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1 **Can soil properties and land use explain glomalin-related soil protein**
2 **(GRSP) accumulation? A nationwide survey in France**

3 Siobhan Staunton¹, Nicolas P.A. Saby², Dominique Arrouays², & Hervé Quiquampoix¹

4 ¹Eco&Sols, INRAE, IRD, Cirad, SupAgro, University of Montpellier, place Viala, 34060

5 Montpellier, France

6 ²INRAE, Unité Infosol US1106, 45075 Orléans 2, France

7 **Corresponding author:** Siobhan Staunton

8 Phone +33 (0) 4 99 61 23 31. E-mail: siobhan.staunton@inrae.fr

9 **Abstract**

10 Organic matter plays essential roles in soil, including physical stabilization, nutrient storage
11 and carbon sequestration. An operationally defined fraction of soil organic matter known as
12 glomalin or glomalin-related soil protein, GRSP, is obtained by autoclaving soil in citrate
13 solution. It is reputed to be of fungal origin, very stable and responsible for enhanced soil
14 physical stability. This is the first nationwide survey of GRSP content in soil and was conducted
15 to test the hypotheses on the origin and identity of GRSP. Nearly 200 archived soils were
16 selected on the basis of organic matter content from the French National Soil Inventory,
17 representing mostly cropland, grassland and woodland land uses. Two fractions of GRSP were
18 measured, easily extractable (GRSP_{EE}) and total (GRSP_T), extraction. The median values of
19 GRSP_{EE} and GRSP_T were 0.7 and 2 g kg⁻¹ respectively, with a strong correlation between the
20 two fractions. Scatter plots and cubist modelling were used to explore the relationships between
21 the contents of the two GRSP fractions and both soil properties and land use. Land use effects
22 were almost entirely attributable to soil characteristics. No evidence was found to support the
23 hypotheses that GRSP is solely of fungal origin, nor that easily extracted GRSP is more recent
24 than the total fraction, although this does not disprove either hypothesis. Soil organic matter was
25 enriched in GRSP C-depleted cropland soils and lower in C-rich woodland, this may result from
26 inherent stability or differences in plant-related composition of carbon input.

27 **Keywords** : soil, glomalin, protein, autoclaved citrate-extractable protein, carbon
28 sequestration, carbon dynamics, fungal tracer

29 **Highlights**

- 30 • Land use effect of GRSP content was indirect and due to soil characteristics
- 31 • Fungal origin of GRSP could not be confirmed
- 32 • Correlation between GRSP_{EE} and GRSP_T did not support claim of contrasting ages
- 33 • Organic matter was enriched in GRSP as soils were depleted in C

34 **1. Introduction**

35 Soil organic matter, SOM, has many important functions including the supply of nutrients,
36 stabilizing soil to prevent erosion and sequestering carbon to limit the build-up of green-house
37 gases. Much attention has recently been given to the possible role of soil to sequester carbon,
38 which requires a knowledge of the storage of soil carbon (Hernandez-Soriano et al., 2018;
39 Houghton et al., 2012; Janzen, 2006; Janzen, 2015; King, 2011; Lal, 2004; Schmidt et al., 2011;
40 Stockmann et al., 2013; Yu et al., 2017). A component of SOM, glomalin or Glomalin Related
41 Soil Protein, GRSP, is claimed to be particularly stable and to convey great physical stability
42 to soils, and has been a focus of research since its first report in 1996 by Wright and co-workers
43 (Wright and Upadhyaya, 1996a). It is an operationally defined fraction, extracted by
44 autoclaving in neutral or alkaline citrate solution to yield Easily Extractable (EE) or Total (T)
45 fractions. The term autoclave citrate-extractable protein has recently been proposed to describe
46 this fraction (Hurriso et al, 2018). The protein is quantified using either an antibody directed
47 against fresh fungal hyphae (Wright et al., 1996; Wright and Upadhyaya, 1996a) or more
48 commonly by the non-specific colorimetric assay developed by Bradford (Bradford, 1976).
49 Other substances, operationally classed as humic substances, are co-extracted particularly at
50 alkaline pH (Bolliger et al., 2008; Gillespie et al., 2011a; Gillespie et al., 2011b; Halvorson and
51 Gonzalez, 2006; Schindler et al., 2007; Whiffen et al., 2007).

52 GRSP is proposed as an indicator of soil physical stability and of fungal activity and turnover
53 (Bedini et al., 2009; Fokom et al., 2012; Gispert et al., 2013; Šarapatka et al., 2019; Young et
54 al., 2012). A strong correlation is often observed between GRSP and organic carbon content,
55 this along with the realization that the composition of GRSP is not as simple as was originally
56 claimed, has led to doubt as to the identity of GRSP as a distinct component of soil organic
57 matter. Nevertheless the seasonal variation of GRSP, its sensitivity to land use change,
58 exhibiting better resistance to depletion than average organic matter, accredit the hypothesis of

59 GRSP as a distinct fraction (Emran et al., 2012; Gispert et al., 2013; Oliveira et al., 2016; Preger
60 et al., 2007; Rillig et al., 2001). It is generally stated that the ease of extraction of the GRSP
61 fractions reflects the period of contact between soil and protein (Koide and Peoples, 2013;
62 Lovelock et al., 2004b) because the strength of association of protein with soil organo-mineral
63 surfaces was expected to increase with time. The easily extractable fraction, obtained at neutral
64 pH, is thus considered to be more recent than the more strongly fixed, total fraction, extracted
65 at pH 8. GRSP is reputed to be a glycoprotein originating from the hyphal walls of arbuscular
66 mycorrhizal fungi (Driver et al., 2005; Wright and Upadhyaya, 1996b).

67 The aim of this study was to assess the evidence to support some of the various claims made
68 concerning the origin and identity of GRSP and the factors that determine its accumulation. An
69 underlying question is whether or not GRSP is a distinct fraction of organic matter. We also
70 consider the validity of the premise that GRSP is largely of fungal origin, and hence is a useful
71 probe of fungal activity. If this were true then there should be a link between soil composition
72 and land-use favourable towards fungal activity and the amount of GRSP. Finally, by
73 comparing the ratio of the two operationally defined fractions of GRSP, GRSP_{EE} and GRSP_T,
74 we aim to determine if there is any reason to presume that they probe soil protein pools of
75 different ages. To achieve these aims we have surveyed a large number (197) of top soils from
76 metropolitan France and compared the GRSP contents to soil properties and land-use. The study
77 was undertaken in the context of the suggestion that carbon sequestration in soil could be
78 engineered, including phytoengineering, to mitigate climate change due to increasing levels of
79 atmospheric greenhouse gases (Janzen, 2006; Janzen, 2015; Lal, 2004; Lorenz et al., 2007).

