Available online at www.sciencedirect.com
SCIENCE DIRECT®
Science of the Total Environment 363 (2006) 245–252
Science of the Total Environment
An International Journal for Scientific Research into the Environment and its Relationship with Humankind
www.elsevier.com/locate/scitotenv

Laser ablation ICP–MS analysis of the radial distribution of lead in the femur of *Alligator mississippiensis*

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Received 22 February 2005; accepted 25 May 2005
Available online 27 June 2005

Abstract

A laser ablation ICP–MS technique has been used to examine the radial distribution of lead in transverse sections of alligator femur. Annual bone growth in the femur results in the deposition of incremental layers of calcified tissue at the periphery of existing bone. Patterns of lead concentration within these layers provide a record of time-dependent accumulation from which exposure history can potentially be deduced. Femur specimens obtained from captive-reared alligators exhibited levels of lead accumulation that were entirely consistent with previously documented clinical signs of lead intoxication. In contrast, femurs obtained from wild alligators contained only minor amounts of lead that were likely accumulated as a result of incidental exposure.
Published by Elsevier B.V.

Keywords: Laser ablation; ICP–MS; Bone; Lead; Alligators

1. Introduction

Elevated levels of lead in bone occur as a direct consequence of long-term exposure to external sources. Lead is sequestered in bone by means of chemical incorporation into the hydroxyapatite mineral matrix (MacDonald et al., 1951). The laminated calcified structures that comprise certain bones contain discrete lead concentrations that are uniquely representative of uptake at the time of laminar construction (Jeffree et al., 2005). Although remobilization may occur under certain pathophysiological circumstances, the residence time of lead in these structures can be decades (Nilsson et al., 1991; Aufderheide and Wittmers, 1992). The total lead concentration of macroscopic bone, determined through bulk elemental analysis, is a reliable gauge of cumulative

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0048-9697/$ - see front matter. Published by Elsevier B.V.
doi:10.1016/j.scitotenv.2005.05.024

exposure over the lifetime of the affected subject. To its disadvantage, this approach reveals little information regarding the momentary intensity of exposure or the duration over which exposure was sustained. However, data of this dimension can conceivably be obtained by examining the spatial distribution of lead in bone that consists of multiple layers of tissue that have been deposited in distinct, chronological sequence. The present investigation explores this notion through micro-analytical examination of lead concentrations in alligator femur bones.

Alligators, and crocodilians in general, are vulnerable to the effects of environmental contamination due to their status as predators at the top of the food chains in their respective ecosystems (Almli et al., 2005). Among inorganic contaminants affecting crocodilians, lead may be second only to mercury (Jagoe et al., 1998) in terms of its ecotoxicological significance (Campbell, 2003). Although lead accumulation in wild crocodilians can be attributed in part, to the phenomenon of biomagnification (Hoffman et al., 2001), evidence of chronic exposure, due to incidental ingestion of prey containing lead shot or bullet fragments, has also been observed (Twining et al., 1999; Jeffree et al., 2001).

Inadvertent dietary intake of lead bullet fragments has been recognized as a cause of lead poisoning in captive-reared, juvenile American alligators (*Alligator mississippiensis*) (Camus et al., 1998). Elevated levels of lead were detected in blood, kidney, and liver tissues of several of the alligators examined during that study. Radiographic imaging confirmed the presence of foreign bodies, later identified as lead bullet fragments, in the stomachs of some animals. Identical fragments were subsequently observed in the supply of nutria meat that was one of several dietary components of the captive-reared alligators in question. Nutria (*Myocastor coypus*) are large fur-bearing rodents that were once commonly harvested using leg traps, but are now increasingly taken by shooting rather than trapping (Camus et al., 1998).

A similar situation of chronic lead exposure was investigated as a possible contributing factor for reproductive failure in captive-reared, adult alligators (Lance et al., unpublished results). Samples of kidney, liver, bone, and yolk obtained from several animals examined during that study contained extremely high concentrations of lead. As in the previous case, lead bullet fragments were retrieved from the stomachs of alligators during necropsies. In this instance however, the alligators were fed nutria meat predominantly, over a period of 15–20 years. Although there exists no documented evidence to substantiate the presence of lead fragments in the nutria meat over this entire period, the exposure circumstances for this group of alligators were nevertheless extraordinary.

