DOI:10.1021/jf901238g

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Tracing and Quantifying Sources of Fatty Acids and Steroids in Amended Cultivated Soils

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Soluble organic fractions from soils of two agricultural sites from Brittany (France) have been analyzed to (i) identify the source of polar compounds in soils and (ii) evaluate the impact of organic fertilization and crop type on the distribution and concentration of polar compounds in soils. The main sources of polar compounds in soils are higher plants; they represent >70% of the polar compounds from the experimental sites and mainly originate from crop residues and animal manure. Crop type and animal manure application significantly increase the polar compound concentrations in soils. Among polar compounds, fatty acids cannot be used as specific markers because their distributions in soils whatever the crop type or organic fertilization type are the same. On the other hand, analysis of steroids provides interesting information. Cow and poultry manure applications increase only the concentration of steroids. Pig slurry fertilization modifies both the concentration and distribution of steroids. The identified pig slurry steroid fingerprint can persist in the soil for 9 years after the slurry application has been stopped. Those compounds are then robust markers to detect pig slurry contribution in soils.

KEYWORDS: Organic matter; animal manures; biomarker; experimental fields; polar compounds; persistence

INTRODUCTION

Animal manure is a valuable resource as a soil fertilizer, providing a high content of macro- and micronutrients for crop growth and representing a low-cost alternative to mineral fertilizers (*1*). However, the overproduction of organic wastes from animal manure has led to their overapplication to soils in many areas, a practice that may raise serious environmental problems. For example, manure application can lead to excessive inputs of potentially harmful trace metals, inorganic salts, and organic pathogens into soils (*2*). Overapplication of animal manure to soils can also generate an excess of nitrate and phosphate in soils, which can ultimately contaminate both groundwater and river waters (*3*). Finally, manure overapplication can strongly increase the amount of soil−water-extractable organic matter, thereby leading to an increase of organic matter fluxes in agricultural landscapes, which may spoil river water quality (*4*, *5*). To manage animal manure application to soils and to detect and prevent contamination of groundwater or river water, a technique that allows revealing and determining quantitatively the contribution of animal manure to soil organic matter must be developed.

Molecular markers can provide such a technique, because they are organic compounds that fingerprint a specific source and resist degradation. Among them, lipids display interesting attributes to make them valuable tracers of animal manure in soils. Although lipids usually represent a small fraction of the total organic carbon, they are robust molecular markers of organic matter production because of the specificity of their biosynthesis and their adaptation of biosynthetic pathways to environmental parameters (*3*, *6*−*15*).

In a previous study (*16*), the use of sterols has been demonstrated to have the potential to detect pig slurry influence in soils. The specificity of these molecular markers was analyzed by comparing their distribution in raw samples of pig slurry and in dairy and poultry manures. Their persistence through time was checked by the analysis of present-day sterol profiles of an experimental field that had received pig slurry input between 14 and 10 years ago. However, this first study suffers from some limitations. It dealt with an experimental field receiving an overdose of pig slurry corresponding to 40 times the agronomic dose usually disposed on agricultural soils in Brittany (France). It is then difficult to extend those results to soils receiving agronomic doses of pig slurry. Moreover, quantification of sterols was not performed in this previous study. As a consequence, it was necessary to conduct further investigations to fulfill these limitations. Investigations on other lipid compounds were also developed to improve the fingerprint of various animal manures in agricultural soils.

This second study aims at (i) identifying the sources of polar compounds in soils from experimental fields, (ii) studying the impact of crop type or animal manure application on the polar compounds of soils, and (iii) evaluating the persistence of pig slurry markers with time. This study will contribute to revealing the impact of soil management (crop type, animal manure) on the chemical distribution and quantification of polar compounds in soils.

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 Published on Web 07/20/2009

MATERIALS AND METHODS

Experimental Fields and Sampling. Two experimental fields, located in Brittany (in western France), have been used.

Champ-Noël Site. This experimental field, established in 1993, is located close to Rennes, France (48° 7′ N; 1° 40′ E). It consists of fields developed on silt loam soils (clay = 16%; silt = 70%; sand = 14%) lying themselves on alterite micaschist. The plots sampled were cropped continuously with corn (*Zea mays*). From 1993, pig slurry has been spread once a year in late spring on three different plots (X, X1, and X2) at various doses. The fertilization dose has been calculated according to the nitrogen requirement of the corn to be grown (i.e., the agronomic dose). On plot X (labeled CN-pig1), it represents a mean load of 0.6 t of organic carbon (OC) ha^{-1} $year^{-1}$. Pig slurry was applied at X + 75 (0.8 t of OC ha^{-1} $year^{-1}$) onto plot X1 (labeled CN-pig2) and at X + 150 (1.1 t of OC ha^{-1} $year^{-1}$) onto plot X2 (CN-pig3). A fourth untreated plot was used as a control soil (CN-ref) to study and quantify the impact of pig slurry application. On this control soil, only corn has been grown since 1993. Pig slurry application onto X1 (CN-pig2) and X2 (CN-pig3) plots was stopped in 1998 and replaced by mineral fertilization (with ammonium nitrate at the recommended rate, 110 kg ha^{-1} $year^{-1}$ $N\text{-}NH_4NO_3$) since 2002. This plot is still currently fertilized with mineral fertilization. On CN-pig1 the pig manure fertilization is still applied today. Soil samples were collected in October 2006.