80 **2 Materials & Methods**

81 Soils had been sampled throughout mainland France as part of the National Soil Quality
82 Monitoring Programme (RMQS) using a 16x16 km grid. Sampling strategy and soil analysis
83 and conservation have been described in detail elsewhere (Arrouays et al., 2002; Meersmans et

84 al., 2012). Soils were air-dried and sieved to $< 200 \mu\text{m}$. Ten percent of the top-soils (0-30 cm)
85 in the collection were selected to be representative of the full set on the basis of soil organic
86 carbon content (median, average and standard deviation of about 20 g kg^{-1}). Several physico-
87 chemical parameters had been measured on each soil during the soil monitoring survey
88 performed by the Soil Analysis Laboratory of INRA (Arras, France,
89 <http://www.lille.inra.fr/las>). Data are available upon request, and a synthesis is given
90 <https://doi.org/10.15454/BNCXYB>). Soil class was not included in statistical analysis because
91 more than half the samples were Cambisols. Figure SI_1 shows the boxplots of relevant soil
92 properties for the complete sample set and for each of the major land-use categories (cropland,
93 grassland and woodland). We calculated the fractions of soil organic carbon and of clay that
94 were complexed or uncomplexed using the relationship proposed by Dexter (Dexter et al.,
95 2008b). This model assumes that organic matter is preferentially complexed with clay minerals
96 in soil and so there is no non complexed organic matter until the saturation limit of clay minerals
97 is reached. This limit is reported to be $10 \text{ g clay g}^{-1} \text{ organic carbon organic matter}$ in European
98 soils.

99 Two operationally defined fractions of glomalin related soil protein, easily extractable and total
100 protein were obtained. The easily extractable fraction was obtained by a single cycle of
101 autoclaving of soil in 20 mM sodium citrate at pH 7. The total GRSP was obtained by a single
102 cycle of autoclaving of soil in 50 mM sodium citrate at pH 8. Many studies employ a variable
103 number of extractions in alkaline citrate which are pooled to obtain the total fraction, using the
104 criterion of colour to decide the number of extractions. This was originally proposed by Wright
105 and co-workers who assumed that the colour was due to the protein, glomalin, and not to humic
106 substances (Wright and Upadhyaya, 1996b). Unpublished data have shown that extracted
107 protein decreases exponentially with extraction rank, and so we chose to use a single extraction.
108 Soil:solution ratio was always 1:8 (0.25 g + 2 ml). After cooling, soil and solution were
109 separated by centrifugation at $15\,000 \text{ g}$ for 20 minutes. Bradford reactive protein was assayed

110 in microplates, after dilution if necessary, using the Bradford Quick Start kit purchased from
111 BioRad (20 μ l sample + 230 μ l reagent). The absorbance was read at 590 nm and bovine serum
112 albumin (0 – 200 mg dm⁻³) used for calibration. Variation between triplicate extractions was
113 about 7 % and average variation between triplicate protein assays was 5 %. Data are presented
114 either as the Bradford reactive protein with respect to mass of air-dry soil, or as the ratio of
115 GRSP to soil organic carbon content, OC. We prefer to report the ratio of GRSP:OC, rather
116 than assuming a C content for GRSP, since the reported C contents are not typical of those of
117 most proteins (53% (Rouwenhorst et al., 1991)). In previous studies a factor of 32% has been
118 used to convert GRSP to GRSP-carbon, and other studies report that the C content of GRSP
119 vary between samples (Lovelock et al., 2004b; Seguel et al., 2008; Woignier et al., 2014).

120 In addition to simple linear regression, we used a Cubist model using the dedicated R package.
121 The advantage of the cubist approach, as with other machine learning approaches, is that no
122 regression type is imposed on data. Regression using Cubist works with condition-based rules
123 where the output is a set of rules, and each rule has a specific multivariate linear model. The
124 algorithm was first described by Quinlan (Quinlan, 1992). A decision tree is constructed and a
125 model is used to calculate the predicted value for each leaf (Minasny and McBratney, 2008).

126 Unlike regression trees, which predict a rigid value for each ‘leaf,’ regression rules build a
127 multivariate linear function. The Cubist model can also use a boosting-like scheme called
128 committees where iterative model trees are created in sequence. To select the best iteration
129 numbers, we used a k-fold cross validation on the dataset with 300 replications. We tested a set
130 of values for the number of committees (1, 2, 5, 10, 15, 50 and 100). The following prediction
131 quality criteria were calculated during the cross validation: Coefficient of determination (R²)
132 and Root mean square error (RMSE). The soil properties considered for these analyses were:
133 organic carbon (OC), total nitrogen (N_T), texture (clay, silt and sand contents), pH (in water),
134 total iron, exchangeable phosphorus, total carbonate, base saturation, exchangeable aluminium,
135 extractable DNA and land use. The influence of different soil properties in predicting the ratio

136 GRSP_{EE}:GRSP_T was also checked. The utility of each variable during the model building was
137 derived. This is known as variable importance (VI) and is computed as the percentage of times
138 where each variable was used in a condition and/or a linear model.

139 **3. Results and Discussion**

140 **3.1 Relations between GRSP and soil properties**

141 The median value of GRSP_T was 2 g kg⁻¹, with the median value of GRSP_{EE} about three times
142 less at 0.7 g kg⁻¹. Figure 1 shows the box plots of GRSP_{EE} and GRSP_T for the full data set and
143 classed by major land-use categories: cropland, grassland and woodland which accounted for
144 more than 90% of the soils studied. The range of values of both GRSP_{EE} and GRSP_T are similar
145 to previously reported values. For example Nichols & Wright (2005) found values of GRSP_T
146 in the range 1.6-4 g kg⁻¹ (with up to 4 successive extractions pooled) for eight US soils with
147 textures ranging from sandy loam to silty clay loam. With some seasonal variation, annual
148 average values of GRSP_T in the range 1-5 g kg⁻¹ have been reported for sandy to sandy loam
149 soils with different land uses in Spain, with GRSP_{EE} being about a third less in the range 0.5-
150 1.3 g kg⁻¹ (Emran et al., 2012). Treseder & Turner (2007b) report much lower average values
151 of immuno-reactive GRSP_T for relevant biomes, agricultural and temperate grassland (0.5±0.1
152 and 0.7±0.3 g kg⁻¹), and greater values for temperate forests (2.5 g kg⁻¹) and even greater for
153 tropical forests (7 g kg⁻¹). When immuno-reactive and Bradford reactive protein can be
154 compared, the former is about three-fold smaller than the latter (Franzluebbers et al., 2000;
155 Nichols and Wright, 2005), bringing the relevant data of Treseder and Turner in line with those
156 presented in this study. The values reported in this study for woodland soils are somewhat lower
157 than those previously reported for ten French mainland forest soils (3.5 – 10.5 g kg⁻¹ for GRSP_T
158 and 0.7-2.1 g kg⁻¹ for GRSP_{EE}) (Jorge-Araújo et al., 2015). The main reason for this difference
159 is that the former study investigated forest soils with organic carbon contents of about 100 g
160 kg⁻¹ whereas the median value for forest soils in this study was considerably less, 28 g kg⁻¹.