Knowledge of the specific patterns of lead accumulation that arise in response to various exposure circumstances may facilitate understanding of the mechanisms by which lead intoxication occurs in animals and humans. To this end, several previous efforts have been made to exploit the archival potential of incrementally deposited bone for examining the uptake of heavy metals in crocodilians. Nuclear microprobe analysis, using particle induced X-ray emission (PIXE), has been employed with some success to examine the lead content of sectioned osteoderms obtained from live freshwater and saltwater crocodiles (Orlic et al., 2002; Hammerton et al., 2003; Orlic et al., 2003). Osteoderms are bony structures composed of annual layers that can be utilized for age determination (Tucker, 1997). The PIXE results appear to have a reasonable degree of correlation with temporal changes in blood lead concentration that occurred in response to systemic exposure (Hammerton et al., 2003). Elemental analyses, having similar objectives, have also been performed using secondary ion mass spectrometry (SIMS) (Twining et al., 1999).

The objective of the present investigation was to examine the utility of laser ablation inductively coupled plasma mass spectrometry (laser ablation ICP–MS) for elucidating the radial distribution of lead in femur bone. Laser ablation ICP–MS is a powerful analytical tool that combines sensitive detection of isotopically resolved elemental species with the versatile solid sampling capability of the laser microprobe (Gunther et al., 2000; Russo et al., 2002). Laser ablation ICP–MS has been used in numerous instances to analyze elemental distribution in growth bands, laminae, and rings present in various hard biological tissues (Outridge et al., 1995). Laser ablation ICP–MS was recently employed in this capacity to profile the concentrations of trace elements in the shell scutes of desert tortoises (Seltzer and Berry, 2005).

An existing collection of preserved alligator femurs, including specimens obtained from chronically exposed alligators (Lance et al., unpublished results), was made available for the present investigation. Unlike osteoderms, femurs can be readily obtained only from deceased or sacrificed animals and therefore, are not likely to find practical application as diagnostic indicators of lead exposure. However, the femurs described above were exceptionally useful as experimental specimens for the present investigation. The histological organization of the alligator femur (Lee, 2004) is much more complex than that of the osteoderm. Adapted for load bearing and locomotion, the cortical portion of alligator femurs consists of a dense network of circumferential lamellae, among which varying numbers of Haversian structures (osteons) may be observed (Lee, 2004). The circumferential lamellae are deposited on an annual basis, primarily around the periosteal (external) margin of the bone cortex (Roberts et al., 1988). Haversian remodeling of cortical bone occurs in response to tensile and compressive strain (Lee, 2004). Since secondary remodeling in alligator femurs is less extensive than that observed in mammals (Enlow and Brown, 1957; de Ricqlès et al., 1991), the primary features (circumferential lamellae) that correspond to successive growth increments are essentially preserved. Accordingly, the spatial profiles of lead concentration revealed using the present methodology should faithfully represent the pattern of lead uptake and accumulation over a specific duration.

2. Methods and materials

The femurs analyzed during the present investigation were provided by the Rockefeller Wildlife Refuge (Grand Chenier, Louisiana). Among these were original specimens obtained from a group of chronically exposed, captive-reared alligators that were subjects of a previous study (Lance et al., unpublished results). The alligators were approximately 27 years of age at the time of sacrifice. Femur specimens from wild adult alligators, having no documented history of lead exposure, were also examined. Transverse sections 3–4 mm in thickness, were taken at mid-diaphysis using an abrasive wafering saw. The anatomical orientation (Lee, 2004) of each femur section was noted. The femur sections were rinsed using de-ionized water following removal of residual marrow and soft tissues. The sections were then dried in a dessicator for several days in order to minimize the amount of water vapor entrained into the argon plasma during sample introduction.

