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Abstract

Per- and poly-fluoroalkyl substances (PFASs) are persistent and bioaccumulative compounds with adverse impacts on the environment and human health. Diet is one of the main sources of exposure to PFASs. Recently, the EFSA established a tolerable weekly intake (TWI) limit (4.4 ng/kg b.w.) for a mixture of the four major PFASs.

Eggs and egg products can contribute to this intake, with their contamination possibly dependent on the husbandry system. Monitoring Italian eggs from backyard chickens revealed a relatively uniform PFAS contamination, with perfluoro-1-octanesulfonate being the most abundant. Contamination was detected to be significantly higher in eggs from backyard chickens than in eggs from commercial laying hens, consistent with a previous Italian study.

According to the recently set TWI value, the consumption of eggs from backyard chickens could contribute significantly to dietary intake of PFASs (up to 29% of the TWI in children, also considering the lower bound approach).

Keywords

PFAS; Eggs from backyard chickens; Hen husbandry system; Dietary intake; LC-MS/MS

Chemical compounds

Chemical compounds studied in this article:

Perfluoro-1-octanesulfonate (PubChem CID: 22483718); Perfluoro-n-octanoic-acid (PubChem CID: 9554); Perfluoro-n-nonanoic acid (PubChem CID: 67821); Perfluoro-1-hexanesulfonate (PubChem CID: 23704962)

Abbreviations

b.w., body weight; EBC, eggs from backyard chickens; ECLH, eggs from commercial laying hens; ESI, electrospray ionisation; LB, lower bound; LC, left-censored; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; PFASs, per- and poly-fluoroalkyl substances; PFOA, perfluoro-n-octanoic acid; PFHxS, perfluoro-1-hexanesulfonate; PFNA, perfluoro-n-nonanoic acid; PFOS, perfluoro-1-octanesulfonate; POP, persistent organic pollutant; QC, Quality control; SPE, solid-phase extraction; TWI, tolerable weekly intake; UB, upper bound; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry

1. *Introduction*

Per- and poly-fluoroalkyl substances (PFASs) are a class of compounds consisting of a hydrophobic alkyl chain of varying length (usually C4–C16), with all the H atoms replaced by F atoms, such that PFASs contain the perfluoroalkyl moiety (C_nF_{2n+1}) and a hydrophilic end group (Buck et al., 2011). Since the 1940s, PFASs have been produced and used in numerous industrial applications because of their characteristic physicochemical properties (Gluge et al., 2020). In fact, the strength of the C-F bond provides these substances with high thermal, chemical, and biological stability, as well as non-flammability and surface-active properties. Therefore, their physicochemical characteristics make these substances extremely persistent and bioaccumulative with potential adverse impacts on human health and the environment (Su et al., 2017).

Among the thousands of commercially manufactured PFASs (Gluge et al., 2020; Sunderland et al., 2019), very few have been monitored in food and the environment and thoroughly investigated with respect to their toxic effects on human health. The most commonly evaluated PFASs are perfluorocarboxylic acids, such as perfluoro-n-octanoic acid (PFOA), and perfluorosulfonic acids, such as perfluoro-1-octanesulfonate (PFOS) (EFSA, 2012; Su et al., 2017). In 2009, PFOS and its salts were included in Annex B of the Stockholm Convention as persistent organic pollutants (POPs), with restrictions placed on their manufacturing and use. Similarly, PFOA and its salts have been included in Annex A of the Stockholm Convention as POPs in 2019, implying that measures should be taken to eliminate their production and use. The European Union Regulation (EU) 2019/1021 on POPs, amended by the Commission Delegated Regulation (EU) 2020/784, has prohibited the manufacturing, commercialisation, and use of PFOS and its salts, along with that of PFOA, its salts, and its derivatives.

