However, this concentration could vary from 0.008 to 0.039 g.L^{-1} according to the
209 analysis of ten jars of different batches with 171 to 305 days before expiry. The AITC
210 solution, used in the scientific method of earthworm sampling, was thus about four times
211 more concentrated in AITC than the mean of commercial mustard solutions used in the
212 participatory method.

213

214 3. 2. Comparison of chemical expellant efficiency

215 Mean abundances of total expelled earthworms over the 22 fields were 6.1 and 14.5
216 individuals per m^2 for mustard and AITC solutions respectively, but the difference was not
217 significant ($p=0.39$) (Fig. 2a). Most values obtained with the AITC solution were higher than
218 with the mustard solution but their variability was also higher (Fig. 2a). Mean biomasses of
219 the total earthworm community over the 22 fields were 1.9 g.m^{-2} and 4.9 g.m^{-2} for mustard
220 and AITC solutions, respectively. Again, this difference was not significant ($p=0.18$) (Fig.
221 2b).

222 Both chemical expellants allowed us to sample seven species of earthworms. Six
223 species were common to mustard and AITC solutions: *Lumbricus terrestris* (Linnaeus, 1758),
224 *Aporrectodea longa* (Ude, 1885), *Aporrectodea giardi* (Ribaucourt, 1901), *Allolobophora*
225 *chlorotica* (Savigny, 1826), *Aporrectodea rosea* (Savigny, 1826) and *Aporrectodea*
226 *caliginosa* (Savigny, 1826) (Fig. 3). The first three are anecic and the last three are endogeic
227 species. *Aporrectodea icterica* (Savigny, 1826), an endogeic species, was only found with the
228 AITC solution and *Lumbricus castaneus* (Savigny, 1826), an epigeic species, was only found

229 with the mustard solution (Fig. 3). AITC appeared to be more efficient than mustard to bring
230 the three dominant species to the soil surface (Fig. 3): for mustard and AITC solutions, *L.*
231 *terrestris* represented 21 and 27% of the total abundance, *A. caliginosa* represented 24 and
232 43% of the total abundance and *A. chlorotica* represented 27 and 20% of the total abundance,
233 respectively.

234

235 **Discussion**

236 This study highlighted that AITC concentration was uniform among different jars of a
237 given batch of commercial hot mustard (Amora® ‘Fine et Forte’), used in the participatory
238 method for earthworm sampling and exported and therefore available worldwide. However,
239 the AITC concentration in mustard jars varied five-fold, depending on their use by date. This
240 can cause problems of standardization of the method since commercial mustards of the same
241 brand may not have the same efficiency. Efficiency does not necessarily increase with AITC
242 concentration (Čoja et al., 2008) but the concentration found in the mustard solution was low
243 compared to the optimal AITC concentrations reported by Zaborski (2003). It is thus
244 preferable to use jars with a long time before expiry. To get reliable and comparable results
245 between fields in a study, it is thus preferable to use mustard jars (i) with a long time before
246 expiry for maximum efficiency and (ii) from the same batch for minimum variation in
247 expellant efficiency.

248 Abundance of extracted individuals was quite low compared to data reported by Čoja
249 et al. (2008) in a meadow or by Pelosi et al. (2009) in cultivated fields. This may be due first
250 to the ploughing of the fields where earthworms were collected: in ploughed fields, endogeics
251 are generally found in majority compared to anecics (Chan, 2001), while chemical expellants
252 are preferentially used to collect the latter ecological group (Bouché, 1972). Secondly, the soil
253 was perhaps slightly too dry for an optimal penetration of chemicals in soil. However, it could

254 have been unnecessary to add water before doing the samples, since Eisenhauer et al., 2008
255 explained that “surprisingly, the efficiency [...] was not improved by beforehand water
256 addition”. The results might have been different if the soil had been wetter. Nevertheless, the
257 values found in this study are in accordance with the data published by Bartlett et al (2006)
258 for mustard. Moreover, a hand sorting of earthworms at the end of the extraction time
259 revealed that individuals were active (i.e. not curled in an inactive stage).

