Therefore, anammox in these waters could account for 10–15% of the marine N_2 production.

The anammox process may be more important than this, with particularly favourable conditions occurring in upwelling areas where anoxic nitrate-rich water reaches the sediment. A good example would be upwelling areas off the Peruvian and Chilean coasts, where sedimentary sulphide is oxidized by sulphide-oxidizing bacteria with nitrate, producing ammonium[23]. This ammonium is released to the water column, probably enhancing the significance of anammox, and N_2 production, in these areas. Thus, the anammox process, with its distinct regulatory characteristics, should be included in studies of nitrogen cycling in the marine environment. □

Methods

Sampling was performed at Station A (8° 34.15″ N, 83° 14.69″ W) and Station B (8° 37.99″ N, 83° 20.62″ W) of Golfo Dulce in November 2001. Profiles of salinity, temperature and oxygen were measured at 10-m intervals with a DataSonde 4 (Hydrolab). All water samples were retrieved with a 5-l Niskin bottle (KC Denmark). For nutrient profiles, water was sampled from the Niskin bottle with a plastic syringe and filtered through a cellulose acetate filter (pore size 0.22 μm) into polypropylene vials that were stored on ice until return to the laboratory where they were frozen for later analysis. For the ^{15}N-labelling experiments, water was sampled at 120, 140, 160 and 180 m depth at Station A, and at 100, 120, 160 and 180 m depth at Station B. Water was transferred from the Niskin bottle via Tygon tubing into the bottom of a 250-ml glass bottle and allowed to flow over for half a volume change. The bottle was closed with a Viton stopper taking care to exclude bubbles, and stored on ice until return to the laboratory. Experiments were started no later than 6 h after sampling.

For each of the four depths from each station experiments were started by the addition of ^{15}N labelled and unlabelled nitrate and ammonium to the 250-ml bottles to the following final concentrations from concentrated stock solutions: 10 μM $^{15}NO_3^-$, 10 μM $^{15}NO_3^-$ + 10 μM $^{14}NH_4^+$ and 10 μM $^{15}NH_4^+$. After addition the amended water was bubbled with helium for 15 min to facilitate the detection of N_2 production, and then transferred to 12-ml glass vials with a 5-mm butyl rubber septum (Exetainers, Labco) through a glass tube leading from the bottle into the bottom of the Exetainer and allowed to overflow. The Exetainers were incubated at *in situ* temperature in a water bath kept anoxic with an alkaline sodium ascorbate solution. The oxygen concentration in the Exetainers was measured with a microelectrode designed for measuring low oxygen concentrations (Unisense) and was below the detection limit of 0.2 μM throughout the experiment. At each time point Exetainers were sampled in triplicate by removing 5 ml of water while replacing it with helium and adding 0.1 ml of 50% (w/v) ZnCl to stop biological activity in the Exetainer. The removed water was filtered for nutrient analysis and stored as described above.

The concentration of $NO_3^- + NO_2^-$ was determined using the vanadium chloride reduction method[24] (NO_x analyser model 42c, Thermo Environmental Instruments Inc.). Nitrite was analysed spectrophotometrically[25] and NH_4^+ was determined using the flow injection method with conductivity detection[26]. The isotopic composition of the nitrate and ammonium pool was estimated from their concentrations before and after amendment. Concentrations of $^{14}N^{15}N$ and $^{15}N^{15}N$ were determined by isotope ratio mass spectrometry and calculated as excess above their natural abundance[2].

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Correspondence and requests for materials should be addressed to T.D. (e-mail: tda@dmu.dk).

Anaerobic ammonium oxidation by anammox bacteria in the Black Sea

Marcel M. M. Kuypers*, A. Olav Sliekers†, Gaute Lavik*, Markus Schmid†, Bo Barker Jørgensen*, J. Gijs Kuenen†, Jaap S. Sinninghe Damsté‡, Marc Strous§ & Mike S. M. Jetten§

** Max Planck Institute for Marine Microbiology (MPI), Department of Biogeochemistry, Celsiusstrasse 1, 28359 Bremen, Germany*
† Department of Microbiology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands
‡ Royal Netherlands Institute for Sea Research (NIOZ), Department of Marine Biogeochemistry and Toxicology, PO Box 59, 1790 AB Den Burg, The Netherlands
§ Department of Microbiology, University of Nijmegen, Toernooiveld 1, 6526 ED Nijmegen, The Netherlands