72 In humans, PFOS and PFOA are rapidly absorbed in the gastrointestinal tract and widely
73 distributed throughout the body. These PFASs are not metabolised and are, instead, excreted
74 through urine and faeces. Human half-lives for PFOS and PFOA are estimated to be approximately
75 5 and 2–4 years, respectively (EFSA, 2018), and in general, the half-life for long-chain PFASs can
76 exceed 3 years (EFSA, 2020). Human exposure studies to PFAS have focused primarily on serum
77 concentrations (DeLuca, Angrish, Wilkins, Thayer, & Cohen Hubal., 2021). Many studies
78 underscore the relationship between PFAS exposure and adverse health outcomes including
79 dyslipidaemia, especially an increase in serum total cholesterol (EFSA, 2018; Sunderland et al.,
80 2019) and increased serum levels of the liver enzyme, alanine aminotransferase (EFSA, 2020).
81 Moreover, adverse immune outcomes have been reported in children; in particular, PFOS is
82 associated with decreased antibody response at vaccination and reduced birth weight (EFSA,
83 2018; Liew, Goudarzi, & Oulhote, 2018).
84 Epidemiological data do not provide sufficient indications for PFOS and PFOA carcinogenicity in
85 humans (EFSA, 2020; Kennedy, & Symons, 2015). However, some studies have reported evidence
86 of carcinogenicity, although these are limited to individuals exposed to extremely high PFAS
87 concentrations due to occupational hazard (Sunderland et al., 2019).
88 Dietary intake is considered to be one of the major pathways for human exposure to PFASs, along
89 with drinking water and airborne sources (DeLuca et al., 2021; Jian et al., 2017). Environmental
90 diffusion of PFASs can contaminate the soil and water used in food production. PFASs can enter
91 into the animal food chain via feed, water, and soil ingestion by foraging farm animals, thereby
92 resulting in contamination of products, such as milk, eggs, and meat (EFSA, 2020; Göckener et al.,
93 2020). Food can also be contaminated through the presence of PFAS in food packaging or
94 processing equipment (EFSA, 2020). Several studies have been conducted to assess PFAS
95 contamination in different food products, with fish and seafood reported to contain the highest

level of these contaminants (Barbarossa et al., 2016; Chiesa et al., 2018; EFSA, 2018; Hlouskova et al., 2013; Jian et al., 2017; Vestergren, Berger, Glynn, & Cousins, 2012).

In 2020, EFSA published a recent scientific opinion on the risk to human health associated with the presence of PFASs in food (EFSA, 2020). The novelty of this opinion lies in the fact that it represents an assessment for the sum of four PFASs (namely PFOS, PFOA, PFNA, and PFHxS), claimed to 'contribute most to the levels observed in human serum' and with similar effects in animals and toxicokinetics.

In recent years, the EFSA has developed a harmonised framework for evaluating the potential 'combined effects' of mixtures of PFASs in food and feed (EFSA, 2019). Based on this approach, the 2020 opinion sets a group tolerable weekly intake (TWI) limit of 4.4 ng/kg b.w. for the mixture of the four PFASs. This value was actually lower than that set in the previous EFSA opinion (EFSA, 2018), where separate values were set for PFOS and PFOA (13 and 6 ng/kg b.w. per week, respectively). The EFSA CONTAM Panel concluded that parts of the European population exceed this new TWI, which is of concern (EFSA, 2020). This study showed that the main food categories contributing to a combined exposure to PFOS, PFOA, PFNA, and PFHxS were 'Fish meat', 'Fruit and fruit products', and 'Eggs and egg products' across all population groups.

Although there are numerous publications on PFAS levels in foods, only few have specifically focused on chicken eggs (D'Hollander, de Voogt, & Bervoets, 2011; Zafeiraki et al., 2016). In addition, most studies have focused on wild bird eggs (Miller, Elliott, Elliott, Lee, & Cyr, 2015; Letcher et al., 2015) or other wild animal species related to environmental biomonitoring studies. Other studies have reported PFAS levels in various food items, including chicken egg, but often with a limited number of samples (Guerranti, Perra, Corsolini, & Focardi, 2013; Hlouskova et al., 2013; Jain, 2018; Jian et al., 2017; Vestergren, Berger, Glynn, & Cousins, 2012).

119 Chicken eggs represent a non-negligible part of the human diet, with a per-capita consumption of
120 approximately 14 kg/year both in the EU and Italy (FAO, 2020). As discussed above, these eggs
121 could be a possible source of PFASs in particular, and POPs in general, for humans. It is noteworthy
122 to consider that the contamination level may depend on the bird husbandry system employed
123 (Pajurek, Pietron, Maszewski, Mikolajczyk, & Piskorska Pliszczynska, 2019). In addition to eggs
124 from commercial laying hens (ECLH), many people consume eggs from backyard chickens (EBC),
125 which are usually raised outdoors with access to a pasture where they can forage for small insects,
126 worms, and soil particles. Such feeding behaviour can represent a route of exposure to
127 environmental pollutants, including PFASs, for the chickens, and could therefore represent a
128 potential source of contamination for chicken eggs (Pajurek, Pietron, Maszewski, Mikolajczyk, &
129 Piskorska Pliszczynska, 2019; Zafeiraki et al., 2016). Eggs from rural flocks may represent a PFAS
130 source, particularly for PFOS (Brambilla et al., 2015).
131 Therefore, the aim of the present study was to characterise the contamination level of the four
132 major PFASs (PFOS, PFOA, PFNA, and PFHxS) in EBC in Italy. The next goal was to compare these
133 results with those previously reported for Italian ECLH (Ghelli et al., 2019) and that reported in the
134 latest EFSA opinion (EFSA, 2020), using the lower bound (LB) and upper bound (UB) approaches.
135 Finally, the results obtained in the present study were used to further evaluate whether EBC
136 represent an important human dietary source of PFASs.
137