The site is an ideal target for two main reasons. It allows (i) quantifying the relationship between the dose of pig slurry spread onto soils and the concentration of molecular markers in soils and (ii) testing the persistence of molecular markers from pig slurry with time by comparing results from plots on which the application of pig slurry was stopped.

Kerguehennec Site. This experimental field, established in 2000, is located near Bignan, France (47° 52′ N; 2° 46′ W). It consists of experimental fields developed on loamy soils (clay = 17%; silt = 46% and sand = 37%), lying themselves on alterite micaschist. On these fields, three different types of crops are grown in rotation: canola seeds, corn, and wheat. At sampling time, canola seeds were grown on the fields. Since 2000, three different types of organic manures have been disposed once a year in autumn, on three different plots of this facility. The fertilization dose has been calculated according to the nitrogen requirement of the canola seeds to be grown (i.e., agronomic dose). It represents a mean load of 1.3 t of OC ha^{-1} $year^{-1}$ for pig slurry (labeled K-pig), 1.1 t of OC ha^{-1} $year^{-1}$ for poultry manure (labeled K-poultry), and 2.8 t of OC ha^{-1} $year^{-1}$ for dairy manure (labeled K-dairy). The results on the amended soils were compared to results obtained on reference soils (labeled K-ref), on which only canola seeds have been grown. Organic fertilization continues today on the soils of this experimental field. The soil samples analyzed for this study were collected in October 2006.

The main interest of this site is to provide data to evaluate the impact of three different organic manures on the chemical composition of soils under organic fertilization at the agronomic dose.

On each site, representative samples were obtained by gathering and mixing several samples taken in the 0−20 cm depth layer. Particulates of > 2 mm were first eliminated; samples were then freeze-dried during 5 days and subsequently crushed and sieved at 500 μm before analyses.

Analytical Methods. The soluble organic fraction (SOF) was extracted by dichloromethane using an automated extractor (Dionex ASE 200). Two to five grams of each freeze-dried sample was extracted twice and then assembled together. The conditions of extraction by ASE were as follows: 11 mL cells, 100 °C, 130 bar, 5 min heat-up time, two cycles of 5 min, static time, 150% flush, 300 s purge with nitrogen. Elemental sulfur was removed by reduction on metallic copper. The SOF was then fractionated into aliphatic, aromatic, and polar compounds by two-step liquid chromatography. High molecular weight (HMW) polar compounds were retained by alumina, whereas hydrocarbons and low molecular weight (LMW) polar molecules were eluted by dichloromethane. HMW polar compounds were then recovered by a mixture of methanol and dichloromethane (1:1, v/v). After exchanging dichloromethane by cyclohexane without drying, hydrocarbons and LMW polar molecules were fractionated on silica column into aliphatic hydrocarbons, aromatic hydrocarbons, and LMW polar compounds by three successive elutions with cyclohexane, a mixture of cyclohexane and dichloromethane (2:1, v/v), and a mixture of methanol/dichloromethane (1:1, v/v). At each fractionation step, the mass of organic matter in the different fractions was weighed.

Table 1. Names and Symbols of the Compounds Identified in the Soil Samples

name	symbol	name	symbol
decanoic acid	C10:0	tricosanoic acid	C23:0
dodecanoic acid	C12:0	tetracosacnoic acid	C24:0
11-methyldodecanoic acid	iC13:0	pentacosanoic acid	C25:0
10-methyldodecanoic acid	aC13:0	hexacosanoic acid	C26:0
tridecanoic acid	C13:0	heptacosanoic acid	C27:0
tetradecanoic acid	C14:0	octacosanoic acid	C28:0
13-methyltetradecanoic acid	iC15:0	nonacosanoic acid	C29:0
12-methyltetradecanoic acid	aC15:0	triacontanoic acid	C30:0
pentadecanoic acid	C15:0	dotriacontanoic acid	C32:0
9-hexadecenoic acid	C16:1n7	5β-cholestan-3β-ol	coprostanol
hexadecanoic acid	C16:0	5β-cholestan-3α-ol	epicop
15-methylhexadecanoic acid	iC17:0	cholest-5-en-3β-ol	cholesterol
14-methylhexadecanoic acid	aC17:0	5α-cholestan-3β-ol	cholestanol
heptadecanoic acid	C17:0	24-ethyl-5β-cholestan-3β-ol	24ethylcop
11-octadecenoic acid	C18:1n7	24-methylcholest-5-en-3β-ol	campesterol
9-octadecenoic acid	C18:1n9	24-methyl-5α-cholestan-3β-ol	campestanol
octadecanoic acid	C18:0	24-ethylcholest-5-en-3β-ol	sitosterol
eicosanoic acid	C20:0	24-ethyl-5α-cholestan-3β-ol	sitostanol
docosanoic acid	C22:0		

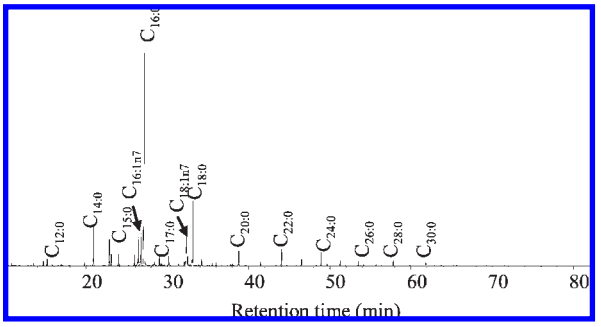

Figure 1. Chromatogram of fatty acids from soils.