The ICP–MS instrument operating conditions and laser ablation methodology employed here are similar to those described previously (Seltzer and Berry, 2005). For the present application, the laser repetition rate was 5 Hz and the laser pulse energy was 0.6 mJ. Concentrations of lead along the laser ablation transect axes were measured via a semi-quantitative analysis scheme (Seltzer and Berry, 2005) that utilizes ion intensities of both the lead analyte and a specified internal standard element of known concentration, and sensitivity factors for both elements. In the absence of appropriate calibration materials, this alternative approach can provide reasonable results, assuming that matrix effects such as elemental fractionation (Chen, 1999) are not excessive.

Calcium was selected for use as an internal standard because of its near-homogeneous distribution throughout the bone mineral matrix. The bone concentration of calcium is variable among alligators, and it was necessary therefore, to determine its value in each of the femur specimens. Three 0.6–0.8 g portions of cortical bone, collected adjacent to the transverse sections, were acid digested by microwave heating in closed Teflon® pressure vessels. Elemental analyses were subsequently performed on solutions of digested bone using inductively coupled plasma atomic emission spectrometry (ICP–AES).

In order to examine the distribution of lead among the lamellar structures that comprise the cortical portion of alligator femurs, laser ablation transects were performed along the surfaces of femur sections. Fig. 1

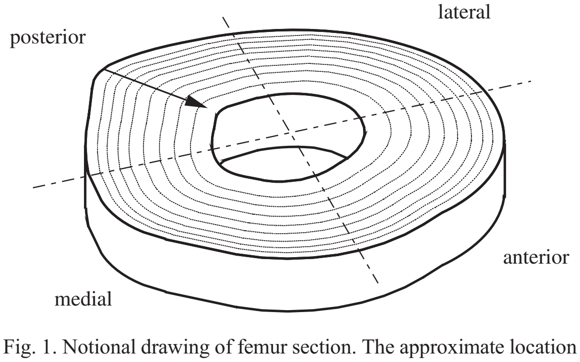

Fig. 1. Notional drawing of femur section. The approximate location of the laser ablation transect is indicated by an arrow.

is a notional diagram of a femur section in which the general anatomical orientation is indicated. Transects were performed within the posterior quadrant (Lee, 2004) on each femur section as illustrated in Fig. 1. It is presumed that radial transects, initiated at the periphery of each femur section, encountered a succession of circumferential lamellae. Clean-up transects were first performed along each axis in order to condition the surface and remove residual contaminants. Multiple analytical transects were performed over the same axis to examine data reproducibility, and to allow identification of spurious responses arising from re-condensation of ablated material.

ICP–MS data was acquired in a time-resolved analysis (TRA) mode, in which ion intensities for individual isotopes were integrated over successive 50-ms dwell increments. Under these circumstances, the data acquired during each increment is associated with a unique time coordinate. During individual transects, data collection was initiated 10 s prior to the start of actual ablation, to enable recording of baseline ion intensities. Net intensities, corresponding to the analyte content of ablated sample material, were later obtained by subtracting average baseline intensities from all ensuing data.

3. Results

3.1. Bulk concentrations of calcium, lead, and strontium

Bulk concentrations of calcium (internal standard), lead, and strontium, measured independently for each femur specimen, are listed in Table 1. The lead concentrations corresponding to captive-reared alligators are lower than those measured in the same femurs during a previous investigation (Lance et al., unpublished results), but are generally consistent with the transect profiles shown below. We attribute this disparity to differences in the size and orientation of the bone samples analyzed in both instances, and the overall inhomogeneity of lead distribution in alligator femur bone.

It is interesting to note that the strontium concentrations listed in Table 1 for femurs obtained from captive-reared animals are consistently lower than those measured in femurs from wild alligators. Relatively low strontium levels in bone are characteristic of diets in which bone-forming minerals are derived almost exclusively from meat (Burton and Wright, 1995). The monotypic diet of the captive-reared alligators represented here is a case-in-point. Wild alligators, on the other hand, are opportunistic and adaptive predators that avail themselves of a diverse diet that includes crustaceans and fish (Valentine et al., 1972). The mineralized tissues of crustaceans and fish contain appreciable concentrations of strontium (Rosenthal et al., 1970). It is likely therefore, that the differences in bone strontium level noted above are a direct consequence of the dietary intake of strontium in each instance.