161 Previous studies have pointed to a link between GRSP with some soil properties, especially soil
162 organic carbon content, although with much smaller data sets (Rillig et al., 2003; Treseder and
163 Turner, 2007a). Figure 2 shows that there are positive but non-linear relationships between each
164 of the GRSP fractions and both OC and total N. There are approximately linear relations at
165 lower values of OC and N_T , with a threshold value reached at about the 95th percentile. The
166 relation with N_T is consistent with a large proportion of soil N being organic and that proteins
167 typically contain about 16% N. There is no relationship with clay content, therefore there is no
168 evidence that GRSP is stabilised by association with clay minerals, or that the measured GRSP
169 content depended on extraction efficiency.

170 Simple regression analysis cannot take into account the complex interrelation between land use
171 and soil properties and so is of limited value for studies of a large number of soils with
172 contrasting properties. In order to avoid bias in the choice of parameters we adopted the Cubist
173 approach to identify the determinants of soil GRSP content. Correlations between observed and
174 predicted values were calculated during the cross validation. They were significant for $GRSP_{EE}$
175 ($r^2=0.54$) and $GRSP_T$ ($r^2=0.75$) with root mean square prediction errors of 0.287 and 0.383
176 respectively (Table SI_1). The agreement between predicted and observed values showed little
177 bias for $GRSP_T$, whereas lower values were overestimated for $GRSP_{EE}$.

178 Figure 3 shows the relative importance of the parameters identified by this approach to explain
179 the measured values of $GRSP_{EE}$ and $GRSP_T$. The most obvious feature is that soil organic
180 carbon and total nitrogen contents are the most important soil parameters to explain both GRSP
181 fractions, which is consistent with N-containing proteins being important components of GRSP.
182 Sand is also important, particularly for $GRSP_{EE}$, which may be related with the ease of
183 extraction of protein. However clay and iron oxide contents are of less importance, lending little
184 support to the widely held assumption that minerals contribute to the protection and hence
185 accumulation of GRSP, like organic matter in general (Six et al., 2002). A striking feature of
186 these rankings is that land use does not appear to be important, as is discussed later. The precise

187 order of importance of the less important soil properties must be subject to caution. It is
188 recognised that the selection of the most relevant features when variables are correlated is
189 challenging (Kursa and Rudnicki, 2011) . We choose to use the VI criteria of the Cubist model
190 in this study.

191 The curvilinear relation between both $GRSP_{EE}$ and $GRSP_T$ and both OC and N_T (Figure 2)
192 suggests a difference between organic matter complexed with soil minerals and free,
193 uncomplexed organic matter. Separate analyses were therefore performed in which either
194 organic carbon was classed as complexed or non-complexed with clay (c. or n.c.) or clay
195 classified as saturated or non-saturated with organic matter (s. or n.s.), or both This
196 classification was obtained using the clay-organic matter relationship proposed by Dexter
197 (Dexter et al., 2008a). Neither r^2 nor RMSPE were markedly changed by these changes to the
198 models (Table SI_1), the relative importances of all parameters for $GRSP_{EE}$ and $GRSP_T$ and
199 each of the variants of the model are shown in Figure SI_2. For both GRSP fractions the sum
200 of relative importances of complexed and non complexed clay or OC fractions was very similar
201 to that of the simpler model. Non complexed OC was always more important than complexed
202 OC when the distinction was made. This may seem surprising, since both fractions of GRSP
203 levelled off at larger values of OC, when more organic matter would be uncomplexed. However
204 the explanation for this is that samples with no uncomplexed organic matter were excluded to
205 avoid a strong weighting of zero, and so only the residual regression contributed.

206 **3.2 Relations between GRSP and land use**

207 In their review, Treseder & Turner (2007b) stated that average immuno-reactive $GRSP_T$
208 increased in the order desert<agricultural≈temperate grassland<boreal forest<temperate
209 forest<tropical forest. Emran (Emran et al., 2012) also reports a strong land use effect on
210 $GRSP_T$, with lowest values for vineyard soil and greatest values for pasture soil. Figure 1 shows
211 a marked land use effect for both GRSP fractions, with median, first and third quartile values
212 being larger for grassland and woodland soils than for cropland soils. However, Figure 2 shows

213 that the relations between GRSP and other soil parameters exhibit little distinction between land
214 use classes, suggesting that the apparent land use effect may arise from other soil properties,
215 and notably OC. Furthermore, the cubist modelling approach showed that land use was of very
216 minor importance, since land use effects have been largely explained by soil properties. It seems
217 likely that for this data set and most previously studied soils, the observed land use effect might
218 be largely explained by soil composition, and in particular organic matter content.

219 **3.3 Is GRSP a distinct fraction of organic matter?**

220 The average values of $GRSP_{EE}/OC$ and $GRSP_T/OC$ were 0.038 and 0.097 respectively, with
221 ranges of 0.006-0.096 and 0.02-0.19. Thus the average value for $GRSP_T$ would account for 3
222 or 5% of OC taking the C content of GRSP to be 32% or 53% (Rouwenhorst et al., 1991; Seguel
223 et al., 2008). The strong correlation of both fractions of GRSP with OC content in this and other
224 studies might suggest that GRSP is not a distinct fraction of organic matter with different
225 patterns of production and accumulation. However the positive correlation of GRSP with N_T
226 supports the assumption that GRSP is largely composed of proteins, that contain on average
227 16% N (Rouwenhorst et al., 1991). Another indication that GRSP is not a uniform fraction of
228 OC is that the positive correlation of GRSP with clay content (Figure 2) was weaker than that
229 of OC with clay (not shown, $r^2=0.155$, $P<0.001$) for these soils. Most importantly, the nonlinear
230 relations between both fractions of GRSP and OC (Figure 2) indicate that GRSP does not
231 simply follow OC. There is a smaller proportion of GRSP in organic matter in soils with large
232 OC contents. This is in line with observations from chronosequences (Preger et al., 2007) that
233 indicate that GRSP resists mineralisation following land-use change better than organic matter.
234 As previously discussed, model predictions of GRSP were not greatly improved when
235 complexed and non complexed OC and clay were considered separately. Another way of
236 considering the distinction between complexed and free organic matter is to compare GRSP
237 with the ratio of organic carbon to clay content. Values of this ratio superior to 0.1 correspond
238 to the presence of free, uncomplexed organic matter, and values less than 0.1 correspond to

239 under-saturated clay, according to the classification of Dexter (Dexter et al., 2008a). Significant
240 positive linear correlations ($P < 0.001$, $r^2 = 0.416$ and 0.345 for $GRSP_{EE}$ and $GRSP_T$ respectively)
241 were observed between both fractions of GRSP and the ratio OC:clay (Figure 4), with no
242 inflection in the trend around the value of 0.1. However these correlations were led by extreme
243 points, correlations were less significant for the first three quartiles and not significant when
244 data from the interquartile range of OC:clay were considered.

