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Using Ninhydrin to Detect Gravesoil

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Abstract: Some death scene investigations commence without knowledge of the location of the body and/or decomposition site. In these cases, it is necessary to locate the remains or the site where the body decomposed prior to movement. We hypothesized that the burial of a mammalian cadaver will result in the release of ninhydrin reactive nitrogen (NRN) into associated soil and that this reaction might have potential as a tool for the identification of clandestine graves. Juvenile rat (*Rattus rattus*) cadavers were buried in three contrasting soil types in Australian tropical savanna ecosystems and allowed to decompose over a period of 28 days. Soils were sequentially harvested and analyzed for NRN. Cadaver burial resulted in an approximate doubling (mean = 1.7 ± 0.1) in the concentration of soil NRN. This reaction has great potential to be used as a presumptive test for gravesoil and this use might be greatly enhanced following more detailed research.

Keywords: forensic science, forensic taphonomy, cadaver decomposition, grave location, clandestine

Victims of fatal crime are often concealed by the perpetrator in an attempt to evade capture (1). One relatively common approach for concealing a corpse is burial in soil (2). Obviously, concealing a cadaver has a confounding effect on the search and location of these bodies and, as a result, several techniques have been developed to assist in the location of buried cadavers. These include probing (3), the use of cadaver dogs (4), geophysics (3), and the measurement of decomposition odors (5). Unfortunately, none of the methods currently used to locate clandestine graves is successful under every scenario. Thus, a need exists for the continual development of new methods for the detection of gravesoil (any soil associated with a decomposing cadaver [6]). Ideally, the location of these sites would be rapid and require little destruction of putative crime scenes, such as that achieved with cadaver dogs.

Much recent research has focused on the decomposition processes in gravesoils (7–11). This work has shown that cadaver decomposition results in a significant pulse of nitrogen (N) into associated soil (7,8), some of which is in the form of ammonium (NH_4^+) and nitrate (NO_3^-). This, in itself, is not surprising, as a body typically comprises approximately 3% N (12). Knowing this, it is logical that cadaver decomposition would also result in the release of organic forms of N, such as protein, peptide, and amino acids. These nitrogenous compounds, in addition to ammonium, represent chemicals that react with ninhydrin.

Since the description of ninhydrin in 1910 (13), the compound has found regular use in food, medical, and agricultural sciences. A forensic application for the reaction of ninhydrin with organic- and ammonium-nitrogen has been recognized since the mid-1950s (14) and ninhydrin is now regularly used as a medium to detect latent fingerprints on porous surfaces such as paper (e.g., 15). As such, ninhydrin is a compound that is readily available to most investigative agencies.

We hypothesized that the decomposition of a body would result in a significant increase in ninhydrin reactive nitrogen (NRN) in soil. To test this hypothesis, we buried mammalian cadavers in soils located at three contrasting sites in Australian tropical savanna ecosystems. Ninhydrin reactive N was measured in gravesoil at 7-day intervals over a period of 28 days.

Materials and Methods

Juvenile rats (*Rattus rattus*) (~20 g) were used as model cadavers in one of three contrasting sites in tropical savanna ecosystems of Queensland, Australia during the dry season (Oct. 2002). These sites were Yabulu (19°12′S, 146°36′E), Pallarenda (19°11′S, 146°46′E), and Wambiana (20°33′S, 146°08′E). Yabulu soil was a Brown Sodosol (16) and had a loamy sand texture (84.2% sand, 11.0% silt, 4.8% clay). Pallarenda soil was a Rudosol (16) and had a sandy texture (97.7% sand, 1.3% silt, 1.0% clay). Wambiana soil was a Grey Vertosol (16) and had a medium clay texture (30.9% sand, 20.8% silt, 48.3% clay). Soil chemical characteristics are presented in Table 1.

Cadavers were buried (2.5 cm) on their right side in the center of 2 m^2 plots of soil. Control (soil without cadaver) graves were dug 2 m away from each cadaver. Gravesoil and control soil was sequentially and destructively sampled at 7, 14, 21, and 28 days following burial (17). Thus, cadavers were buried and exhumed only once. This harvesting regime avoided the effects of soil disturbance. This experiment was replicated six times, which resulted in the collection of 48 soil samples at each site.