3.2. Semi-quantitative analysis

Ion intensities for $^{208}Pb^{+}$(analyte) and $^{43}Ca^{+}$(internal standard) were recorded as counts per second (cps) throughout each of the analytical laser ablation transects performed on femur sections. Following the acquisition of analytical data, raw ion intensities

Table 1
Bulk elemental analysis of femur specimens

Femur specimen[a]	Alligator length (m)	Sex	Bulk calcium concentration (wt.%)	Bulk lead concentration (ppm)	Bulk strontium concentration (ppm)
C4	3.82	M	27.6 ± 1.5	105 ± 16	367 ± 22
C27	3.67	M	25.6 ± 1.1	163 ± 20	368 ± 30
C46	2.69	F	25.0 ± 1.3	138 ± 31	377 ± 13
C50	3.81	M	23.8 ± 2.2	386 ± 98	329 ± 10
W88	2.24	Unknown	26.6 ± 2.3	1.28 ± 0.54	1065 ± 32
W90	2.29	Unknown	23.5 ± 2.1	<1	961 ± 22
W98	2.49	Unknown	25.9 ± 1.1	3.57 ± 0.66	898 ± 13

[a] "C" denotes captive alligators; "W" denotes wild alligators.

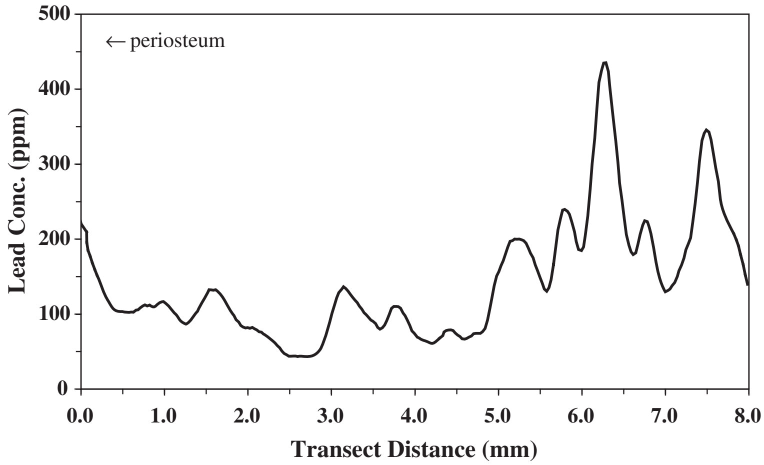

Fig. 2. Transect profile of lead concentration in femur section from captive-reared alligator C4.

and corresponding time coordinates were transferred to electronic spreadsheets. Lead concentrations, corresponding to the measured $^{208}Pb^{+}$ ion intensities, were calculated on a point-by-point basis using a semi-quantitative scheme that incorporates empirically determined elemental sensitivity factors (Seltzer and Berry, 2005) and known concentrations of calcium internal standard (Table 1). The elemental sensitivity factors for calcium and lead measured under the present conditions and adjusted for isotopic abundance were 1020 and 5150 cps/ppm, respectively. Because laser ablation was performed at a fixed transect rate (100 μm/s) along the sample surface, it was possible to transform the time coordinates of individual data points into transect distances. Transect profiles, displaying elemental concentration vs. distance, were generated accordingly.

3.3. Elemental transect profiles

The sub-periosteal region of each femur section represents a well-defined starting point for examining the radial distribution of lead. Laser ablation transects initiated within this region yield transect profiles that represent the sequence of accumulation in reverse, but definite, chronological order. Fig. 2 is a radial transect profile of lead concentration in a section obtained from femur specimen C4. The number and appearance