Quantitative analyses of polar fractions were carried out by capillary gas chromatography−mass spectrometry (GC-MS) after derivatization using a mixture of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMSC) (99:1, v/v). GC-MS analyses were performed with a Hewlett-Packard G1800A equipped with a capillary column DB 5-MS (5% diphenyl−95% dimethylpolysiloxane, 60 m; 0.25 mm i.d., 0.1 μm film thickness, Supelco) coupled with a mass spectrometer operating in the full-scan mode. Samples were injected in the splitless mode at 300 °C. The oven temperature was programmed from an initial temperature of 70 °C (held for 2 min) to 130 °C at 15 °C min^{-1} and then from 130 to 315 °C (held for 15 min) at 3 °C min^{-1}. Helium was the carrier gas with a flow rate of 1.4 mL min^{-1}. All of the compounds were identified by their mass spectra by comparison with standards or with the library of spectra.

The quantification of polar compounds (fatty acids and sterols) was performed by the addition of internal standards in the solutions: $^2H_6$-cholestane and perdeuterated *n*-alkanes (^{2}H-hexadecane, ^{2}H-eicosane, ^{2}H-tetracosane, and ^{2}H-triacontane). Perdeuterated *n*-alkanes were purchased from Carlo-Erba SDS (Val de Reuil, France), and $^2H_6$-cholestane was purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada).

The results of the quantification are given in parts per million, which represents the concentrations of polar compounds in soils in micrograms per gram of dry weight of soil. In the following text, the results are expressed with the units "ppm". Analysis of variance (ANOVA) was used to test for differences in the lipids content according to the fertilization type, and the resulting mean separations were performed using the protected least significant differences (PLSD) Fisher test. All statistical analyses were performed using Statistica version 7 (Statsoft).