Measurement of NRN in soil followed the method developed by Amato and Ladd (18). Two grams of soil (dry weight) was amended with 8 mL potassium chloride (KCl) (2 M) and shaken (150 rpm) for 30 min. Following shaking, the solution was filtered through a Whatman No. 42 filter paper into a sterile test tube. To 1 mL of filtrate, 0.5 mL ninhydrin reagent (0.8 g ninhydrin [Sigma N6014], 0.12 g hydrindantin [Sigma H2003], 30 mL dimethyl sulfoxide, 10 mL lithium acetate) was added, mixed, and incubated at 100°C for 25 min

Table 1. Physical, chemical, and biological characteristics of soils at Yabulu, Pallarenda, and Wambiana, Queensland, Australia.

Determinant	Soil		
	Yabulu	Pallarenda	Wambiana
Bulk density (ρ_b) (mg/cm^3)	1.50	1.40	1.05
Particle density (ρ_s) (mg/cm^3)	2.48	2.37	2.23
Total porosity (θ) (cm^3/cm^3)	0.40	0.41	0.55
% Coarse sand (2.0–0.2 mm)	35.2	69.6	8.2
% Fine sand (0.2–0.02 mm)	49.0	28.1	22.7
% Silt (0.02–0.002 mm)	11.0	1.3	20.8
% Clay (< 0.002 mm)	4.8	1.0	48.3
Soil texture	Loamy sand	Sand	Medium clay
pH (H_2O)	3.4 (0.2)	4.9 (0.1)	6.1 (0.1)
Total C (%)	1.09 (0.1)	1.35 (0.1)	1.18 (0.1)
Organic C (%)	0.84 (0.0)	1.30 (0.1)	0.99 (0.0)
Total N (%)	0.04 (0.0)	0.10 (0.0)	0.07 (0.0)
Total P (%)	0.01 (0.0)	0.03 (0.0)	0.01 (0.0)
Total S (%)	< 0.01 (0.0)	0.01 (0.0)	0.01 (0.0)
Cation exchange capacity (cmol/kg)	2.30 (0.1)	6.40 (0.1)	31.7 (0.2)
Electrical conductivity (mS/cm)	0.02 (0.0)	0.05 (0.0)	0.10 (0.0)
Microbial biomass C (µg/g soil)	810 (0)	839 (0)	766 (50)
Protease activity (µg tyrosine/g soil/h)	418 (46)	714 (45)	134 (63)
Phosphodiesterase activity (µg p-nitrophenol/g soil/h)	7.1 (0.7)	13.2 (1.3)	25.0 (2.4)

Numbers in brackets represent standard errors where $n = 6$.

at the laboratory. Samples were then removed and allowed to stand at room temperature (~20 min). The solution was then amended with 10 mL 50% ethanol–water (v/v) and absorbance was read at 570 nm (Genesys 10 Vis spectrophotometer, Thermo Scientific, Waltham, MA). The concentration of NRN was calculated against a leucine standard that was processed in the same manner as the soil samples. To make leucine standard, 0.469 g leucine was dissolved into 1 L distilled water. This contained 50 µg N/mL. Separate 100 mL volumetric flasks were amended with 0, 5, 10, 15, 20, and 30 mL leucine solution, 50 mL of 2 M KCl, and water to make up to 100 mL. These standards contained 0, 2.5, 5, 7.5, 10, and 15 µg N/mL.

Statistical Analysis

Data analysis was conducted using SPSS v.15. Normality and homogeneity of variance were tested using the Kolmogorov–Smirnov test and Levene's test, respectively. NRN data were not normally distributed, so means were compared using the Mann–Whitney U-statistic.

Results

Cadaver burial resulted in a 1.4–2.2-fold increase in soil NRN (Figure 1). Significantly greater NRN was observed in Yabulu and Wambiana gravesoil within 7 days of burial. However, this increase did not occur in Pallarenda gravesoil until day 14. Once elevated, the concentration of NRN in gravesoils remained constant until the end of the experiment (day 28), by which time the cadaver had been skeletonized for a minimum of 14 days.