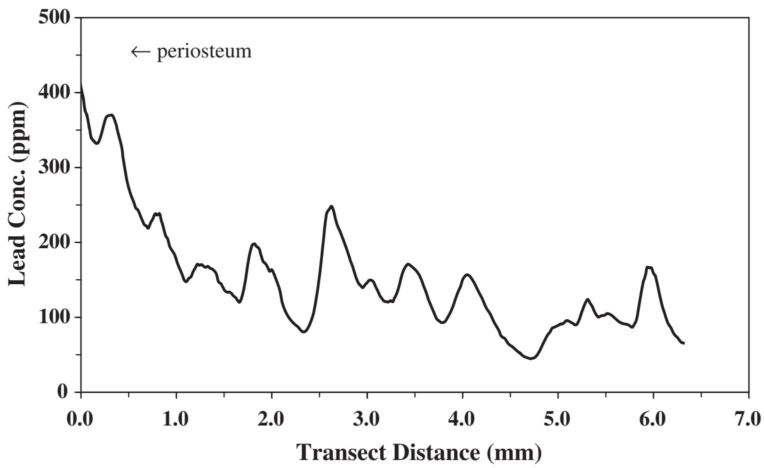

Fig. 3. Transect profile of lead concentration in femur section from captive-reared alligator C27.

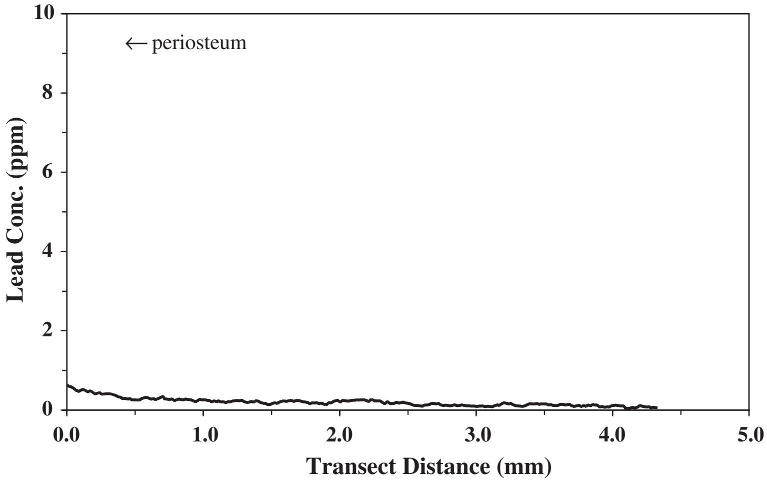

Fig. 4. Transect profile of lead concentration in femur section from wild alligator W90.

of distinct features in this profile suggests that the peak concentrations represented in each instance correspond to accumulation of lead within individual lamellae. Concentration features of similar appearance are commonly observed in conjunction with laser ablation ICP–MS analysis of other incrementally grown biological structures (Outridge et al., 1995; Seltzer and Berry, 2005). It is important to note that lines of arrested growth that define the margins of adjacent lamellae (Woodward and Moore, 1992) were not easily discernible in the present specimens. Therefore, explicit correlations between transect distances and individual lamellae were not established. Histological staining techniques (Roberts et al., 1988), that are often used to visualize these features, were not implemented during the current investigation. The transect profile shown in Fig. 2 suggests that lead exposure was substantial in past years, but later diminished in intensity. The elevated lead concentration observed near the beginning of the transect is consistent with the observation of sub-periosteal lead deposition in cases of severe lead intoxication in humans (Aufderheide and Wittmers, 1992), and is probably indicative of a substantial lead burden at the time of death. The transect profile shown in Fig. 3 conveys a pattern of lead accumulation in femur specimen C27