260 Both expellant solutions used the same compound, AITC, to extract earthworms but in
261 one case it was pure and in the other case, it was only a component of a commercial product.
262 Although (i) a greater quantity of chemical is sprayed on the soil per square meter (Table 1)
263 and (ii) the concentration of AITC was four times higher in the AITC solution, both solutions
264 seemed to have almost the same efficiency to bring earthworms to the soil surface. While both
265 expellant solutions retrieved the same number of species, we found a trend towards collecting
266 more individuals of the three dominant species with the AITC solution. This could be due
267 either to the more irritating character of the solution, given the difference in AITC
268 concentration, or to the pure compound being more evenly spread in the water, thanks to early
269 isopropanol dilution (Pelosi et al., 2009; Zaborsky, 2003). Indeed, in the field, it was
270 necessary to mix the mustard very thoroughly in water before application to prepare an
271 adequate suspension of commercial mustard. Finally, commercial mustard’s components,
272 other than AITC, may have participated to the efficiency of this expellant for earthworms.

273 We here compared advantages and drawbacks of two extraction methods that are
274 currently used by scientists and citizens for earthworm sampling in France and elsewhere
275 (Table 2). The area sampled with the mustard solution was 6.25 times larger than that
276 sampled with AITC solution. Thus, the amount of material and water needed was
277 proportionally higher.

278

279 **Conclusion**

280 We here described a new effective method to measure allyl isothiocyanate
281 concentration in mustard. Testing commercial mustard jars from different batches and jars
282 from the same batch, we found that AITC concentration in mustard varied five-fold,
283 depending on its use by date. The comparison of the AITC concentration in two earthworm
284 expellant solutions (diluted commercial mustard and pure AITC solution, used in a French
285 participatory method and a scientific method respectively), revealed that AITC concentration
286 was about 4 times higher in the AITC solution than in the mustard one. Moreover, AITC and
287 commercial mustard solutions had the same earthworm extracting efficiency.

288 To improve the efficiency of earthworm samplings with commercial mustard (as in
289 participatory method), it is thus recommended to use jars of commercial hot mustard Amora®
290 ‘Fine et Forte’ from the same batch and with a long time before expiry. It would also be
291 useful to note the expiry date of jars used for earthworm sampling when using the
292 participatory method.

293

294 **Acknowledgements**

295 We would like to thank everybody who participated in earthworm sampling and more
296 especially Lucile Toutous, Jodie Thénard, Félix Fraillon, Françoise Elsass and Nathalie
297 Cheviron. Financial support was provided by the ‘Fédération Île-de-France de Recherche sur
298 l’Environnement’ (FIRE, FR3020) and the ‘Agence Nationale pour la Nature et la
299 Biodiversité en Île-de-France’ (Natureparif). We would also like to thank Marjolaine
300 Deschamps for initial informations on AITC analysis and Alan Scaife for revising the
301 English.

302

303 **References**

304 Bartlett, M.D., Harris, J.A., James, I.T., Ritz, K., 2006. Inefficiency of mustard extraction
305 technique for assessing size and structure of earthworm communities in UK pasture. *Soil*
306 *Biol. Biochem.* 38, 2990-2992.

307 Bartlett, M.D., Briones, M.J.I., Neilson, R., Schmidt, O., Spurgeon, D., Creamer, R.E., 2010.
308 A critical review of current methods in earthworm ecology: from individuals to populations.
309 *Eur. J. Soil Biol.* 46, 67-73.

310 Bouché, M.B., 1972. *Lombriciens de France: Ecologie et Systématique*. INRA Ann Zool Ecol
311 Anim Publication, Paris.

312 Chan, K.Y., 2001. An overview of some tillage impacts on earthworm population abundance
313 and diversity - implications for functioning in soils. *Soil Tillage Res.* 57, 179-191.