The availability of fixed inorganic nitrogen (nitrate, nitrite and ammonium) limits primary productivity in many oceanic regions[1]. The conversion of nitrate to N_2 by heterotrophic bacteria (denitrification) is believed to be the only important sink for fixed inorganic nitrogen in the ocean[2]. Here we provide evidence for bacteria that anaerobically oxidize ammonium with nitrite to N_2 in the world's largest anoxic basin, the Black Sea. Phylogenetic analysis of 16S ribosomal RNA gene sequences shows that these bacteria are related to members of the order Planctomycetales performing the anammox (anaerobic ammonium oxidation) process in ammonium-removing bioreactors[3]. Nutrient profiles, fluorescently labelled RNA probes, ^{15}N tracer experiments and the distribution of specific 'ladderane' membrane lipids[4] indicate that ammonium diffusing

upwards from the anoxic deep water is consumed by anammox bacteria below the oxic zone. This is the first time that anammox bacteria have been identified and directly linked to the removal of fixed inorganic nitrogen in the environment. The widespread occurrence of ammonium consumption in suboxic marine settings[5–7] indicates that anammox might be important in the oceanic nitrogen cycle.

The Black Sea is the world's largest anoxic basin and is a model for both modern and ancient anoxic environments. It is characterized by a high ammonium concentration in the deep waters (up to 100 μM), whereas only trace amounts of fixed inorganic nitrogen are present in the 'suboxic' zone[6,8] where the reduction of nitrate, manganese oxide or iron oxide occurs[9]. This apparent ammonium sink in the suboxic zone strongly suggests[7,10,11] that ammonium is oxidized anaerobically to N_2. Indeed, bacteria able to oxidize ammonia anaerobically have recently been discovered in laboratory bioreactors and wastewater treatment systems[3,12]. These so-called 'anammox' bacteria belonging to the order Planctomycetales directly oxidize ammonia to N_2 with nitrite as the electron acceptor (Fig 1a, b):

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (1)$$

During an R/V *Meteor* cruise in December 2001 we investigated the role of anammox in the Black Sea water column by using microbiological and biogeochemical techniques. In accord with with earlier studies[6,8,11] we observed a nitrate maximum at the bottom of the oxic zone in the western basin (site 7605; 42° 30.71′ N, 30° 14.69′ E; Fig. 2a). This maximum is caused by the mineralization of phytoplankton-derived organic nitrogen coupled to aerobic nitrification (Fig 1a). Ammonium concentrations are high in deep waters but decrease to background values above 97 m water depth (Fig. 2a). Aerobic nitrification cannot account for the consumption of ammonium because O_2 is absent below 80 m (Fig. 2b). However, nitrate penetrates 15 m deeper in the water column, indicating that nitrate could be the oxidizer of ammonium[11]. Alternatively, anammox bacteria could be using nitrite instead of nitrate to oxidize ammonium. Nitrite is an intermediate of denitrification and a nitrite peak is present at the base of the nitrate peak (Fig. 2a). Anammox in the suboxic zone could be coupled to nitrate reduction to nitrite (Fig 1a) by denitrifiers[13], similarly to the process in anammox bioreactors[14].

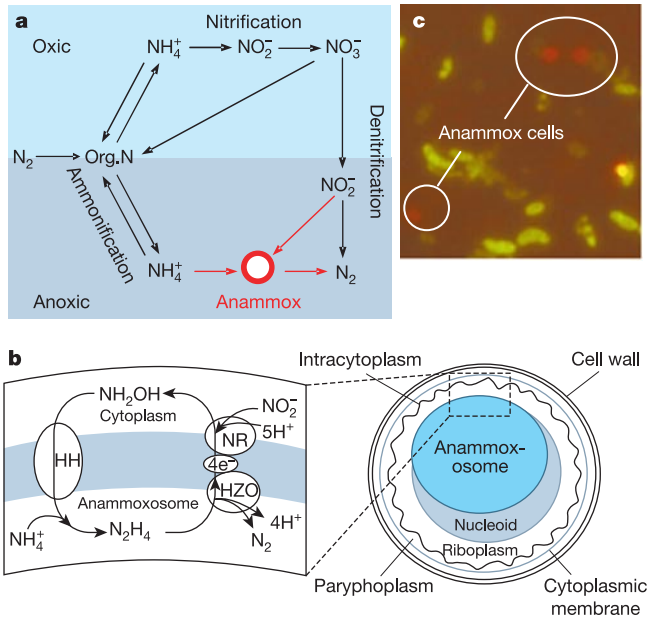

Figure 1 Morphology and physiology of anammox bacteria and their role in the marine nitrogen cycle. **a**, Simplified marine nitrogen cycle including the anammox 'sink'. Org.N, organic nitrogen. **b**, Morphology of the anammox cell and proposed model for the anammox process. HH, hydrazine (N_2H_4) hydrolase; HZO, hydrazine oxidizing enzyme; NR, nitrite reducing enzyme. **c**, Fluorescence *in situ* hybridization of filter material from station 7617 (142 m water depth). Green cells are total Eubacteria stained with EUB338 probe; red cells (encircled) are anammox bacteria stained with a new specific probe (AmxBS820).