245 Another way of exploring the non linear relationship between GRSP and OC is to study the
246 trends of the ratio GRSP:OC as a function of soil composition and land-use. Figures 5 a) and
247 b) show that GRSP as a fraction of OC decreases non linearly as OC increases. Although Preger
248 (Preger et al., 2007) attributed a similar trend following land use change to the greater stability
249 of GRSP, the trend could arise if GRSP represented a component of organic matter continuously
250 produced by plant growth. OC content is an integrated measurement that takes no account of
251 changes in the quality and composition of organic matter. Similar trends (not shown) were
252 observed for both GRSP fractions and the soil nitrogen content. No land use effect could be
253 separated from the overall trends.

254 Figure 5 c) and d) show that there was a strong trend for the ratio GRSP:OC to decrease with
255 increasing clay content. Both OC and the GRSP fractions are positively correlated with clay
256 content ($r^2 = 0.155$ for OC, and 0.0083 and 0.0076 for $GRSP_{EE}$ and $GRSP_T$ respectively). The
257 slope of the correlation of OC is greater than that of GRSP (when normalised to the average
258 value of each). The inverse relation is thus a mathematical consequence of the different slopes
259 of the correlation of OC and GRSP *vs* clay content. This illustrates that, contrary to previous
260 assumptions, GRSP is not more strongly protected by association with clay minerals than is
261 organic matter in soil. No land use effect was observed for $GRSP_{EE}$ within this trend. For
262 $GRSP_T$, correlations for soils from each of the three major land use categories differed slightly,
263 with cropland soils having larger fractions of GRSP/OC at lowest clay content and a steeper
264 decline with increasing clay content. This observation has important implications for the

265 understanding of the chemical nature and biological origin of GRSP and for the hypothesis that
266 this fraction could be exploited as a sink to mitigate rising levels of atmospheric carbon. It is
267 possible that land use could indirectly affect the extent of mineral protection of organic matter.
268 For example, it has been suggested that the solubilization of SOM by chelating ligands
269 contributes to the priming effect (Keiluweit et al., 2015). The strong adsorption of proteins on
270 organo-mineral surfaces would not be influenced by chelating ligands (Cheshire et al., 2000;
271 Dümig et al., 2012; Quiquampoix and Burns, 2007). These trends thus converge to suggest that
272 the greater the pressure on soil organic matter, due to various processes leading to organic
273 matter depletion, including land management and absence of the stabilizing potential of clay
274 minerals, the more favoured will be GRSP accumulation.

275 **3.4 Is GRSP of fungal origin?**

276 If GRSP were largely of arbuscular mycorrhizal fungal (AMF) origin, then strong relations
277 would be expected with land use and management that influence fungal activity. Fungal activity
278 is favoured by low pH (Joergensen and Wichern, 2008; Rousk et al., 2010). In this study pH
279 was not identified as being very important for either fraction of GRSP. The importance of pH
280 was greater for GRSP_{EE} than for GRSP_T, leading to pH being the most important factor for the
281 ratio GRSP_{EE}/GRSP_T. Another influence on mycorrhizal abundance and activity is the nutrient
282 status, in particular the phosphorus status of soils (Abbott et al., 1984). Neither the cubist model,
283 nor simple regression analysis found a strong effect of available P on either GRSP fraction.
284 AMF are reported to be reduced by cropping, mainly due to tillage effects, biocidal treatments,
285 monoculture and enrichment in P due to fertilization, and this could be invoked to explain the
286 lower GRSP contents of cropland soils (Galvez et al., 2001; Gosling et al., 2006; Oehl et al.,
287 2003; Pereira et al., 2018; Treseder and Allen, 2002). However, although both the OC and
288 GRSP contents of cropland soils were lower than for other soils, their organic matter appears
289 to be enriched in GRSP. For example, the median values of the ratio GRSP_T/OC decrease in
290 the order cropland (0.116)>grassland (0.094)>woodland (0.075) soils. This is in contradiction

291 with the hypothesis that GRSP is predominantly of AMF origin (Driver et al., 2005; Wright and
292 Upadhyaya, 1996b). Furthermore, in forest soils where fungal activity is strong, even if the
293 dominant mycorrhizal fungi associated with temperate climate tree species are ectomycorrhizal
294 (Joergensen and Wichern, 2008), GRSP is under-represented in organic matter. These data
295 therefore provide no evidence to support the hypothesis that GRSP is predominantly of AMF
296 fungal origin, and so further investigation would be necessary to confirm this assumption.

297 **3.5 Do EE and Total fractions represent different ages?**

298 On average the ratio between $GRSP_{EE}$ and $GRSP_T$ was 0.40 ± 0.15 (Figure 1) with no significant
299 difference between land use classes although the median value was larger in woodland soils
300 than in crop or grassland soils. There is a significant linear relation between the two fractions
301 of GRSP ($r=0.757$, $P<0.01$, Figure 6) but with no difference between land use categories.
302 Previous studies have also reported linear relations between these fractions, albeit never for
303 such a large sample size nor such a wide range of soil properties (Jorge-Araújo et al., 2015;
304 Koide and Peoples, 2013; Lovelock et al., 2004a). An important consequence of the correlation
305 between both operationally defined fractions of GRSP is that both are equally good probes of
306 soil quality or stable soil organic matter content.

307 The cubist model identifies a simple situation for the ratio $GRSP_{EE}/GRSP_T$ with pH being the
308 most important parameter, followed by sand and extractable P contents (Figure 3). There is no
309 obvious explanation for relation between the ratio $GRSP_{EE}/GRSP_T$ and pH, which was also
310 observed with simple linear regression (Figure SI_3). One explanation is the positive relation
311 between pH and clay content, particularly for non calcareous soils (not shown). Since extraction
312 of proteins, and indeed many organic compounds, is more difficult from clays than from sand
313 due to strong binding processes, then relative extraction yield of the less harsh extraction would
314 be expected to decrease with increasing clay, as observed with simple linear regression.