314 Čoja, T., Zehetner, K., Bruckner, A., Watzinger, A., Meyer, E., 2008. Efficacy and side
315 effects of five sampling methods for soil earthworms (Annelida, Lumbricidae). *Ecotoxicol.*
316 *Environ. Saf.* 71, 552-565.

317 Edwards, C.A., Bohlen, P.J., 1996. *Biology and Ecology of Earthworms*, 3rd ed. Chapman and
318 Hall, London.

319 Eisenhauer, N., Straube, D., Scheu, S., 2008. Efficiency of two widespread non-destructive
320 extraction methods under dry soil conditions for different ecological earthworm groups. *Eur.*
321 *J. Soil Biol.* 44, 141-145.

322 Evans, A.C., McL. Guild, W.J., 1948. Studies on the relationships between earthworms and
323 soil fertility. *Ann. Appl. Biol.* 35, 485-493.

324 Hils, A.K.A., Verlomme-Fried, A., Höfler, N., 2001. Bestimmung von Allylisothiocyanat in
325 Speisesenfens und Meerrettichzubereitungen. *Z. Arzn. Gew. Pfl.* 6, 25-28.

326 Högger, C.H., 1993. Mustard flour instead of formalin for the extraction of earthworms in the
327 field. *Bull. Bodenkundl. Gesellsch. Schweiz.* 17, 5-8.

328 ISO 23611-1:2006. Soil quality - Sampling of soil invertebrates - Part 1: Hand-sorting and
329 formalin extraction of earthworms.

330 Lawrence, A.P., Bowers, M.A., 2002. A test of the “hot” mustard extraction method of
331 sampling earthworms. *Soil Biol. Biochem.* 34, 549-552.

332 Muramoto, J., Werner, M.R., 2002. Mustard powder vermifuge: an alternative to formalin
333 expulsion method for earthworm sampling. *Edaphologia* 70, 7-11.

334 Oberholzer, H.R., Knuchel, R.F., Weisskopf, P., Gaillard, G., 2012. A novel method for soil
335 quality in life cycle assessment using several soil indicators. *Agron. Sustain. Dev.* 32, 639-
336 649.

337 Paoletti, M.G., 1999. The role of earthworms for assessment of sustainability and as
338 bioindicators. *Agr. Ecosyst. Environ.* 74, 137-155.

339 Pelosi, C., Bertrand, M., Capowiez, Y., Boizard, H., Roger-Estrade, J., 2009. Earthworm
340 collection from agricultural fields: comparisons of selected expellants in presence/absence of
341 hand-sorting. *Eur. J. Soil Biol.* 45, 176-183.

342 R Development Core Team (2005). *R: A Language and Environment for Statistical*
343 *Computing*. R Foundation for Statistical Computing, Vienna, Austria.

344 Raw, F., 1959. Estimating earthworm population by using formaline. *Nature* 184, 1661-1662.

345 Sims, R.W., Gerard, B.M., 1999. *Earthworms*. FSC Publications, London.

346 Valckx, J., Govers, G., Hermy, M., Muys, B., 2011. Optimizing earthworm sampling in
347 ecosystems, in: Karaca, A. (Eds.), *Biology of Earthworms*. Springer, Berlin, pp. 19-38.

348 Zaborski, E.R., 2003. Allyl isothiocyanate: an alternative chemical expellant for sampling
349 earthworms. *Appl. Soil Ecol.* 22, 87-95.

Table 1. Characteristics of the two earthworm sampling methods.