138 ## 2. *Material and Methods*

139 ### *2.1 Sample collection*

140 A total of 208 eggs were collected during the 2018-2019 period from 26 sites distributed along the
141 Italian peninsula, which were classified according to their geographical position into two groups:
142 North (12 sites) and Centre/South (14 sites), as shown in Figure S1. After collection, the eggs were

143 boiled, and the yolks were separated from the egg whites. For each sampling site, yolks were
144 pooled to obtain 78 pools of 2 or 3 yolks each, based on their availability. These pooled samples
145 were stored at -20 °C until the analysis.
146
147 *2.2 Reagents and chemicals*
148 Mass spectrometry grade acetonitrile, ammonium acetate, formic acid, and methanol were
149 acquired from Fluka /Honeywell (St. Louis, MO, USA). Sodium hydroxide pellets (>98%) were
150 obtained from Fluka/Honeywell, and ammonium hydroxide (33%) and sodium acetate were
151 procured from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (37%) was purchased from
152 Carlo Erba Reagents (Cornaredo, MI, Italy). Oasis WAX (Weak Anionic eXchange) 3 cc Vac
153 Cartridges, 60 mg (Waters Corp., Milford, MA, USA) were used for solid-phase extraction (SPE).
154 Ultrapure water (18.2 MΩ/cm) was obtained using a Human Power I lab water purification system
155 (Human Corp., Seoul, South Korea).
156 PFOS, PFOA, PFNA, and PFHxS standards and the corresponding isotopically labelled standards (IS),
157 sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]octanesulfonate (M-PFOS), perfluoro-n-[1,2,3,4-$^{13}C_4$]octanoic
158 acid (M-PFOA), perfluoro-n-[1,2,3,4,5-$^{13}C_5$]nonanoic acid (M-PFNA), and perfluoro-1-
159 hexane[$^{18}O_2$]sulfonate (M-PFHxS) were purchased from Wellington Laboratories (Guelph, ON,
160 Canada). An appropriate amount of each standard was combined and diluted in methanol to
161 obtain a 'PFAS working solution' at a concentration of 50 ng/mL. The 'IS working solution' was
162 prepared using the same procedure.
163
164 *2.3 Sample Preparation*
165 Sample preparation was performed according to a previously published method (Ghelli et al.,
166 2019), based on an earlier protocol (Zafeiraki et al., 2016).

167 Briefly, 1 g of homogenised yolk was fortified with 50 µL of 'IS working solution'.

168 The sample was then digested with a sodium hydroxide solution and homogenised using Ultra

169 Turrax. In the next step, the sample was extracted by the addition of methanol, followed by HCl

170 (37%). The sample was centrifuged, and the supernatant was transferred into a new tube

171 containing ultrapure water.

172 The extract was purified by SPE using Oasis WAX cartridges. Finally, the eluate was dried and

173 redissolved in 20 mM ammonium acetate:methanol (90:10) and analysed by UPLC-MS/MS.

174 All procedure steps were performed using polypropylene (PP) materials to prevent possible

175 interactions of PFAS with glass.

176

177 *2.4 Analytical conditions*

178 The analytical system included an ACQUITY UPLC system consisting of a binary pump, solvent

179 degasser, autosampler, and column heater fitted with a Waters BEH C18 column (1.7 µm, 2.1 x 50

180 mm) equipped with a guard column (Waters Corp.). To avoid contamination by LC system, an

181 isolator column for PFAS (2.1 x 50 mm) (Waters Corp.) was used for all analyses. The mobile phase

182 consisted of 20 mM ammonium acetate aqueous solution (A) and methanol (B), and the elution

183 gradient started with 10% B for 1.50 min, followed by a 1 min linear gradient to 80% B, and finally

184 a hold for 1.50 min at 80% B. The flow rate was 0.4 mL/min, and the injection volume was set at

185 10 µL. A Quattro Premiere XE tandem quadrupole mass spectrometer equipped with an ESCi™

186 Multi-Mode Ionization Source (Waters Corp.) and operated in the negative electrospray ionisation

187 (ESI −) mode was used for the analysis.