315 The cubist analysis was also performed for the difference of the two GRSP fractions GRSP_T-
316 GRSP_{EE}. This difference has been proposed to represent the older fraction of GRSP, assuming
317 the more easily extractable fraction to be recent, it should thus be favoured by soil components
318 that protect the protein (Koide and Peoples, 2013). In each case (Figure 3) the most important
319 variable was N_T, twice as important as the next most important variable. The order of relative
320 importance of the other parameters varied only slightly between models (organic matter or clay
321 or both separated onto complexed and non complexed categories). The importance of the clay
322 content on the ratio GRSP_{EE}/GRSP_T was greater than for either GRSP fraction. This results
323 from the greater difficulty in extracting protein from clay than sand due to the strong interaction
324 of proteins with organo-mineral surfaces (Helassa et al., 2011; Quiquampoix and Burns, 2007).

325 The trends observed do not lend support to the hypothesis that the easily extractable fraction is
326 more recent than the total fraction. It should be noted that there is no independent verification
327 of this hypothesis either for GRSP or for proteins in general in soil on the time scales of seasons
328 or years. It is known that proteins may become increasingly strongly adsorbed on mineral
329 surfaces with increasing contact period. This phenomenon has been studied on clean surfaces
330 for short periods, never for complex surfaces such as soils over longer periods of months or
331 years (Nakanishi et al., 2001). For complex surfaces such as soils, it is difficult to separate the
332 effects of time dependent fixation that renders extraction less efficient, protein breakdown and
333 loss of biological or biochemical activity (Helassa et al., 2011; Hung et al., 2016; Kedi et al.,
334 2013). Hung and co-workers (Hung et al. 2016) attributed the loss of insecticidal activity of a
335 Cry protein in contact with soil to fixation, but this appeared complete within a period of about
336 2 weeks. Koide & Peoples cite the poor correlation between easily extracted GRSP and the
337 difference between total and easily extractable as confirmation of this hypothesis (Koide and
338 Peoples, 2013). However, in the absence of marked land use change, it seems likely that GRSP
339 accumulated from previous years would be a function of annual production, and hence strongly
340 related to the most recent season.

341 In conclusion, the previously reported land-use effect on GRSP content appears to be explained
342 by soil organic matter content. Differing trends in OC and GRSP contents lend support to the
343 existence of GRSP as a distinct fraction of SOM. However none of the observed relations
344 between GRSP and either land-use or soil composition could support the hypothesis that GRSP
345 is solely of arbuscular mycorrhizal fungal origin.

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359

360 **List of Figure captions**

361 **Figure 1**

362 Box plots of values of $GRSP_T$, $GRSP_{EE}$ and $GRSP_{EE}/GRSP_T$ for the full data set and for each
363 of the major land use categories.

364 **Figure 2**

365 Various simple regressions of $GRSP_{EE}$ and $GRSP_T$ as a function of (from top to bottom)
366 organic carbon content (OC), pH, total nitrogen content, N_T , and clay content with different
367 symbols chosen to represent each of the major land uses; \circ cropland, \square grassland, Δ
368 woodland and \blacklozenge other land uses. Left column for $GRSP_{EE}$ and Right column for $GRSP_T$.

369 **Figure 3**

370 Relative importance parameters identified by Cubist modelling. a) $GRSP_{EE}$, b) $GRSP_T$, c)
371 $GRSP_{EE}/GRSP_T$ and d) $GRSP_T - GRSP_{EE}$.

372 **Figure 4**

373 GRSP as a function of the ratio of contents of OC and clay, OC:Clay with different symbols
374 chosen to represent each of the major land uses a) $GRSP_{EE}$ and b) $GRSP_T$; \circ cropland, \square
375 grassland, Δ woodland and \blacklozenge other land uses.

376 **Figure 5**

377 GRSP as a fraction of soil organic carbon, OC as a function of OC; a) $GRSP_T$ and b) $GRSP_{EE}$
378 and as a function of clay content; c) $GRSP_{EE}$ and d) $GRSP_T$ with different symbols chosen to
379 represent each of the major land uses; \circ cropland, \square grassland, Δ woodland and \blacklozenge other land
380 uses.

381 **Figure 6**

382 Relation between $GRSP_{EE}$ and $GRSP_T$ with different symbols chosen to represent each of the
383 major land uses; \circ cropland, \square grassland, Δ woodland and \blacklozenge other land uses. The straight line
384 shows the best fit linear regression through all the data.

385 **Supplementary Information**

386 **Table caption**

387 **Table SI 1**

388 Cross validation results for the different models, showing the coefficients of determination and
389 root mean square prediction errors obtained for each of the four models for $GRSP_{EE}$ and $GRSP_T$
390 the ratio of the two fractions and their difference. Model 1 (OC, Clay); Model 2 (cOC, ncOC,
391 Clay); Model 3 (sClay, nsClay, OC); Model 4 (cOC, ncOC, sClay, nsClay) where s/ns are
392 abbreviations for saturated and non saturated and c/nc for complexed and non complexed.

393 **Figure captions**

394 **Figure SI_1**

395 Box plots of values of various soil properties for the full data set and for each of the major land
396 use categories.

397 **Figure SI_2**

398 Effect on relative importance parameters identified by Cubist modelling of separating organic
399 matter into complexed (c) and non-complexed (nc) classes and/or clay as saturated (s) in
400 organic matter or non-saturated OC/clay (ns) for $GRSP_{EE}$ (left-hand columns) and $GRSP_T$
401 (right-hand columns). For ease of comparison, data of Model 1 (OC and Clay not subdivided)
402 already presented in Figure 4 are shown.

403 **Figure SI_3**

404 Ratio of the two GRSP fractions, $GRSP_{EE}/GRSP_T$ as a function of a) pH and b) Clay content
405 with different symbols to indicate each of the major land uses; \circ cropland, \square grassland, Δ
406 woodland and \blacklozenge other land uses. There is a significant ($P < 0.001$, $r^2 = 0.31$) inverse linear
407 relationship between the ratio and pH.