Discussion

The current results demonstrated that cadaver decomposition released a significant amount of NRN into associated soil. This rapid and persistent increase in NRN has great potential to become a useful investigative tool for the location of clandestine graves especially considering that, upon arrival to the laboratory, the analysis of NRN in gravesoil can be conducted in approximately 2 h using materials that are widely available to most crime laboratories throughout the world. However, the inclusion of this method into the standard forensic science toolkit should require the investigation of several other factors, including the effect of soil type, burial depth, cadaver mass, ambient temperature and soil moisture, the innate spatial and temporal heterogeneity of NRN, and the persistence of NRN in gravesoil.

The delayed significant increase of NRN in Pallarenda soil is probably due to the high sand content of this soil. Burial in sandy soil tends to promote desiccation rather than decomposition (19) (especially in conditions such as tropical dry seasons), which is primarily due to the increased rate of gas diffusion in sand relative to finer textured soils (i.e., silt, clay) (20). A lack of available water can retard the activity of enzymes (21) associated with the cycling of N, as many of these are hydrolytic. Recent research (11,22) demonstrated that soil type can have significant effect on cadaver decomposition in soil. This effect apparently extends to the concentration of NRN in gravesoil.

One logical avenue of future investigation is determining the time required for NRN to be released into the soil. Bodies can be moved from the original site of death (and subsequent scenes), so it is conceivable that a body in fresh stage decomposition (23) could come into contact with the soil without affecting the concentration of NRN. At a later stage of decomposition, however, a removed human may leave a persistent effect in former gravesoil. Also, the current data show that a cadaver might not immediately affect concentrations of NRN. These phenomena must be considered when using NRN as a means to locate clandestine graves and sites of death or decomposition on the soil's surface.

The current study clearly shows that a non-human cadaver can release NRN into soil. This is important, as non-human cadavers regularly die and decompose in terrestrial ecosystems (6). Thus, the presence of high concentrations of NRN in soil does not confirm the presence of a human cadaver. It simply confirms the presence of an area of elevated organic- and/or ammonium-N. Effects of cadaver mass should also be investigated. The biological load of a juvenile rat is relatively small,