188 The instrument was operated in the multiple reaction monitoring mode (MRM), and the two

189 transitions monitored for each analyte are reported in Table S1.

190 The capillary voltage was 2.0 kV, extractor voltage 2.00 V, source temperature 150 °C and
191 desolvation temperature 450 °C. Data acquisition and processing were performed using the
192 MassLynx 4.1 software (Waters Corp.).
193
194 *2.5 Calibration and validation*
195 The isotope dilution technique was employed to ensure sufficient correction of the response for
196 reliable quantification based on the mass spectrometry data.
197 Matrix-matched calibration curves were prepared for each day of analysis. A 1 g aliquot of blank
198 yolk was spiked with appropriate amounts of the 'PFAS working solution' to obtain 5 levels of
199 concentration (in the range of 0-10 µg/kg) and 50 µL of the 'IS working solution'. Analysis of
200 samples prepared this way showed a good linear response, with linear regression coefficient (R^2)
201 values always greater than 0.99.
202 The protocol was validated in accordance with the current European guidelines set by Decision
203 2002/657/EC (European Commission, 2002). The specificity of the method was demonstrated by
204 establishing the absence of potential interferences around the retention times of the analytes in
205 chromatograms obtained from non-contaminated yolk samples. Quality control (QC) samples
206 were prepared at three different concentrations (0.5, 2, and 5 µg/kg) in four replicates, and were
207 used to demonstrate the accuracy of the method; the maximum relative standard deviation to the
208 mean (CV%) ranged from 9-19 %, and trueness (relative difference between the measured mean
209 value and the spiked concentration) was always lower than 10%. Limits of quantification (LOQs)
210 and limits of detection (LODs) of the method, defined as the concentrations providing a
211 chromatographic signal with a signal-to-noise (S/N) ratio equal to 10 and 3, respectively, were
212 estimated to be 0.25 and 0.10 µg/kg, respectively, for all analytes.
213

214 *2.6 Weekly intake estimation*

215 Dietary intake of the mixture of four PFASs via EBC was estimated for the whole Italian population,
216 as well as for that in each geographical area (North and Centre/South) considered in this study.
217 The Comprehensive European Food Consumption database (EFSA, 2015), which reports on food
218 consumption by the Italian population in the period of 2005-2006 (based on a survey by INRAN-
219 SCAI (Leclercq et al., 2009)), was used for calculating the dietary intake. The survey reports the
220 average daily consumption of eggs expressed in g/kg b.w. for five different age groups: 0-2, 3-9,
221 10-17, 18-64, and 65-97 years.
222 Weekly intake was estimated based on the assumption that the yolk represents approximately
223 30% of the whole egg and that egg consumption was exclusively represented by those sourced
224 from EBC.
225

226 *2.7 Statistical analysis*

227 A descriptive statistical approach (i.e., median value, standard deviation, range) was applied to the
228 data on pooled EBC samples. The concentrations of PFAS compounds in EBC measured in this
229 study and those reported in Italian ECLH (Ghelli et al., 2019) were compared with the data
230 reported in the latest EFSA Opinion (EFSA, 2020). The LB values were obtained by assigning a value
231 of zero to all samples reported to be lower than the LOD (<LOD) or LOQ (<LOQ). The UB values
232 were obtained by assigning the numerical value of LOD (0.10 µg/kg) to values reported as <LOD
233 and LOQ (0.25 µg/kg) to values reported as <LOQ. These data were analysed using the Student's *t*-
234 test considering the type of husbandry system as a factor (commercial vs. backyard). The presence
235 of one or more PFASs in the analysed samples (both ECLH and EBC) was determined and
236 expressed as a percentage. Within the EBC group, egg samples were further classified according to
237 the geographical location of the collection site (North or Centre/South Italy), as indicated in Figure

238 S1). In addition, the relative concentrations of PFAS compounds in EBC were analysed using the
239 Student's *t*- test, considering the geographical location of the collection site (North vs.
240 Centre/South) as an experimental factor.
241