408 **Graphical highlight**

409 Geological map of France showing GRSP_{EE} contents of soils

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Figure 1

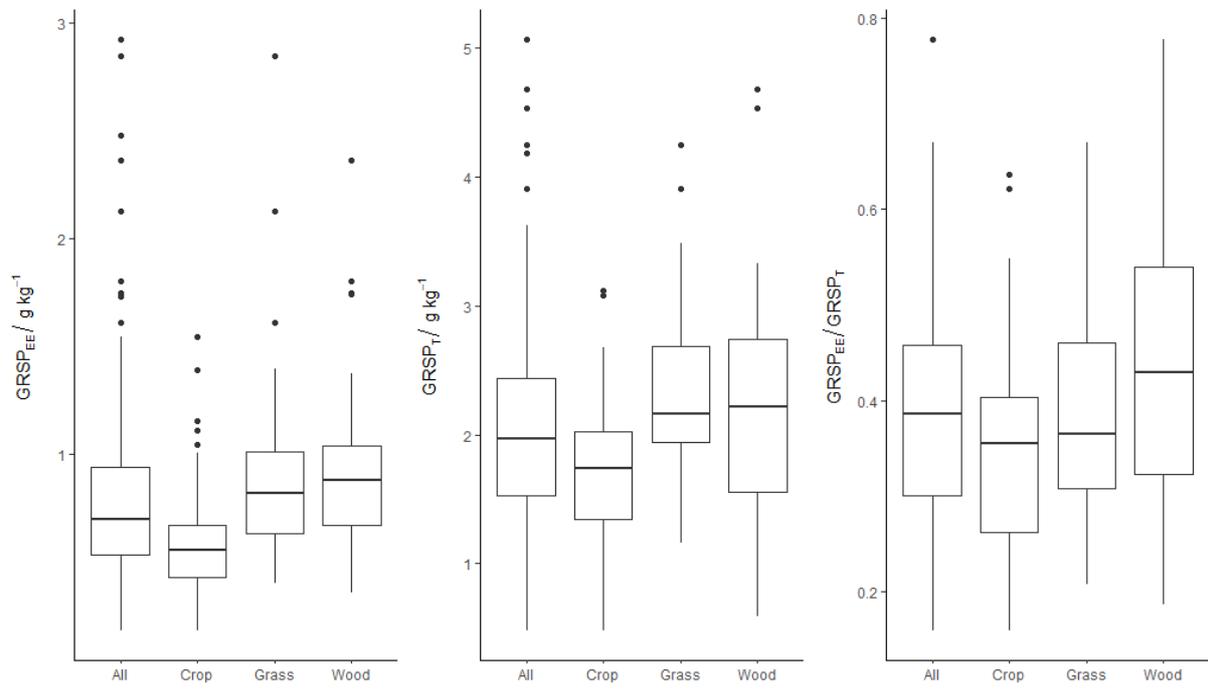


Figure 2