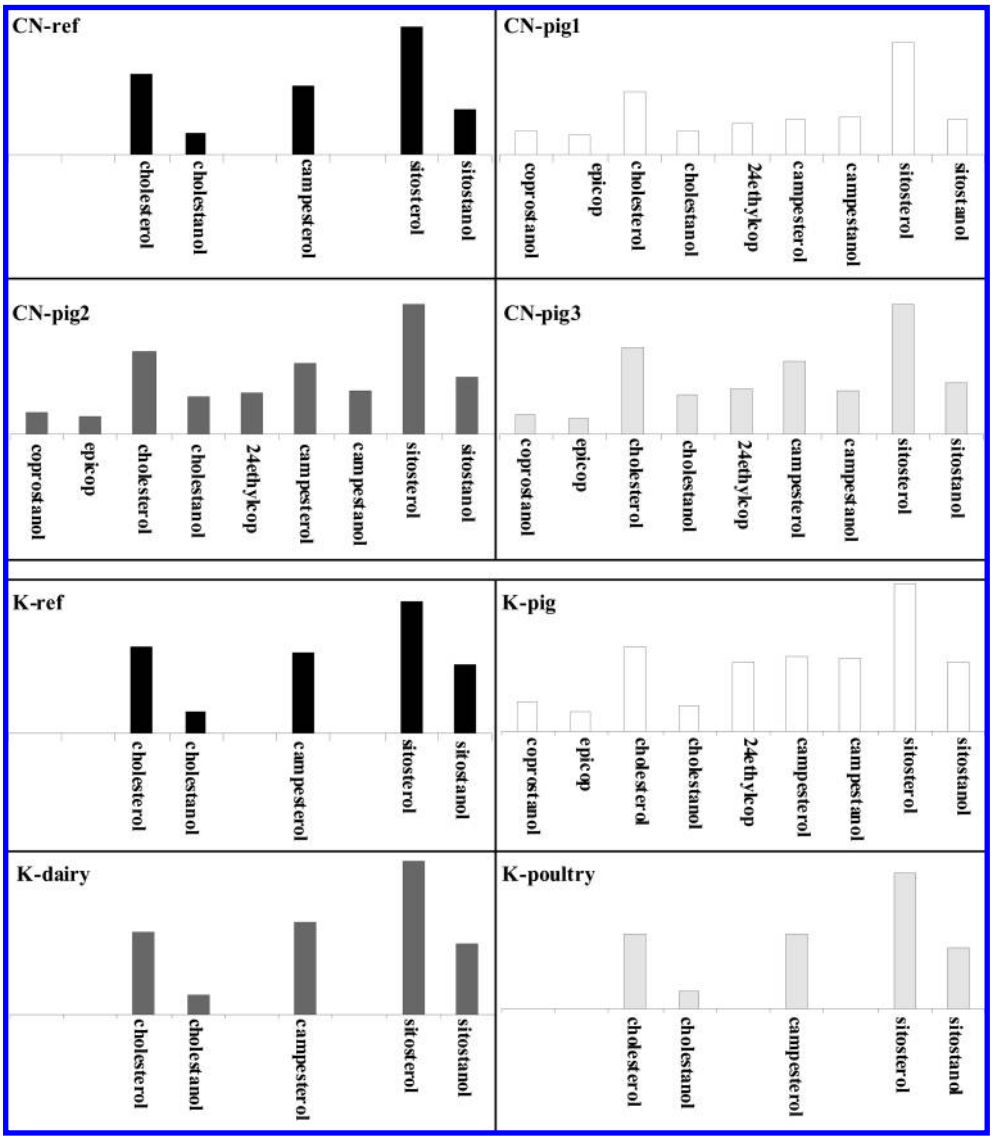

Figure 2. Distribution of steroid compounds in Champ-Noël (CN) and Kerguehennec (K) soils.

Table 1 presents the names and symbols of the polar compounds identified in the soils from the two experimental fields.

RESULTS AND DISCUSSION

Molecular analysis of lipid compounds of soil samples allows (i) identification of the sources of the soil's polar compounds from experimental fields, (ii) study of the impact of crop type, animal manure application, and organic fertilization dose on the soil's polar compounds, and (iii) evaluation of the persistence of pig slurry markers with time.

Identification of Sources of Polar Compounds in Soil Samples. *Fatty Acids*. **Figure 1** displays the representative chromatogram of fatty acid distribution of soil samples. The distribution of fatty acids is the same in the soils of the two experimental fields. Saturated $C_{12}-C_{30}$ alkanoic acids, branched saturated alkanoic acids, and unsaturated alkanoic acid were identified separately in the chromatograms.

Identified saturated alkanoic acids were dominated by even compounds, in the range of n-$C_{12}-n$-C_{30}, and especially by hexadecanoic acid and octadecanoic acid. The main sources of even fatty acids in the range of n-$C_{10}-n$-C_{32} in soils are crop residues and animal manures (*8*, *11*, *12*).

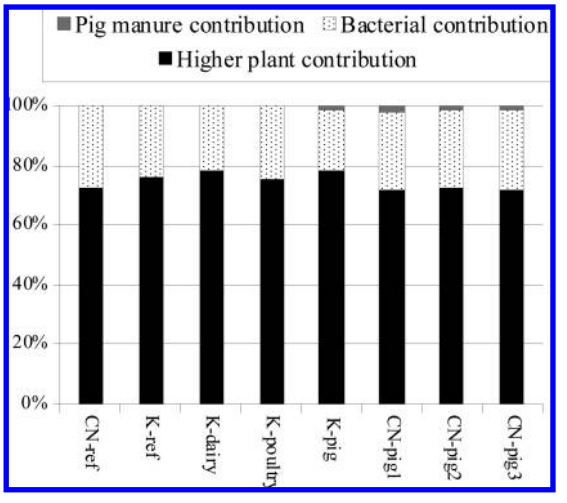

Figure 3. Sources of polar compounds in soil samples.

Branched saturated alkanoic acids were dominated by *iso* and *anteiso* acids with 13, 15, and 17 carbon atoms. Hexadecenoic acid and octadecenoic acid were the unsaturated alkanoic acids present in the soil sampled from the experimental fields. Branched

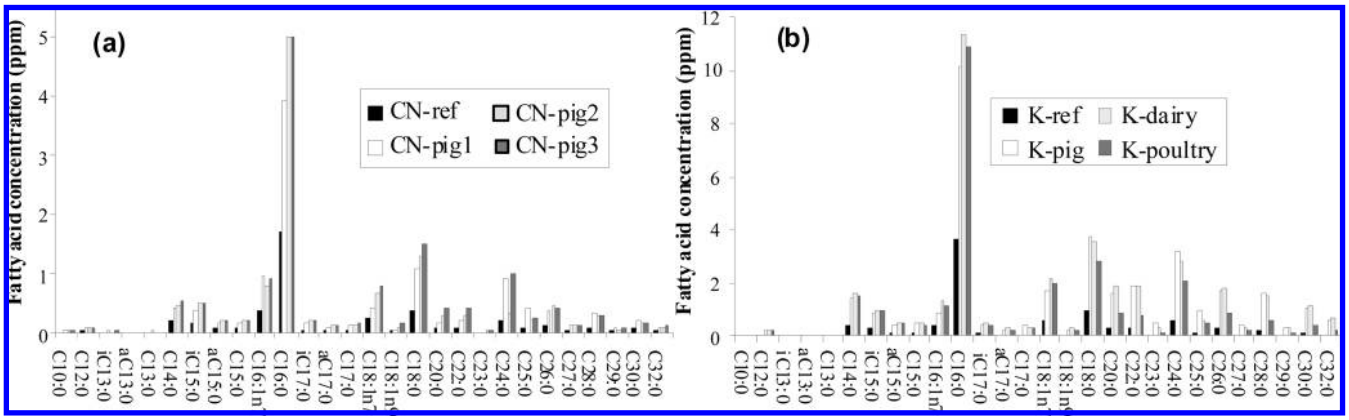

Figure 4. Concentration (ppm) of fatty acids of (**a**) Champ-Noël (CN) and (**b**) Kerguehennec (K) soils.

and unsaturated alkanoic acid identified in the soil samples originated from soil micro-organisms (*8*).