242 ## 3. Results and Discussion

243 ### *3.1 PFAS levels in eggs from EBC*

244 Contaminant levels were measured in the yolk, since earlier studies have demonstrated that PFASs
245 are primarily found in the egg yolk rather than in the egg white (Göckener et al., 2020; Zafeiraki et
246 al., 2016).
247 The concentrations of PFOS, PFOA, PFNA, and PFHxS measured in the pooled EBC samples are
248 presented in Table 1.
249 Of the 78 pools analysed, 58 (74.4%) showed the presence (>LOD) of PFASs, and PFAS levels were
250 quantifiable (≥LOQ) in 41 (52.6%) pools. PFOS was the most frequently detected contaminant
251 (65.4%), with the highest concentration (up to 3.47 µg/kg and a median value of 1.29 µg/kg)
252 among the quantifiable samples.
253 The highest level of PFOS contamination in EBC were in agreement with that reported in previous
254 studies. In particular, the PFOS median value measured in this study aligns with the value reported
255 for eggs collected in Greece (1.1 µg/kg) while it is considerably lower than that obtained in the
256 Netherlands (3.1 µg/kg) (Zafeiraki et al., 2016) and Belgium (6.8 µg/kg) (D'Hollander, de Voogt, &
257 Bervoets, 2011). In addition, the range of PFOS concentrations obtained in this study for the
258 quantifiable samples (0.25-3.47 µg/kg) is narrower than those found in similar studies conducted
259 in Greece (<0.5–8.9 µg/kg), the Netherlands (<0.5–24.8 µg/kg), and Belgium (0.4–3473 µg/kg).
260 The other three PFASs showed similar frequencies, range values, and concentrations among them
261 (Table 1).

262

263 ***Table 1.*** *Frequency and concentration of PFOS, PFOA, PFNA, PFHxS, and Σ(PFASs) analysed in*

264 *pooled EBC samples*

	Detected samples (>LOD)		**Non-quantifiable samples (LOD>X>LOQ)**	**Quantifiable samples (X>LOQ)**			
	Number	Fraction (%)	Number	Number	Fraction (%)	Range µg/kg	Median value
PFOS	51	65.4	12	39	50.0	0.25-3.47	1.29
PFOA	16	20.5	15	1	1.3	0.62	-
PFNA	29	37.2	25	4	5.1	0.25-1.20	0.46
PFHxS	19	24.4	15	4	5.1	0.25-0.50	0.49
Σ(PFASs)	58	74.4	17	41	52.6	0.25-4.64	1.28

265

266 Next, a comparative analysis of the contaminant levels in EBC collected in northern and

267 central/southern Italy was carried out. These results are provided in Table 2.

268

269 ***Table 2.*** *Concentration of PFOS, PFOA, PFNA, PFHxS, and Σ(PFASs) in EBC collected from sites in*

270 *North or Centre/South regions of Italy*

	LB (µg/kg)		**UB (µg/kg)**		***P*-value LB**	***P*-value UB**
	North	Centre/South	North	Centre/South		
PFOS	0.79 ± 0.13	0.49 ± 0.13	0.86 ± 0.12	0.57 ± 0.12	0.11	0.07
PFOA	0.01 ± 0.01	0.00 ± 0.00	0.15 ± 0.01	0.11 ± 0.01	0.38	0.02
PFNA	0.03 ± 0.03	0.04 ± 0.02	0.19 ± 0.03	0.16 ± 0.02	0.21	0.34
PFHxS	0.05 ± 0.02	0.00 ± 0.00	0.16 ± 0.02	0.13 ± 0.01	0.07	0.39
Σ(PFASs)	0.88 ± 0.16	0.53 ± 0.14	1.36 ± 0.15	0.97 ± 0.14	0.07	0.01

271

272 The most significant region-wise difference was observed in the total contaminant level

273 (corresponding to a mixture of the four PFASs), which was significantly higher in EBC from

274 northern Italy (UB value: $P<0.01$). Similarly, PFOA levels were significantly higher in EBC collected

275 from northern Italy (UB: 0.15 vs. 0.11 µg/kg; $P<0.02$). These results could be ascribed to the

276 greater concentration of industrial sites in regions of Northern Italy, which could have resulted in

277 greater contamination of soil and environment and, in turn, to higher PFAS levels in EBC.