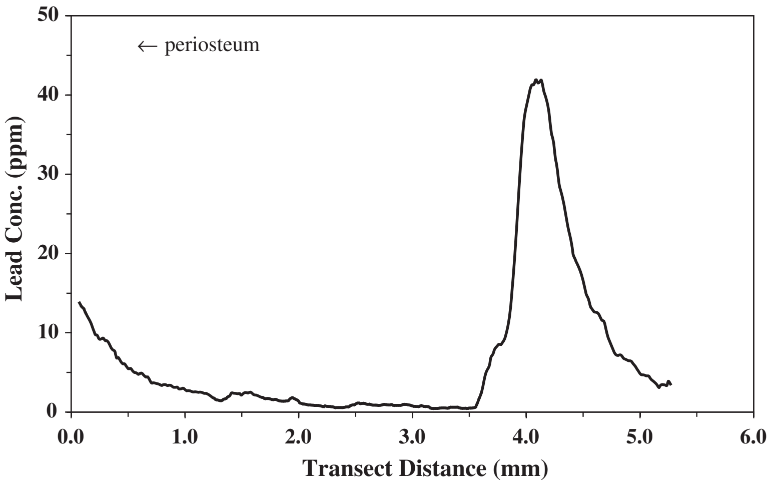

Fig. 5. Transect profile of lead concentration in femur section from wild alligator W98.

that differs significantly from that shown in Fig. 2. Although lead uptake appears to be sustained throughout the transect shown in Fig. 3, peak accumulation during the most recent growth cycles is clearly evident.

The femur specimens associated with the transect profiles shown in Figs. 2 and 3 were obtained from captive-reared alligators that exhibited clinical signs of lead intoxication (Lance et al., unpublished results) as a consequence of the exposure circumstances described above. In distinct contrast, the transect profile depicted in Fig. 4, corresponding to a femur obtained from a sacrificed wild alligator (W90), indicates the virtual absence of lead accumulation. It is reasonable to conclude therefore, that consequential exposure did not occur during deposition of the lamellar bone tissues interrogated along this transect. The transect profile shown in Fig. 4 provides a useful baseline against which elevated patterns of lead accumulation in other femur specimens can be compared. All of the wild alligators represented in this investigation were shorter in length (Table 1) and presumably younger than the captive-reared animals. Typically, alligators of 2.2 m and above are at least 10 years of age, but could be as old as 20 (Chabreck and Joanen, 1979). Femurs obtained from the wild alligators were of correspondingly smaller cross-section; hence, the abbreviated transect lengths. Fig. 5 illustrates the radial distribution of lead in a femur section obtained from a second wild alligator (W98). An isolated, but distinct concentration feature, appearing relatively late in the transect, suggests that acute uptake and accumulation of lead, occurring in early years, was not sustained over more than one or two growth cycles.

4. Discussion

The transect profiles of lead presented here represent the first successful analysis of the radial distribution of lead in the femur bone of American alligators. It is important to note that the laser ablation technique, as described above, was not alone capable of associating lead concentrations along the transect axis with specific lamellae. It is almost certain that the identification and mapping of these structures would have added a more meaningful spatial dimension to the acquired data, thereby realizing the true archival potential of the femur specimens.

The transect profiles presented above nevertheless represent patterns of lead accumulation over the period of time during which the corresponding layers of bone tissue were deposited. The data clearly demonstrate instances of both acute and sustained accumulation of lead. For the captive-reared alligators, the transect profiles are probably indicative of lead accumulation occurring over the most recent 10–15 years of bone growth. Older layers may be gradually lost through perimedullar erosion (de Ricqlès, 1976) and consequently, are not accessible for analysis. However, several of the transect profiles obtained using femur specimens from captive-reared alligators exhibit concentration maxima that correspond to lead accumulation during, and subsequent to, the earliest period of growth represented. This substantiates the notion that lead exposure in these alligators may have been sustained over that duration. It is reasonable to conclude therefore, that lead bullet fragments were present in nutria meat, at least on an intermittent basis, during the entire time that this diet was maintained.

The records of lead accumulation revealed in femur specimens obtained from both captive-reared alligators and wild alligators are uniquely representative of the exposure circumstances that brought about the corresponding lead burdens in each instance. The exposure endured by the captive-reared alligators was extensive in comparison to that of the wild alligators for which lead accumulation in femur bone was at most, relatively minor. The source of exposure in the wild alligators was presumably finite, and possibly associated with the ingestion of prey, containing lead shot or remnants of lead fishing sinkers, or having intrinsic lead burdens acquired through dietary consumption. In all instances, the distinct patterns of lead accumulation were undoubtedly influenced by both the intensity and duration of exposure. The ability to examine these patterns, using the analytical techniques described above, may play a useful role in further understanding the pathologic consequences of lead exposure and accumulation.

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