	Sampling with AITC	Sampling with commercial mustard
Sample size	40 x 40 cm x 20 cm depth	100 x 100 cm
Number of replicates	3	3
Distance between replicates	7-10 m	7-10 m
Chemical expellant	AITC with isopropanol (5 g.L ⁻¹), diluted with water (0.1 g.L ⁻¹ AITC)	2 jars of 150g of commercial hot mustard Amora® 'Fine et Forte' in 10 l of water
Quantity of chemical applied to the soil	2 applications of 3.2 L at 10-min interval	2 applications of 10 L at 15-min interval
Quantity of chemical applied to the soil per m ⁻²	40 L	20 L
Collection duration	20 min	30 min

Table 2. Comparison of earthworm sampling using AITC or commercial mustard solutions

	Sampling with AITC	Sampling with commercial mustard
Principle of the method	AITC (with isopropanol) solution	Commercial hot mustard solution
Safety for handlers	Pre-preparation (AITC and isopropanol) in the lab (pure AITC is highly volatile, risk of burning because of high concentration) and dilution in water in the field	Can be prepared in the field, safety for handlers
Safety for environment	Same expellant molecule (allyl isothiocyanate) Additives: isopropanol	Same expellant molecule (allyl isothiocyanate) Additives: manufacturer' trade secret
Easy access to chemical expellants	Bought from a supplier	Available in many shops, exported worldwide
Amount of water to be transported in the field	6.4 L per replicate	20 L per replicate
Time needed	Approximately 35 min per person per replicate	Approximately 50 min per person per replicate

Fig. 1.

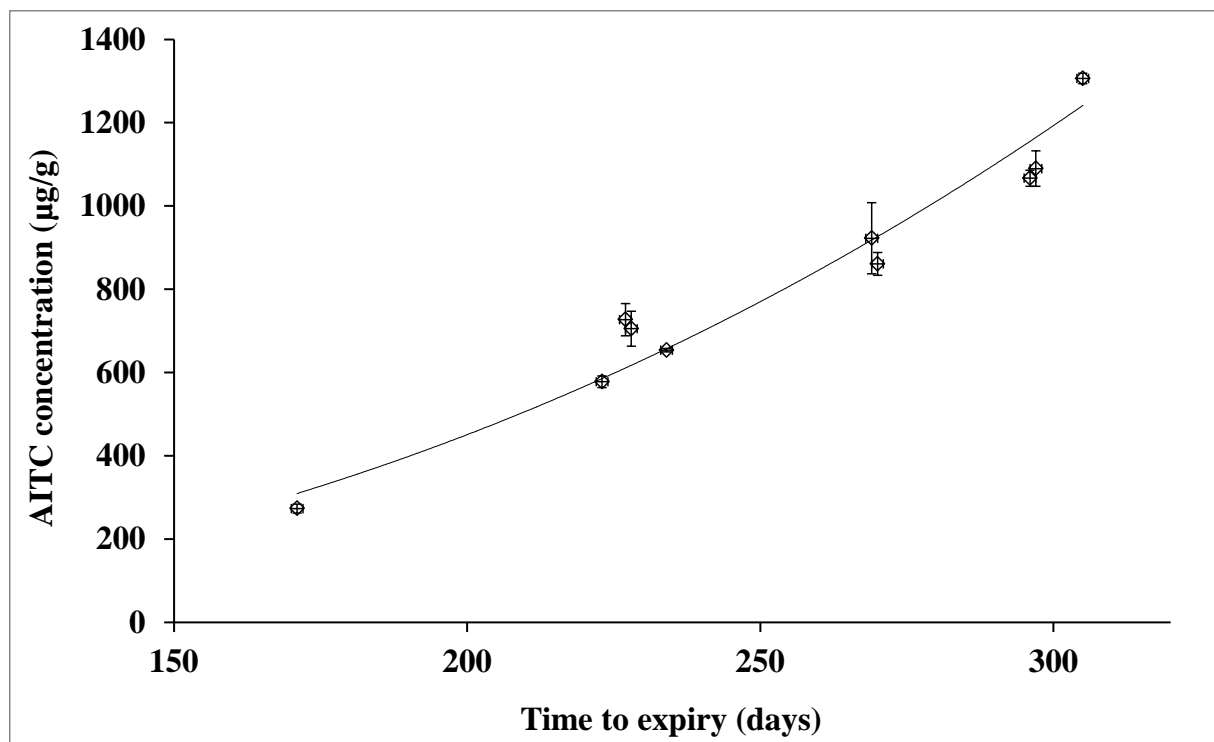


Fig. 1. Allylthiocyanate concentration evaluated as a function of mustard time to expiry (mean of triplicate analyses; error bars correspond to standard deviation).