To check for anammox activity in the suboxic zone we anaerobically incubated water samples from various depths after the addition of [^{14}N]nitrite and [^{15}N]ammonium. Because the anammox process combines 1 mol of [^{15}N]ammonium and 1 mol of [^{14}N]nitrite to form 1 mol of single-labelled dinitrogen gas ($^{14}N^{15}N$) (equation (1)), the depth distribution of $\delta^{14}N^{15}N$ (Fig. 2c) expresses the potential anammox activity. The $\delta^{14}N^{15}N$ record shows a clear peak in the zone of nitrite and ammonium disappearance, whereas no significant anammox activity is observed outside the suboxic zone.

Specific biomarkers, so-called ladderane lipids, were used to trace anammox bacteria in particulate organic matter collected from various depths across the suboxic zone. Ladderane lipids[4] are the main building blocks of a unique bacterial membrane that surrounds the anammoxosome, a special compartment of the anammox cell, in which the anaerobic oxidation of ammonium to N_2 takes place (Fig. 1b). Three different ladderane lipids were detected in the saponified total lipid extracts with a depth distribution (Fig. 2d and e) similar to that of the potential anammox activity (Fig. 2c), indicating that anammox bacteria could indeed be responsible for the anaerobic oxidation of ammonium. A clone library was generated from DNA extracted from Black Sea water at the depth of maximum ladderane abundance (90 m), after amplification of the 16S ribosomal RNA gene with primers specific for Planctomycetes[15]. Phylogenetic analysis of the 16S rRNA sequences confirms that the Planctomycetes, tentatively named *Candidatus* 'Scalindua sorokinii', from the suboxic zone of the Black Sea are related to bacteria known to be capable of the anammox process (87.9% sequence similarity to *Kuenenia*, 87.6% to *Brocadia*; Fig. 3). In fact, the sequence obtained from the Black Sea is nearly identical (98.1%) to a sequence recently obtained from a bioreactor shown to have anammox activity (M. Schmid, K. Walsh, R. Webb, W. I. Rijpstra, K. T. van de Pas Schoonen, T. C. J. Hill, B. F. Moffett, J. A. Fuerst, J.S.S.D., J. A. Harris, P. J. Shaw, M.S.M.J. and M. Strauss, unpublished observations). On the basis of the sequence obtained from the Black Sea, we designed an oligonucleotide probe, labelled with Cy3 fluorochrome, for fluorescence *in situ* hybridization (FISH). This probe gave a bright and specific signal with cells that have the unusual doughnut shape characteristic for anammox bacteria in bioreactors. Ladderane biomarkers and cells hybridizing with the new FISH probe (Fig. 1c) were also found in the suboxic zone at the shelf break (Station 7617, 43° 38.04′ N, 30° 02.54′ E), indicating that anammox bacteria are not restricted to the strongly stratified central basin but are also present in the more dynamic peripheral current[8]. The combined results clearly indicate that anammox bacteria are abundant and active in the Black Sea. Could these anammox bacteria be responsible for the observed ammonium sink in the suboxic zone of the Black Sea?

If we assume that the concentration profile of ammonium represents a steady state, an anaerobic ammonium oxidation rate of ~0.007 μM day^{-1} was calculated for the suboxic zone of the central basin by using a reaction diffusion model. This rate is comparable to aerobic ammonium oxidation rates (0.005–0.05 μM day^{-1}) determined for the nitrate maximum of the western central basin of the Black Sea[16]. An anammox rate of 2–20 fmol ammonium per cell per day was found in laboratory bioreactors[3]. Assuming a similar range of cell-specific activity for the Black Sea, 300–3,000 anammox cells ml^{-1} would be needed to account for the observed ammonium oxidation rates in the suboxic zone. Counts of