Steroids. **Figure 2** presents the distribution of steroids in the reference and amended soils of Champ-Noël and Kerguehennec.

Steroid distribution of soil samples, whatever the type of crop and fertilization, was characterized by the occurrence of compounds with 27, 28, and 29 carbon atoms. Some differences in the distributions of these three families of steroids appeared according to the samples.

Reference soils from Champ-Noël and Kerguehennec were characterized by the presence of cholesterol (cholest-5-en-3β-ol), its derivative cholestanol (5α-cholestan-3β-ol), campesterol (24-methylcholest-5-en-3β-ol), sitosterol (24-ethylcholest-5-en-3β-ol), and its derivative sitostanol (24-ethyl-5α-cholestan-3β-ol). These profiles evidenced the contribution of higher plants to the reference soils because campesterol, sitosterol, and sitostanol are common phytosterols widely distributed within the plant kingdom and are the most common steroids in the waxes of higher plants (*17*).

Cholesterol (cholest-5-en-3β-ol) and its derivative cholestanol (5α-cholestan-3β-ol) are found in both the vegetal and animal kingdoms (*10*, *18*, *19*). Steroid profiles of soils from the Kerguehennec site amended with poultry (K-poultry) and dairy (K-dairy) manures mimic those found in control soils, being also dominated by cholesterol, cholestanol, campesterol, sitosterol, and sitostanol (see **Figure 2**). These compounds were also present in soils receiving pig slurry (K-pig).

Besides plant-derived compounds, steroid profiles of CN-pig and K-pig show particular extra compounds. As displayed in **Figure 2**, coprostanol (5β-cholestan-3β-ol), its derivative epicoprostanol (5β-cholestan-3α-ol), and 24-ethylcoprostanol (24-ethyl-5β-cholestan-3β-ol) appeared on the steroid profiles of K-pig. Coprostanol has widely been used as a molecular marker of fecal organic matter (*7*, *9*, *10*, *14*, *15*, *20*, *21*). It is formed by the hydrogenation of the double bond between C_5 and C_6 in the steroid skeleton of cholesterol. Cholesterol is converted to coprostanol in the gut of warm-blooded animals and is found in the feces (*22*, *23*). Similarly, 24-ethylcoprostanol is formed from the biohydrogenation of sitosterol (24-ethylcholest-5en-3b-ol) in the gastrointestinal tract of herbivores (*7*).

The molecules identified by GC-MS provide information on their sources, which can be useful in distinguishing between the different inputs of polar compounds in agricultural soils. The polar compounds were classified according to their origin into vascular plants, bacterial, and pig manure markers. Polar compounds that can derive from several origins (for example, cholesterol) were considered to be nonspecific compounds and have not been included in the calculations. In each sample, the proportion of each source was calculated as the ratio between the concentrations of source-specific polar compounds to the concentration of the classified polar compounds (i.e., the sum of polar compounds originating from vascular plants, bacteria, and pig manure). Sitosterol, campesterol, and the even saturated fatty acids have been classified as higher plant compounds. Unsaturated and iso/anteiso branched alkanoic acids have been classified as bacterial compounds and coprostanol, epicoprostanol, and 24-ethylcoprostanol as pig manure markers.

As shown in **Figure 3**, polar compounds in soil samples originate from higher plants, bacterial, and pig manure metabolite sources. The main source of polar compounds in soils is higher plants. Higher plant contribution is evidenced by the presence of typical even fatty acids and phytosterols. It represents between 71% (CN-pig1) and 78% (K-dairy) of the polar compound distribution in the soils from Champ-Noël and Kerguehennec. Higher plant contribution mainly originates from crop residues and animal manure (*11*). Bacterial contribution has mainly been evidenced by the presence of branched iso and anteiso fatty acids and monounsaturated alkanoic acid. Bacterial contribution ranges from 19.9% (K-pig) to 27.6% (CN-ref) depending on the samples. Such bacterial contribution in soils from experimental fields has also been described by Jandl et al. (*13*) and Bull et al. (*24*). There is no difference between the distributions of these two sources whatever the crop type, fertilization type, or fertilization dose. Pig manure contribution has only been evidenced in soils receiving pig slurry fertilization. This contribution represents approximately 2% of the polar compounds in the samples amended with pig slurry.