278

279 *3.2 Comparison of PFAS contamination in EBC and ECLH*

280 The results of the present study were compared to those reported earlier in 132 ECLH reared in

281 Italy using the same analytical method (Ghelli et al., 2019) and re-calculated according to the LB

282 and UB approaches or to those reported in the latest EFSA Opinion (EFSA, 2020) as a reference

283 (shown in Table 3).

284

285 ***Table 3.*** *Comparison of the mean levels of PFOS, PFOA, PFNA, PFHxS, and Σ(PFASs) measured in*

286 *EBC or ECLH with those reported in the 2020 EFSA Opinion on 'eggs and egg products'*

	PFOS			**PFOA**			**PFNA**			**PFHxS**			**Σ(PFASs)**	
	LC (%)	LB (µg/kg)	UB (µg/kg)	LC (%)	LB (µg/kg)	UB (µg/kg)	LC (%)	LB (µg/kg)	UB (µg/kg)	LC (%)	LB (µg/kg)	UB (µg/kg)	LB (µg/kg)	UB (µg/kg)
EBC (Present study)	50	0.66 ± 0.10	0.73 ± 0.09	99	0.01 ± 0.01	0.14 ± 0.01	95	0.03 ± 0.02	0.18 ± 0.02	95	0.03 ± 0.01	0.15 ± 0.01	0.73 ± 0.11	1.20 ± 0.11
ECLH (Ghelli et al., 2019)	98	0.01 ± 0.01	0.11 ± 0.01	100	0.00 ± 0.00	0.11 ± 0.01	100	0.00 ± 0.00	0.11 ± 0.01	98	0.01 ± 0.01	0.12 ± 0.01	0.02 ± 0.01	0.45 ± 0.02
EBC vs. ECLH *P*-value		<0.001	<0.001		0.45	0.10		0.13	<0.001		0.43	0.04	<0.001	<0.001
2020 EFSA Opinion	92	0.27	0.35	92	0.11	0.21	100	0.00	0.10	97	0.00	0.06	-	-

287 LC: Left-censored (%)

288

289 From this comparison, it is evident that the contaminant level in EBC is generally higher than that

290 reported for ECLH (Ghelli et al., 2019) and in the 2020 EFSA opinion.

291 The number of quantifiable samples for PFOS in EBC was considerably higher and the mean values

292 of contaminants more than doubled for both LB and UB compared with those reported in the EFSA

293 opinion. On the contrary, ECLH showed a smaller percentage of quantifiable samples and lower

294 contaminant levels than those reported in the 2020 EFSA opinion.

295 PFNA and PFHxS were quantifiable in 5% of the EBC samples and, in this case as well, the values of
296 both LB and UB were markedly higher than those reported in the 2020 EFSA opinion. On the other
297 hand, ECLH samples had a similar percentage of quantifiable samples and showed similar levels of
298 contamination for PFNA and a higher level for PFHxS than those reported in the EFSA opinion.
299 In EBC samples, PFOA was quantified in only one pool, and both LB and UB values were found to
300 be lower than those reported in the EFSA opinion. Overall, this finding agrees with the study on
301 ECLH.
302 A direct comparison of the data on EBC and ECLH highlighted the significantly ($P<0.01$) higher total
303 contaminant level (corresponding to a mixture of the four PFASs), as well as that for PFOS (both LB
304 and UB values) and PFNA (only UB values) in EBC (Table 3).
305 Figure 1 shows the distribution of PFASs in eggs produced in the two husbandry systems (EBC and
306 ECLH). A total of 52.6% of the analysed EBC samples showed the simultaneous presence of more
307 than one PFAS, whereas at least one PFAS was detected in 21.8% of the samples. On the other
308 hand, only 4.5% of the ECLH samples were contaminated by one PFAS, while none of the ECLH
309 samples were reported to contain two or more types of PFAS.
310
311