Fig. 2.

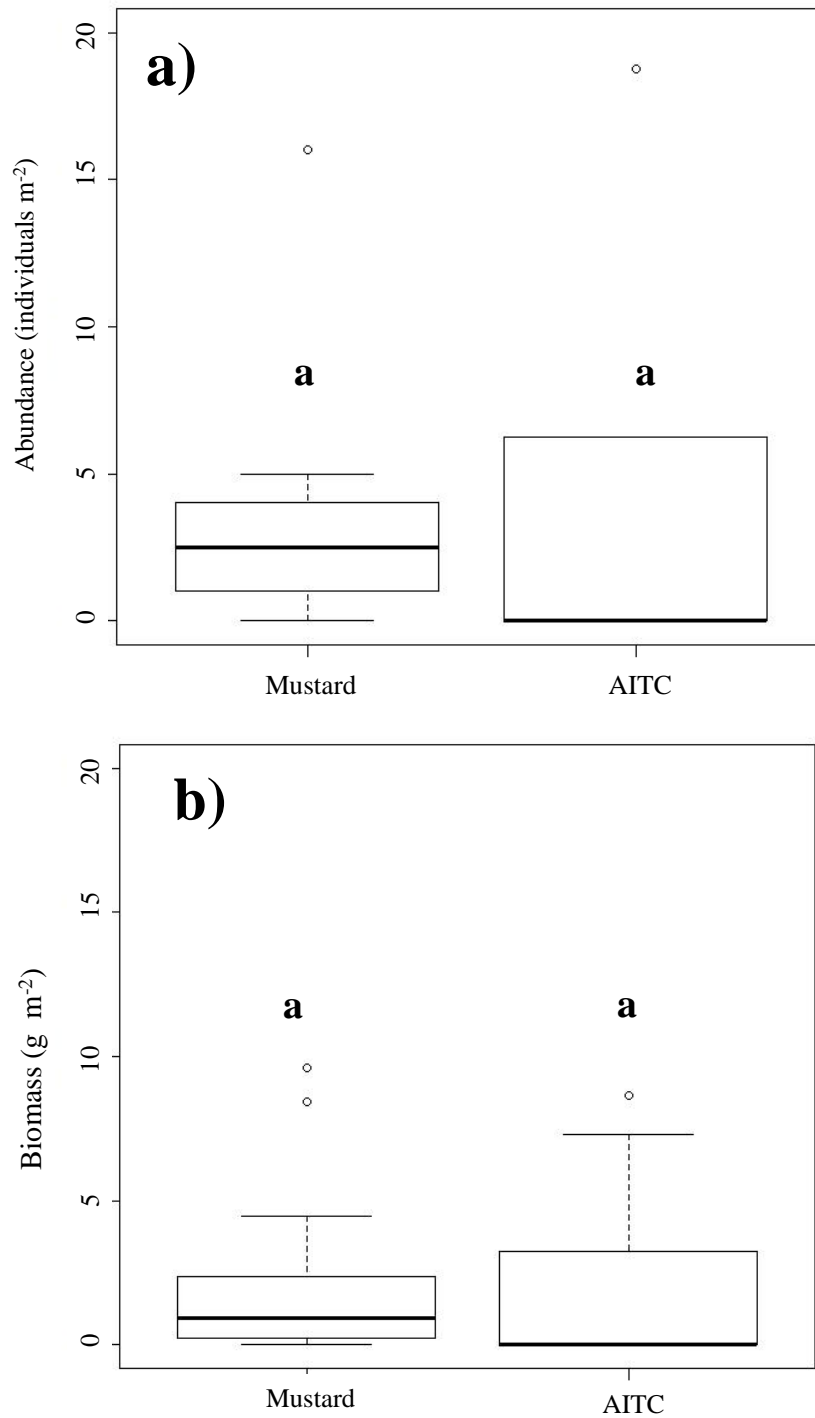


Fig. 2. Boxplots of earthworm abundance (a) and biomass (b) for commercial mustard and AITC solutions. Boxes with the same letter are not different at $P = 0.05$. Values higher than 20 individuals m^{-2} for abundance (three values for mustard