Impact of the Crop Type, Animal Manure Application, and Organic Fertilization Dose on the Polar Compounds of Soils. **Figure 4** displays the concentration of fatty acids in soil samples. The concentration of the individual saturated alkanoic acids ($C_{10:0}$−$C_{32:0}$) in the soil samples from Champ-Noël and Kerguehennec ranged from 0.01 to 11.4 ppm. In soil samples, the summed concentration of fatty acids ranged from 4.3 ppm (CN-ref) to 37.9 ppm (K-dairy). Those results are in agreement with previous study on the fatty acid pool in agricultural soils (*11*). Animal manure application increased the fatty acid concentration in amended soils. In the Champ-Noël field, the concentration of fatty acids in amended soils with pig slurry ranged from 11 ppm (CN-pig1) to 13.9 ppm (CN-pig3), that is, 2.5−3 times higher than in the reference soil (CN-ref, 4.3 ppm). In the Kerguehennec field, the concentration of fatty acids in amended soils ranged, by order of increase, from 29 ppm (K-poultry) to 35.7 ppm (K-pig) and 37.6 ppm (K-dairy), that is, 3−4 times higher than the concentration of fatty acids in the reference soil (K-ref, 9.7 ppm).

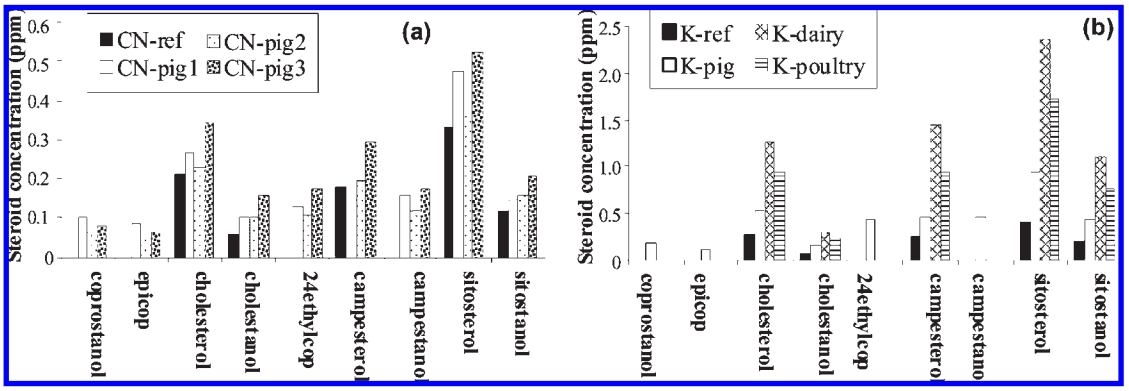

Figure 5. Concentration (ppm) of sterol and stanol compounds of (**a**) Champ-Noël (CN) and (**b**) Kerguehennec (K) experimental fields.

Fatty acid distribution with regard to animal manure type showed similar profiles. This is probably linked to the animal breeding diet, which is mainly composed of plants, leading to the same fatty acid profiles in reference and amended soils. These results also show that there are no fatty acids that could be used as specific markers of animal manures to trace them into the soil. The distributions of fatty acids in amended soils reflected only a "higher plant" fingerprint such as in the reference soils.

The concentrations of steroids (in ppm) are presented in **Figure 5** for reference and amended soil samples from Champ-Noël (**Figure 5a**) and Kerguehennec (**Figure 5b**). The concentration of individual compounds in the soil samples ranged from 0.05 to 2.36 ppm. The summed concentration of steroids ranged from 0.90 ppm (CN-ref) to 6.45 ppm (K-dairy). Those results are in agreement with previous results presented by Bull et al. (*24*) and Shah et al. (*25*). As previously noted for the concentration of fatty acids, the concentrations of steroids in Kerguehennec soils were always higher than their counterparts in Champ-Noël soils. Moreover, animal manure application increased the steroid concentration in amended soils. In the Champ-Noël field, the concentration of steroids in amended soils with pig slurry ranged from 1.6 ppm (CN-pig1) to 2 ppm (CN-pig3), that is, approximately twice the concentration of steroids in the reference soil (CN-ref, 0.9 ppm). In the Kerguehennec field, the concentration of steroids in amended soils ranged, by order of increase, from 3.7 ppm (K-pig) to 4.6 ppm (K-poultry) and 6.5 ppm (K-dairy). These concentrations are 3−5 times higher than the steroid concentration in the reference soil (K-ref, 1.2 ppm).

Steroid distribution with regard to animal manure type exhibited contrasting results. The results evidenced the impact of pig slurry on the distribution of steroids, whereas no effects have been incurred with dairy or poultry manure application. The effect on the distribution of steroid compounds is linked to the occurrence of specific compounds from pig feces (coprostanol, epicoprostanol, and ethylcoprostanol). This result confirms the interest of these compounds to be used as pig slurry markers, even when pig slurry is applied at agronomic dose on soils. It is important to note that even at a low fertilization dose (the fertilization dose represents an annual load of 0.6 t of OC ha^{-1} onto CN-pig1), it has been possible to detect pig slurry specific compounds. To illustrate the differences of steroid compound distribution with regard to animal manure fertilization, two ratios have been calculated on the basis of the concentration of the individual steroids (*7*, *16*). These ratios are as follows:

$5\beta/C_{27}+C_{29}$ is the sum of coprostanol and 24-ethylcoprostanol (5β) divided by the sum of cholesterol (C_{27}) and sitosterol (C_{29}). This ratio represents the proportion of stanols that have been produced by the degradation in the digestive tract of animals of the two major sterols (i.e., cholesterol and sitosterol) of soils.