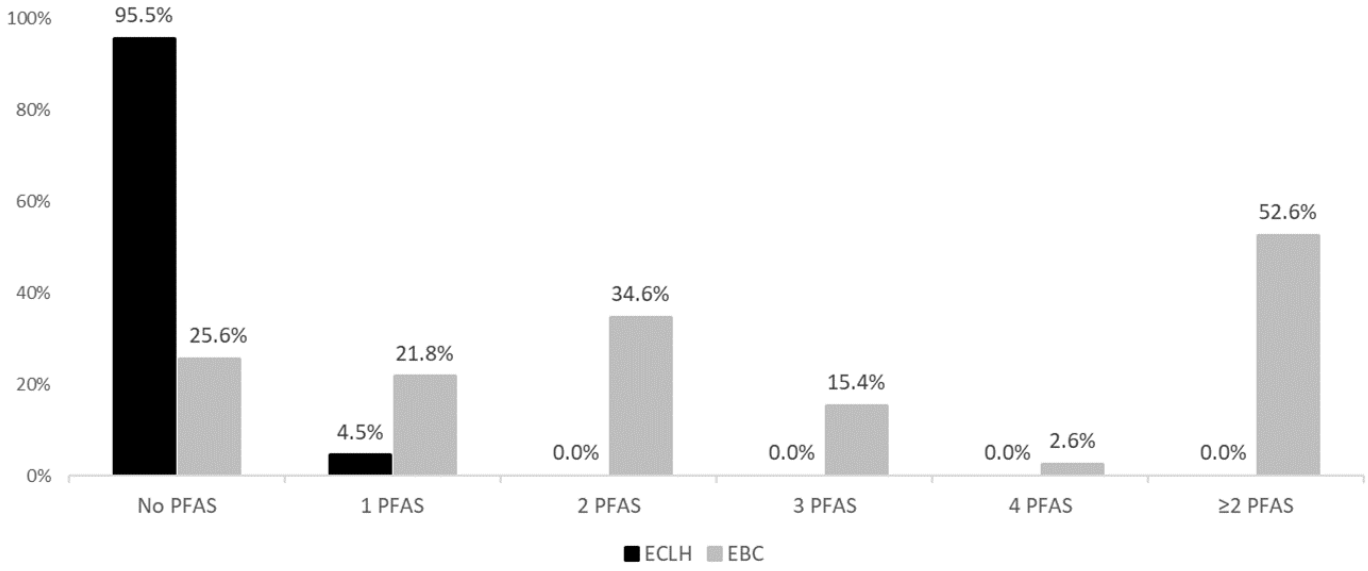

Fig. 1. *Fraction (%) of eggs from ECLH and EBC reared in Italy contaminated with one or more PFASs*

The observed differences between contamination levels in EBC and ECLH samples could be related to the outdoor rearing technique of hens in the former system, in which birds live in constant contact with the soil and consequently ingest soil-based particles, worms, and insects. It is well known that PFASs accumulate in the environment because of their stability and resistance to chemical and physical degradation. The Italian Institute for Environmental Protection and Research performed a screening to check for PFAS contamination in surface and underground water bodies in Italy (ISPRA, 2019). Among the perfluoroalkyl substances found in water, PFOS and PFOA were the most common.

PFASs can be adsorbed by the soil and sediment particles due to the interaction of the perfluoroalkyl tails with the organic carbon fraction of the soil, and to a lesser extent, through electrostatic interactions between the hydrophilic terminal group of PFAS and charged moieties present in the clay fraction (Higgins & Luthy, 2006). Longer-chain PFASs are, therefore, more easily

329 adsorbed into the soil, and sulfonated ones are more abundant than the carboxylated analogues.
330 PFOA is mainly encountered in the liquid phase, whereas PFOS has a higher adsorption capacity.
331 A number of other studies (Amundsen et al., 2008, Das, Megharaj, & Naidu 2015; D'Hollander, De
332 Bruyn, Hagenaars, de Voogt, & Bervoets, 2014; Lasier, Washington, Hassan, & Jenkins, 2011) have
333 evaluated the transfer of PFASs from soil to earthworms. Even in these cases, it was found that the
334 tendency for bioaccumulation increases with increasing chain length and in the presence of a
335 sulfate group. Higher accumulation of sulfate PFASs in soil and earthworms could explain the
336 greater presence of PFOS than PFOA in EBC.
337 An earlier review (Brambilla, D'Hollander, Oliaei, Stahl, & Weber, 2015) modelled PFOS transfer
338 from water to extensive and free-range food-producing animals, while taking into account the
339 spread of contaminated sludge on agricultural land. Although the modelling indicated that
340 foraging behaviour led to 78% of exposure to contaminants in ruminating animals, the soil
341 accounted for >80% of exposure in the case of poultry and pigs raised outdoors. For free-range
342 hens, a soil-to-egg bioaccumulation ratio of 8.9 has been reported. Backyard hens generally live
343 longer than animals reared intensively, and therefore, they are exposed to contaminants for a
344 longer period of time. Moreover, the lower egg deposition rate of backyard hens than that of
345 commercial hybrids with higher reproductive potential could also lead to a higher PFOS content in
346 EBC, as these contaminants accumulate in a lower number of eggs. According to these authors
347 (Brambilla, D'Hollander, Oliaei, Stahl, & Weber, 2015), 'eggs from rural flocks may represent a
348 PFOS source'. Consistently, a study conducted in Italy with 500 women of reproductive age
349 revealed higher plasma levels of PFAS in those who consumed domestic eggs (Defelip et al., 2015).
350