and three values for AITC) and 20 $g m^{-2}$ for biomass (one value for AITC) are not represented.

Fig. 3.

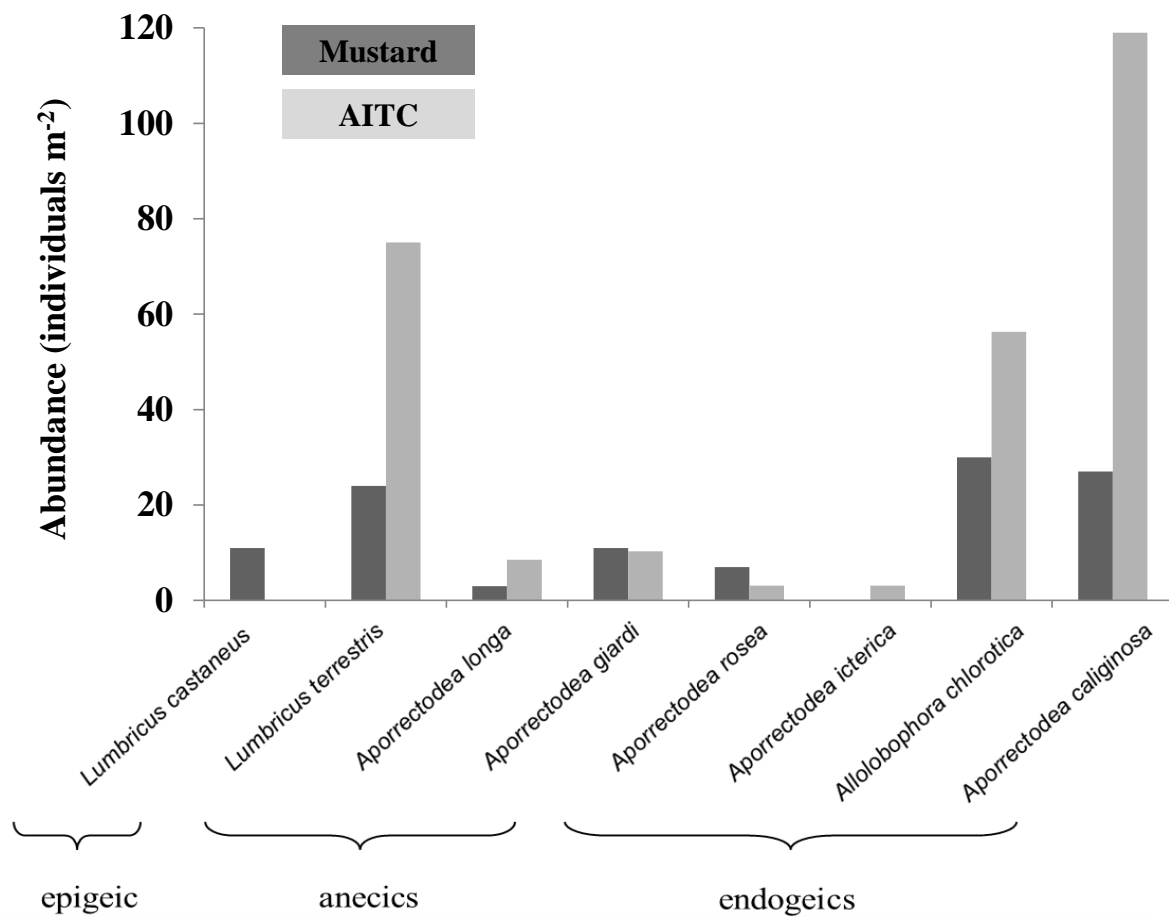


Fig. 3. Cumulative abundance (earthworms m⁻²) of the species found in the 22 agricultural fields for commercial mustard and AITC solutions.