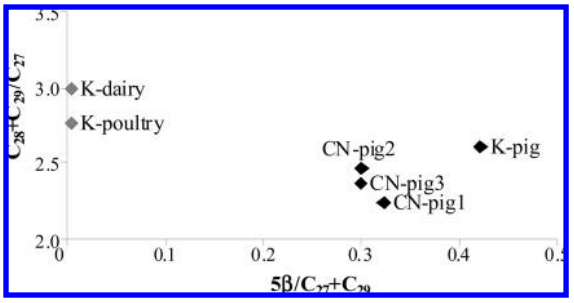

Figure 6. Plot of $C_{28}+C_{29}/C_{27}$ and $5\beta/C_{27}+C_{29}$ ratios comparing the values obtained for amended soils with pig slurry (squares) and poultry or dairy manure (triangles) in Kerguehennec (K) and Champ-Noël (CN) experimental fields.

$C_{28}+C_{29}/C_{27}$ is the sum of campesterol (C_{28}) and sitosterol (C_{29}) divided by cholesterol (C_{27}). This ratio represents the proportion of sterols from the plant kingdom over the proportion of sterols from the animal kingdom.

Figure 6 presents the plots of these steroid ratios. This figure displays two main groups corresponding to amended soil samples from Kerguehennec and Champ-Noël fields. The first one is composed of soils receiving dairy and poultry manure and is characterized by a $C_{28}+C_{29}/C_{27}$ ratio of >2.7 and $5\beta/C_{27}=0$, because no coprostanol, epicoprostanol, or 24-ethylcoprostanol was identified in soils receiving dairy or poultry manure. The second one is formed by soils receiving pig slurry and characterized by a $C_{28}+C_{29}/C_{27}$ ratio of <2.5 and a $5\beta/C_{27}+C_{29}$ ratio of >0.25. Soils receiving pig slurry are clearly distinguished from soils receiving dairy or poultry manure on the basis of these two ratios. This figure also shows that samples from Champ-Noël and Kerguehennec, receiving the same organic fertilization, are gathered together.

Figure 7 displays the results of polar compound concentration in soils of the two experimental fields. When the effect on polar compound concentration is considered, our results show that soils under corn (Champ-Noël field) had lower (significantly lower, $p<0.001$) polar compound concentrations than soils under canola seed (Kerguehennec field). This difference is particularly underscored by the results on reference soils from Kerguehennec and Champ-Noël, to which no animal manure was supplied. According to Jandl et al. (*11*), such differences could be explained by the more intensive aeration due to tillage in corn cropping, which resulted in a net mineralization of soil organic matter.

Moreover, animal manure application increased the polar compound concentrations in amended soils, with polar compound concentrations in amended soils 2−4 times higher than their concentrations in reference soils. The differences in the polar compound concentrations between soils receiving animal

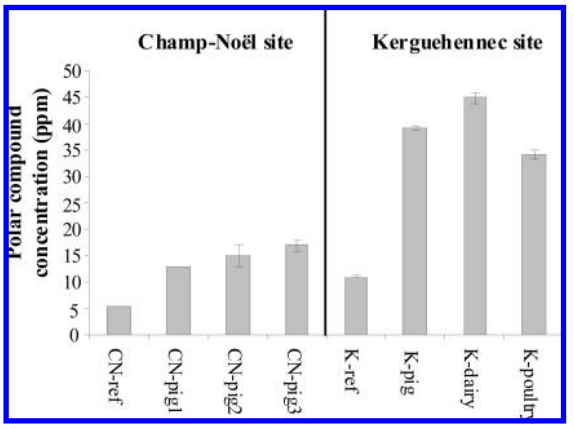

Figure 7. Polar compound concentration (ppm) in soils from Champ-Noël and Kerguehennec sites.

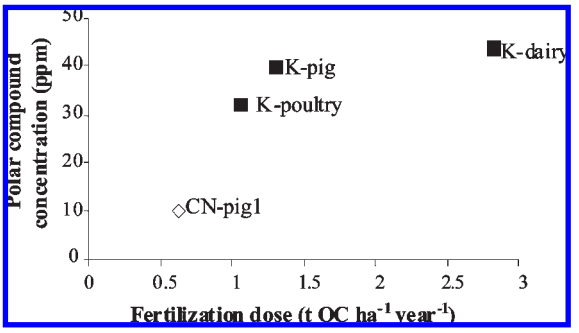

Figure 8. Fertilization dose (in t of OC ha^{-1} $year^{-1}$) versus polar compound concentrations in Champ-Noël (CN) and Kerguehennec (K) soils.

manures were statistically significant ($p < 0.001$; except between K-pig and K-poultry, $p < 0.005$). As shown in **Figure 7**, dairy manure application induced the highest increase in concentration (significantly higher, $p < 0.001$) in comparison with the other animal manures.