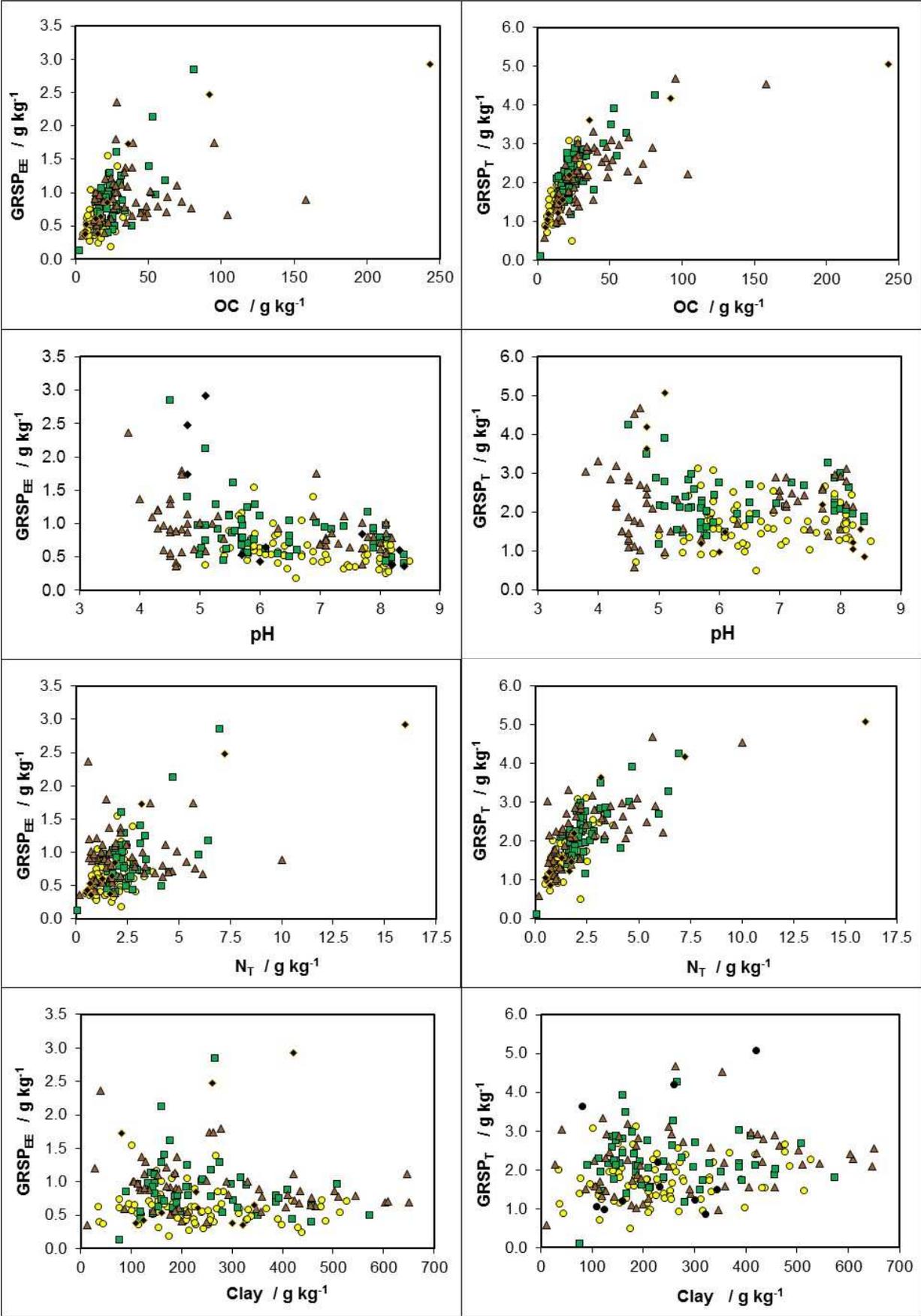


Figure 3

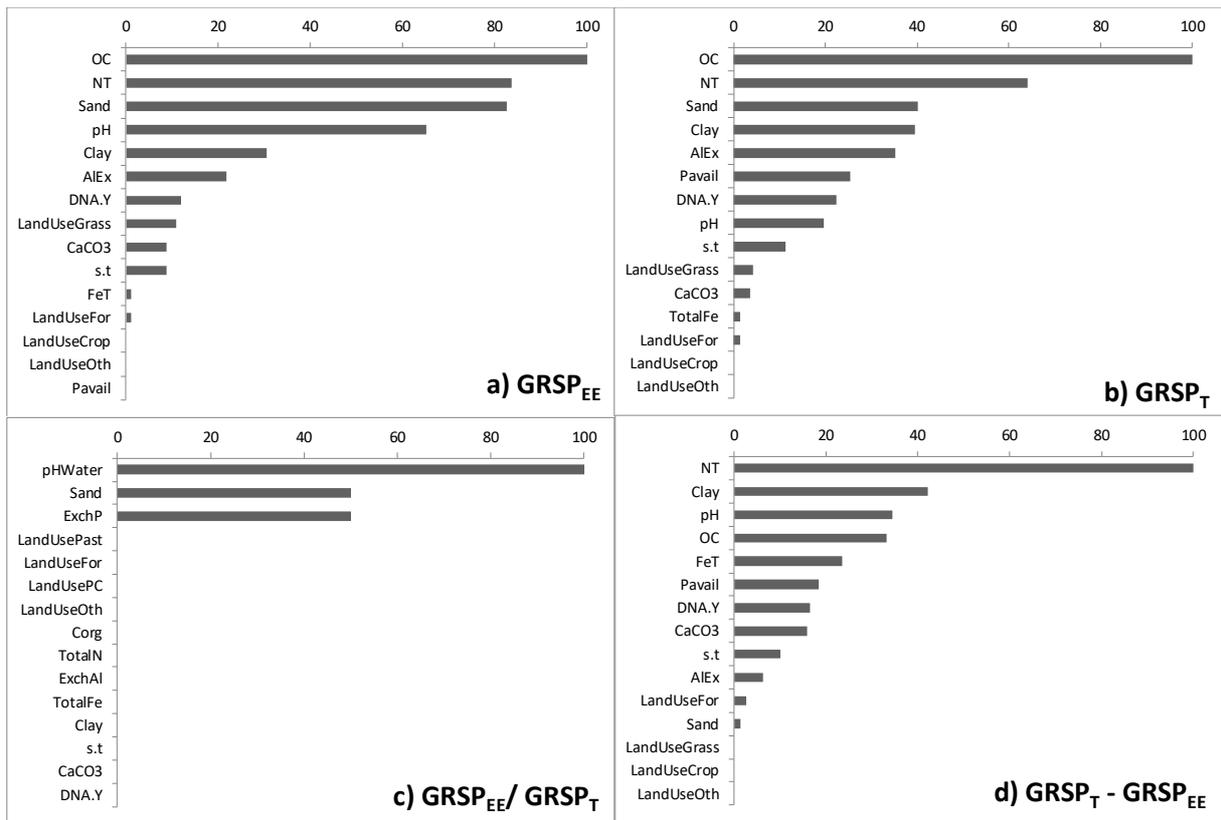


Figure 4

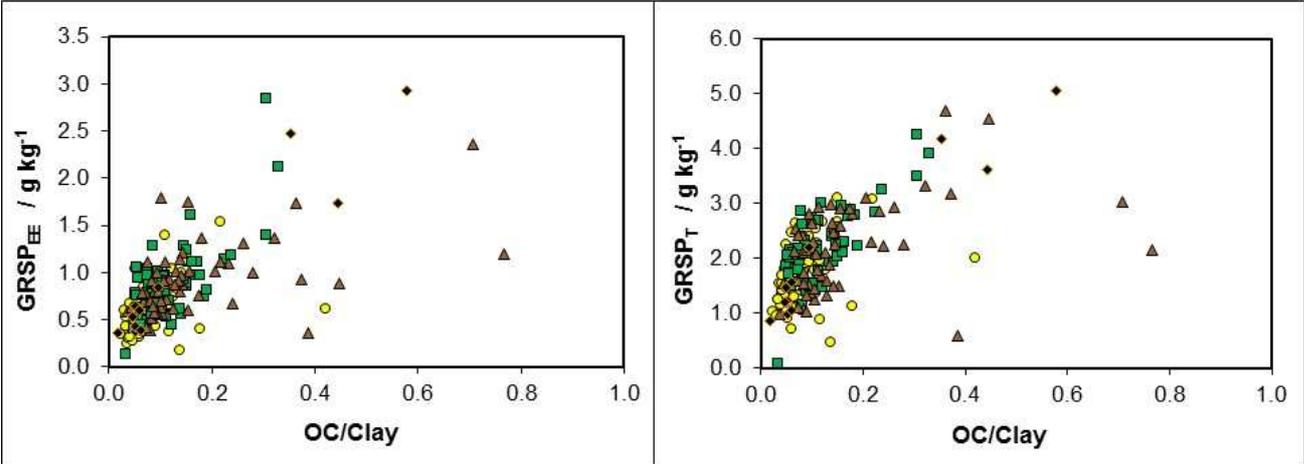


Figure 5

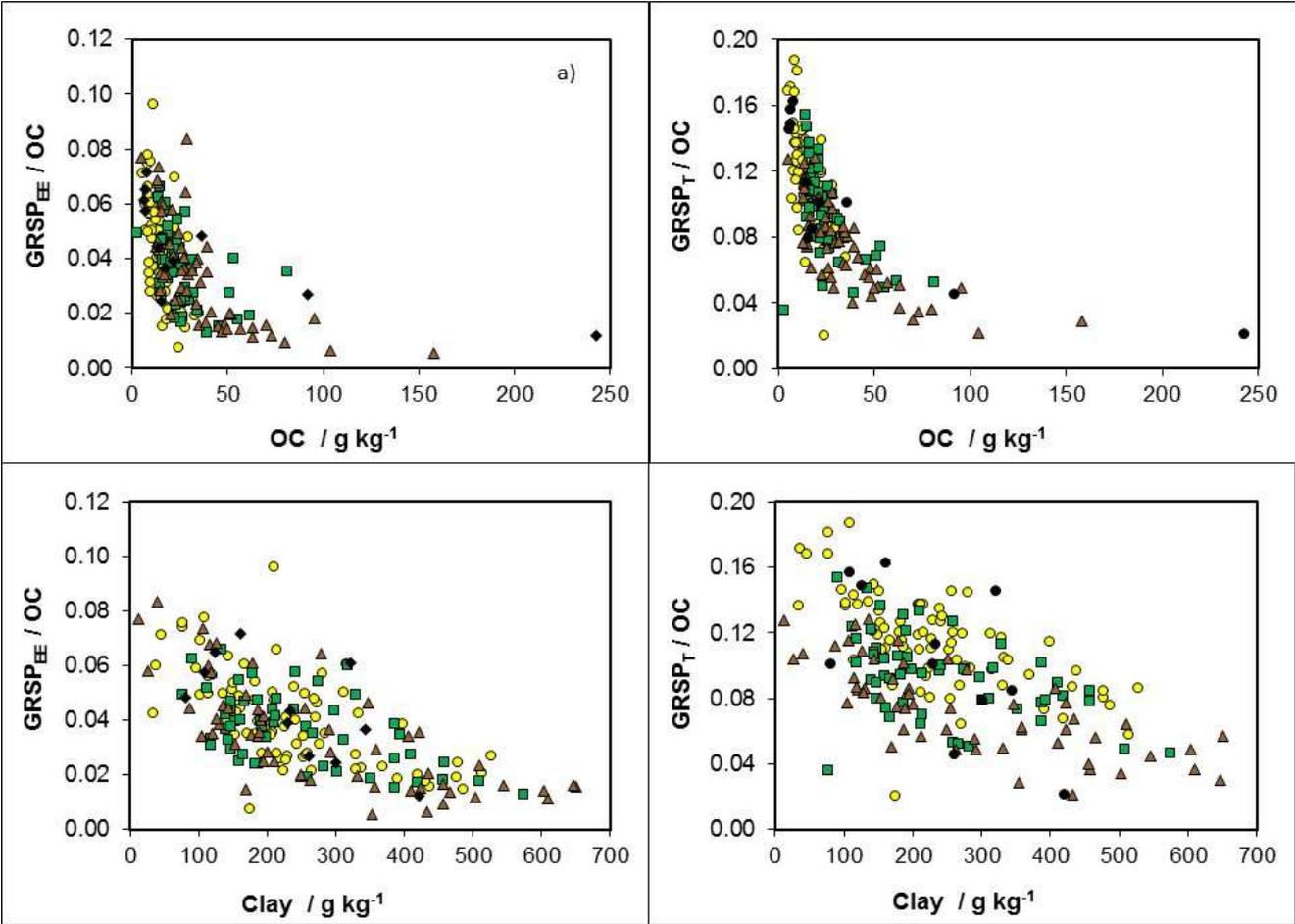
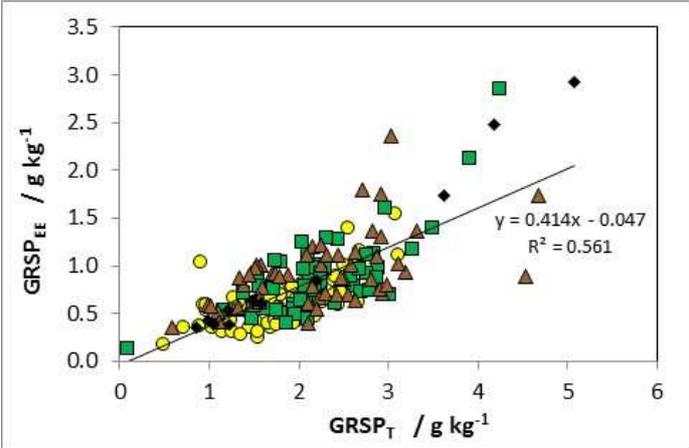