351 *3.3 Weekly intake estimation*

352 Table 4 shows the estimated weekly intakes of the mixture of PFOS, PFOA, PFNA, and PFHxS
353 associated with the consumption of EBC for five different age groups in the Italian population.
354 Similar to the previous analysis, these estimates have also been provided as LB and UB values. The
355 resulting weekly intake was compared with the TWI limit of 4.4 ng/kg b.w. recently set by the EFSA
356 for the mixture of the four PFASs (EFSA,2020). It is noteworthy that no data regarding the actual
357 consumption of EBC are available for the Italian population. Therefore, the weekly intakes were
358 estimated considering that egg consumption was exclusively EBC and represented the average
359 exposure for the entire Italian population, as well as the population in each geographical area
360 (North and Centre/South), according to the different age groups.
361 These results showed that the consumption of EBC could substantially contribute to the dietary
362 intake of PFASs. In particular, this is true for the 3-9 years age group, where this intake would
363 represent 29 and 48% of the LB and UB values, respectively. Following the greater contamination
364 of eggs produced in northern Italy, the resulting weekly intake for this region is worse for the
365 same age group, with percentage values reaching 39% (LB) and 55% (UB) of the established limit.
366 Notably, according to the latest EFSA scientific opinion (EFSA, 2020), the calculated LB exposure is
367 likely to be more realistic than UB exposure.

368

369 ***Table 4.*** *Estimated weekly intake of the mixture of 4 PFASs via EBC in the Italian population for*
370 *different intervals of age (data in italics refers to North or Centre/South of Italy), calculated in LB*
371 *and UB concentrations and reported as a percentage of the TWI value set by EFSA (EFSA, 2020)*

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373

Age group (year)		Weekly intake (ng/kg b.w.)			
		LB	% TWI	UB	% TWI
0-2	Italy	0.70	16	1.16	26
	North	*0.86*	*19*	*1.32*	*30*
	Centre/South	*0.52*	*12*	*0.94*	*21*
3-9	Italy	1.29	29	2.11	48
	North	*1.56*	*36*	*2.41*	*55*
	Centre/South	*0.94*	*21*	*1.72*	*39*
10-17	Italy	0.64	15	1.06	24
	North	*0.78*	*18*	*1.21*	*27*
	Centre/South	*0.47*	*11*	*0.86*	*20*
18-64	Italy	0.47	11	0.78	18
	North	*0.58*	*13*	*0.89*	*20*
	Centre/South	*0.35*	*8*	*0.64*	*14*
65-97	Italy	0.46	10	0.76	17
	North	*0.56*	*13*	*0.86*	*20*
	Centre/South	*0.34*	*8*	*0.61*	*14*
Total population	Italy	0.54	12	0.88	20
	North	*0.65*	*15*	*1.01*	*23*
	Centre/South	*0.39*	*9*	*0.72*	*16*

374
375

376 ## *4. Conclusion*

377 To our knowledge, this study is the first to monitor levels of PFAS contamination in eggs produced
378 from backyard chicken reared in Italy, which represent a non-negligible share of total egg
379 consumption in the region. Our results revealed a relatively uniform level of contamination, with
380 PFOS representing the most abundant and widespread contaminant in EBC. Overall, the presence
381 and levels of PFAS contamination in EBC were remarkably higher than those previously observed
382 for ECLH. A plausible explanation for this difference could be that PFAS are persistent
383 environmental pollutants, and, therefore, backyard chickens, which live outdoors and are known
384 to forage in the soil, have higher exposure to these substances than do commercially raised hens
385 with no outdoor access. Furthermore, EBC raised in northern regions of Italy had higher PFAS
386 contamination than those raised in the central/southern regions. This could be related to the
387 greater contamination of the soil and environment in northern Italy due to different concentration
388 of industrial sites along the Italian peninsula. Finally, the weekly intake estimation revealed that

389 EBC could represent an important dietary source of PFASs. This is especially true for children (3-9
390 years) where, even considering the LB approach, the dietary intake could contribute to 29% of the
391 TWI value for PFASs.
392
393
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397
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