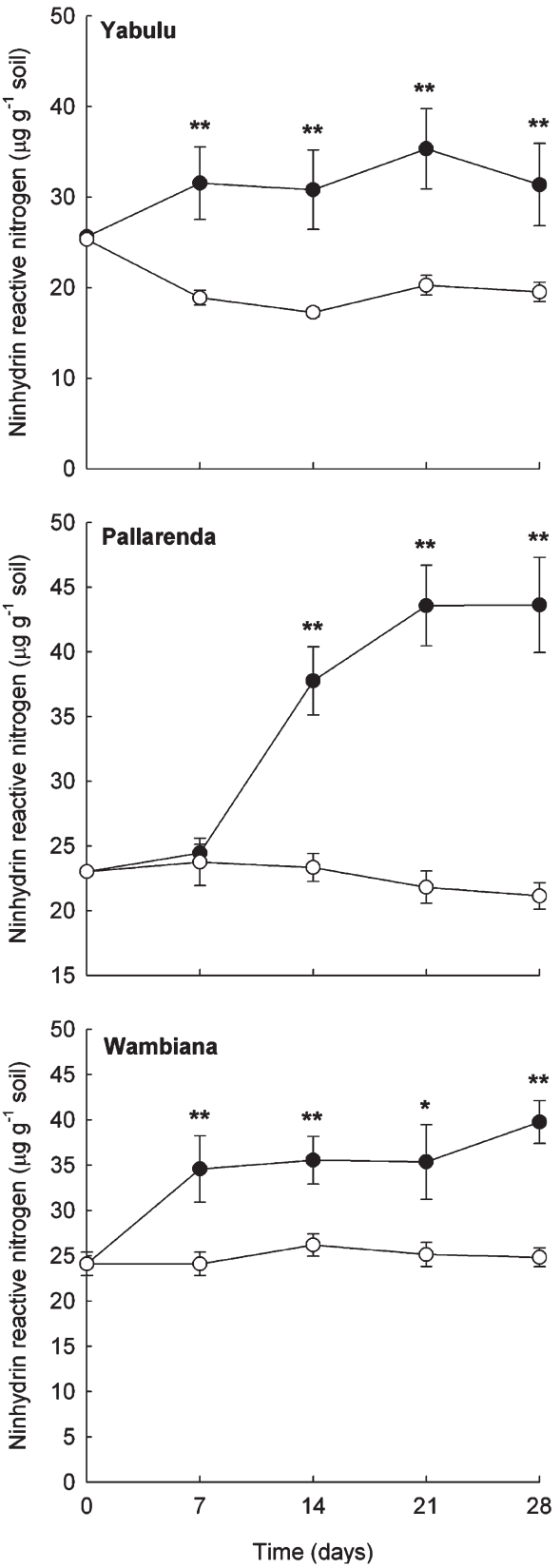

Figure 1. Concentration of ninhydrin reactive nitrogen following the burial (2.5 cm) of a juvenile rat (*Rattus rattus*) cadaver (•) (~20 g) and in control (○: no cadaver) soil in Yabulu, Pallarenda, or Wambiana, Queensland, Australia. * represent significant differences between treatment within time where * = $p < 0.05$ and ** = $p < 0.01$. Bars represent standard errors where $n = 6$.

so the impact of a small mammal on soil NRN will probably be less than a large body such as a human. In addition, other organic resources, such as fecal matter and plant litter can be associated with elevated levels of NRN (24). While it is unlikely that these resources would be associated with levels of NRN as high as those found with a cadaver, it is necessary to explore these relationships in greater detail. Until this is done, or the ability to extract and identify human DNA from putative gravesoil is developed, the presence of elevated concentration of soil NRN will remain a presumptive test for gravesoil. At this stage, we view this use as being similar to the detection of acid phosphatase as a presumptive marker for semen: acid phosphatase is found in several areas of the environment, such as microorganisms (25), plants (26), and soil (27), that can come into contact with locations (e.g., clothing, genitalia) typically tested for the presence of semen.

Also requiring investigation is the lateral and vertical diffusion of NRN away from the decomposition site and burial depth. Decomposition fluids diffuse laterally and vertically away from a body (6) and we would expect a decrease in the concentration of NRN with increasing distance from a cadaver. Understanding this gradient will allow investigators to determine the proximity in which a soil sample must be collected from a body and, possibly, the spatial frequency with which putative gravesoil should be collected when searching for a clandestine grave. NRN might also be influenced by burial depth. Greater burial depth tends to result in a slower rate of cadaver decomposition, which would affect the time required for the release of NRN into gravesoil.

Further studies should investigate the persistence of NRN in gravesoil. Some aspects of chemistry can provide an accurate estimate of postmortem interval (PMI) (7) and measurement of NRN certainly holds this potential. It would be necessary to examine the concentration of NRN over time, possibly as a function of temperature, initial body mass, and soil moisture content, as conducted by Vass et al. (7). The current data show that NRN remains elevated for at least 2 weeks following skeletonization and this might have significant implications for forensic science. There is currently no one method that can accurately estimate PMI in every given circumstance. Arguably, the most accurate means to estimate PMI is through the use of entomological evidence (28), particularly after the initial 24 h of death when the state of the cadaver itself can be quite unpredictable. However, because this approach is best when live immature flies are collected from a scene, insects lose much of their forensic value once the initial colonizers have reached the adult stage. It is during this period, the extended PMI, that the measurement of NRN might make its most significant contribution to estimates of PMI. More detailed research is required before NRN can be used to accurately estimate PMI.

In conclusion, NRN concentrations that are approximately two times greater than control soils can represent the presence of a nitrogenous resource, probably a mammalian cadaver and possibly a human corpse. While it is unlikely that the observed reaction will ever be accepted as a confirmatory test of gravesoil, a fundamental understanding of the processes associated with gravesoils will certainly strengthen its use as a presumptive test. Future studies should investigate the effect of cadaver mass, time, clothing, burial depth, and the diffusion of NRN in soils. Once a robust understanding of these relationships is achieved, the use of ninhydrin to detect gravesoil should become recognized as a reliable contributor to the reconstruction of death scenes in terrestrial ecosystems.

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