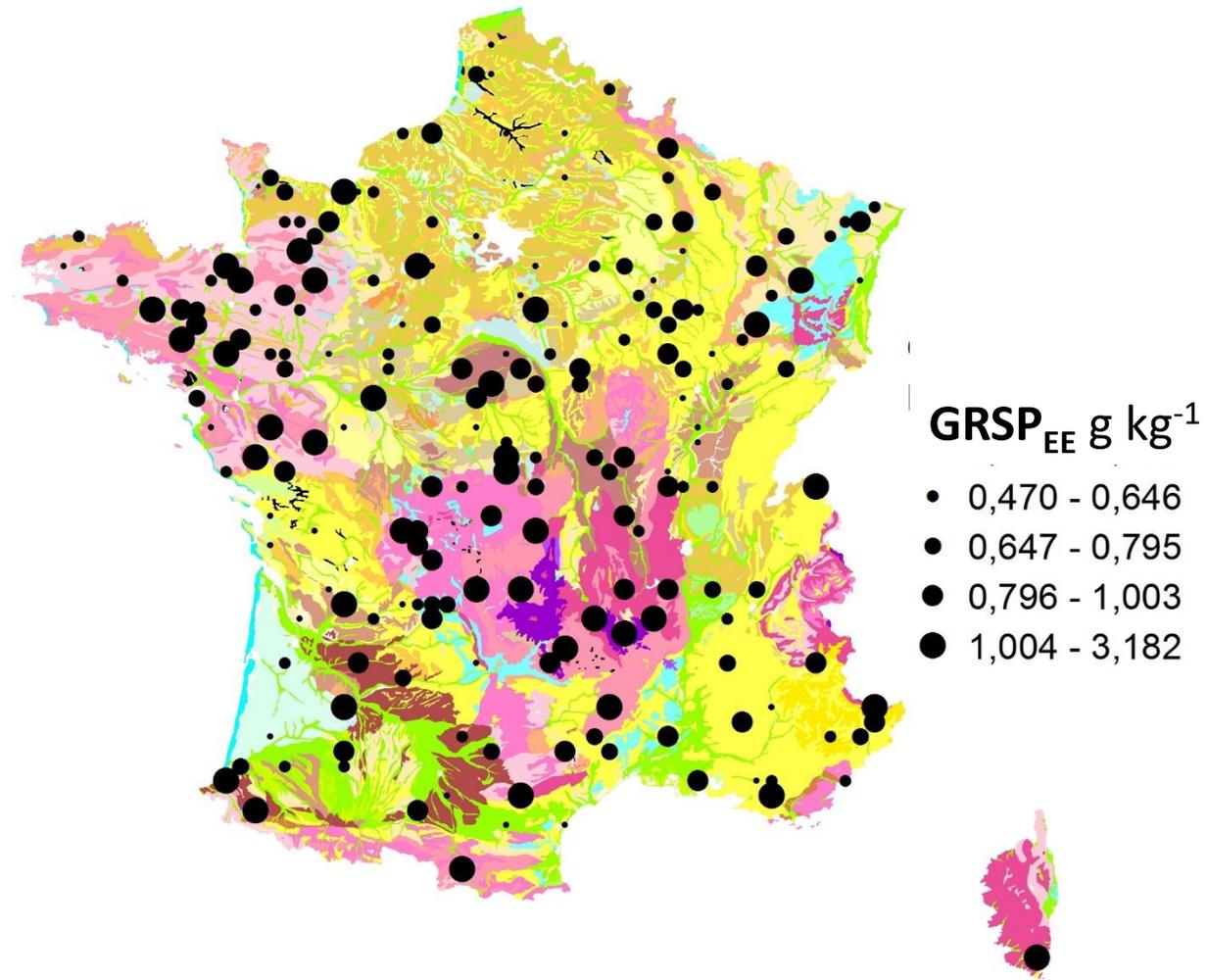


Figure 6





Geological map of metropolitan France showing the measured values of GRSP_{EE}, the size of the spots indicating the range of GRSP_{EE} content