1 Appendix A. Site and soil characteristics of the 22 sampled agricultural field plots in Seine-et-
 2 Marne (all under winter wheat).

Plot name	GPS Coordinates		Cropping system	Clay (g kg ⁻¹)	Silt (g kg ⁻¹)	Sand (g kg ⁻¹)	Organic matter (g kg ⁻¹)	CaCO ₃ (g kg ⁻¹)	C/N ratio	pH
Org1	N 48°79,909'	E 3°13,267'	Organic	261.0	646.0	26.0	19.3	1.0	10.2	7.4
Org2	N 48°66,961'	E 3°17,820'	Organic	242.0	696.0	16.0	19.5	1.0	9.8	7.8
Org3	N 48°67,099'	E 3°17,904'	Organic	299.0	653.0	12.0	19.5	1.0	9.8	7.5
Org4	N 48°68,602'	E 2°78,570'	Organic	174.0	664.0	65.0	22.7	1.0	11.9	7.8
Org5	N 48°43,157'	E 3°32,136'	Organic	193.0	284.0	77.0	25.7	1.0	11.2	8.5
Org6	N 48°70,640'	E 2°67,897'	Organic	178.0	733.0	33.0	32.7	1.0	13.6	7.3
Org7	N 48°76,876'	E 3°15,272'	Organic	194.0	751.0	25.0	23.2	1.0	10.8	7.5
Org8	N 48°76,566'	E 3°14,826'	Organic	170.0	765.0	32.0	19.5	1.0	10.7	7.5
Org9	N 48°64,506'	E 3°04,909'	Organic	227.0	689.0	19.0	23.7	1.1	10.0	7.6
Org10	N 48°29,850'	E 2°87,968'	Organic	256.0	347.0	175.0	26.0	1.7	11.6	7.6
Org11	N 48°84,641'	E 3°10,906'	Organic	165.0	774.0	24.0	19.0	1.0	10.7	7.9
Conv1	N 48°61,808'	E 2°96,832'	Conventional	204.0	704.0	28.0	18.1	1.0	9.9	7.9
Conv2	N 49°03,467'	E 2°84,154'	Conventional	213.0	723.0	12.0	18.2	5.5	9.9	8.1
Conv3	N 49°06,166'	E 2°94,686'	Conventional	180.0	756.0	10.0	16.9	1.1	9.9	7.9
Conv4	N 48°45,583'	E 3°14,232'	Conventional	221.0	658.0	43.0	20.7	7.9	9.9	8.3
Conv5	N 48°43,775'	E 3°04,751'	Conventional	160.0	580.0	135.0	16.1	1.0	10.5	6.4
Conv6	N 48°50,036'	E 3°12,826'	Conventional	228.0	667.0	25.0	16.0	1.0	9.9	8.2
Conv7	N 49°02,709'	E 2°98,335'	Conventional	298.0	648.0	7.0	17.6	1.0	9.4	7.4
Conv8	N 48°40,579'	E 3°32,293'	Conventional	270.0	457.0	123.0	26.9	7.5	10.9	8.2
Conv9	N 48°79,928'	E 3°13,529'	Conventional	209.0	692.0	23.0	17.3	1.0	10.4	7.2
Conv10	N 48°68,684'	E 2°78,558'	Conventional	197.0	646.0	55.0	18.3	1.0	10.2	7.1
Conv11	N 48°84,380'	E 3°10,896'	Conventional	244.0	662.0	41.0	23.8	5.9	10.4	8.0

3

4

5