The effect of animal manure on the polar compound concentrations of soils seems to be controlled by the dose applied onto soil. Animal manure was applied at different doses onto Champ-Noël and Kerguehennec soils. **Figure 8** represents the polar compound concentrations (in ppm) versus fertilization dose applied onto soils from Kerguehennec and Champ-Noël. The concentration of polar compounds increases with the amount of animal manure applied onto soils; CN-pig1 has the lowest polar compound concentration and K-dairy the highest one. This seems to be controlled by the fertilization dose of animal manure spread onto soils, because the dose applied onto K-dairy was 5 times higher than that applied onto CN-pig1.

When the particular case of soils amended with pig slurry is considered, the concentration of pig slurry markers is twice higher in K-pig than in CN-pig1 and the fertilization dose increases in the same order of magnitude. These results show a positive correlation between pig slurry markers (coprostanol, epicoprostanol, and ethylcoprostanol) and the fertilization dose applied onto soils and confirm the potential to use these compounds to trace pig slurry once applied onto soils.

Persistence of Pig Manure Marker with Time. Pig slurry application onto CN-pig2 and CN-pig3 plots was stopped in 1998. The steroid compounds specific to pig slurry contribution to soil are, however, still present in samples CN-pig2 and CN-pig3, in which pig slurry fertilization was stopped 9 years before sampling. As shown in **Figure 6**, there is no obvious difference in

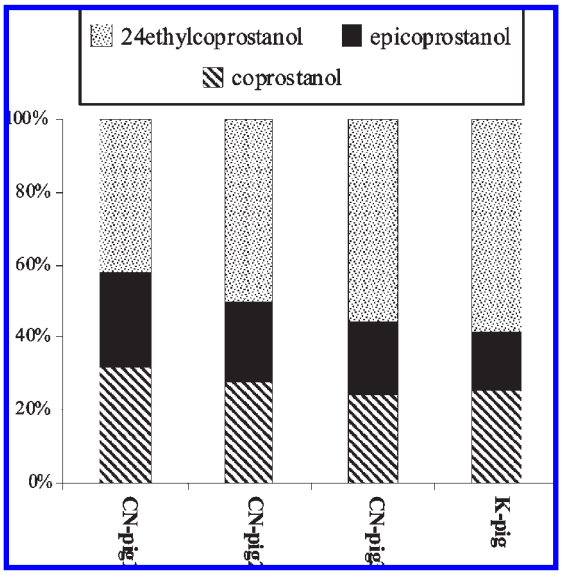

Figure 9. Relative distribution of sterols derived from pig slurry in soils amended with pig slurry.

the $5\beta/C_{27}+C_{29}$ ratio between CN-pig1, CN-pig2, and CN-pig3. **Figure 9** displays the relative distribution (proportions calculated regarding dry weight of soil samples) of steroid derived from pig slurry application onto CN-pig1, CN-pig2, CN-pig3, and K-pig. This relative distribution was fairly invariant, whatever the management type of the soil (i.e., crop type, crop rotation, received dose of pig slurry), thereby confirming the specificity of these compounds as biomarkers of pig slurry input in soils (*7*).

On the basis of the concentrations of pig slurry specific compounds (i.e., coprostanol, epicoprostanol, and 24-ethylcoprostanol), it was possible to estimate the loss in concentrations linked to the fertilization stop. The results showed that in 9 years, 55−57% of coprostanol and epicoprostanol was degraded in CN-pig2 and CN-pig3. The degradation rate was slower for 24-ethylcoprostanol, as only 30 and 35% of this compound was degraded in 9 years in CN-pig3 and CN-pig2, respectively. These results show that stanols are degraded through time in soils by the bacterial community. However, it seems that degradation rates for coprostanol or epicoprostanol and 24-ethylcoprostanol were different. As previously mentioned by Canuel and Martens (*26*), steroid degradation occurs at different rates depending on environmental factors. Moreover, Ahmed et al. (*27*) and Richnow et al. (*28*) showed that the susceptibility of lipids to biodegradation decreases as the number of aromatic rings and the number of alkyl subsistents increase. Thus, the slower degradation rates of 24-ethylcoprostanol could be linked to the occurrence of the ethyl group.

Our results demonstrate that pig slurry specific markers are still present in experimental fields 9 years after the last pig slurry application onto soils. This result fulfills one of the limitations of our previous study (*16*), by demonstrating that a pig slurry steroid fingerprint is persistent through time on soils, even when pig slurry is applied on soils at recommend rates. The persistence of steroids is sufficient to give them effective diagnostic value in determining whether a soil has been contaminated or not by pig slurry.

ACKNOWLEDGMENT

We thank the organizations that manage the two experimental fields: Institut National de la Recherche Agronomique (INRA), Rennes, for the Champ-Noël site and Chambre d'Agriculture du Morbihan, for the Kerguehennec site.

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Received April 20, 2009. Revised manuscript received July 3, 2009. Accepted July 3, 2009.