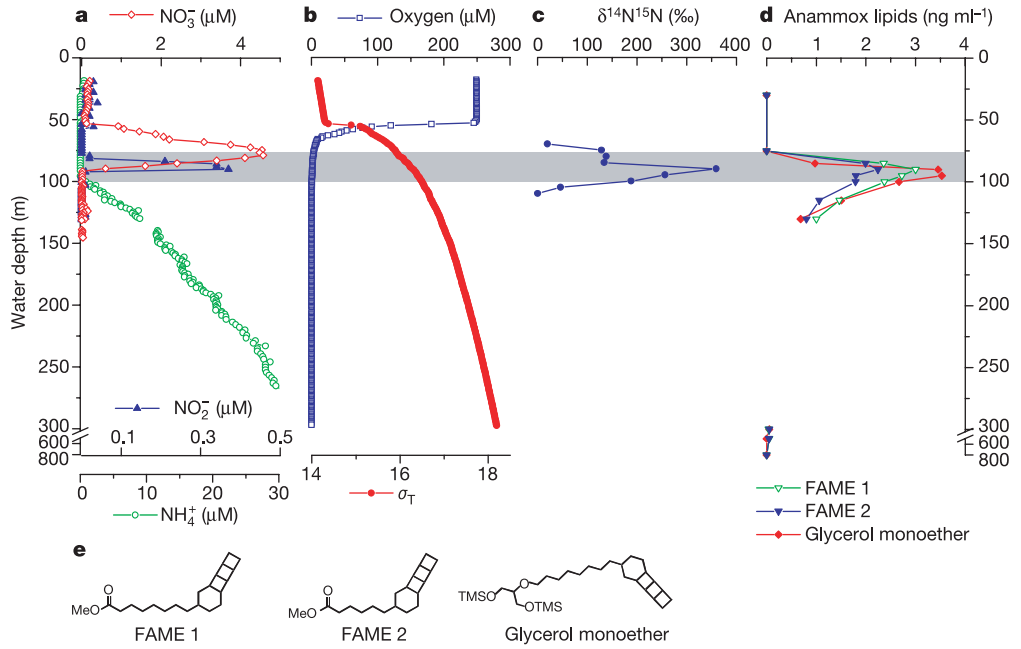

Figure 2 Chemical zoning and distribution of anammox indicators across the Black Sea chemocline. **a**, Fixed inorganic nitrogen species; **b**, water density and oxygen concentrations; **c**, interface peak of potential anammox activity expressed as anaerobic $^{15}NH_4^+$ oxidation by $^{14}NO_2^-$ to $^{14}N^{15}N$; **d**, peak of three ladderane membrane lipids specific for anammox bacteria; **e**, molecular structures of the three ladderane membrane lipids specific for anammox bacteria presented in **d**. The suboxic zone is indicated by grey shading. Density (σ_T, the density of seawater in kg m^{-3} − 1,000), nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and oxygen profiles from Station 7605 (42° 30.71′ N, 30° 14.69′ E). Ladderane lipid data from Stations 7605 and 7620 (42° 55.56′ N, 30° 03.65′ E) were used to create a composite plot for the ladderane glycerol monoether and for the fatty acid methyl esters (FAMEs) 1 and 2.

cells stained with the newly designed FISH probe (Amxbs820) gave an anammox cell density of ~1,900 ± 800 cells ml^{-1} (0.75% of all cells counted by 4,6-diamidino-2-phenylindole (DAPI)) at the nitrite peak.

Although we acknowledge the uncertainty involved in the extrapolation of laboratory-derived anammox activities to the natural environment, the rates of net ammonium and nitrate consumption calculated as a function of depth indicate that nitrate reduction by denitrifiers coupled to anammox accounts for a substantial loss of fixed inorganic nitrogen. In fact, the downward flux of nitrate (~7 μmol m^{-2} h^{-1}) is sufficient to oxidize all the ammonium (~5 μmol m^{-2} h^{-1}) diffusing up into the suboxic zone. If we assume that the area (3×10^5 km^2) below the shelf break (<200 m)[8] represents the total surface area of the suboxic zone, 0.3 Tg of fixed inorganic nitrogen per year might be lost through nitrate reduction coupled to anammox. For comparison, the annual primary production of phytoplankton in the whole basin is ~80 Tg carbon (ref. 8), which is equivalent to 14 Tg of fixed organic N if we assume an atomic C/N ratio of 6.6 for phytoplankton[17]. Because more than 95% of this phytoplanktonic organic nitrogen is recycled in the upper 80 m (ref. 18), anammox might consume more than 40% of the fixed nitrogen that sinks down into the anoxic Black Sea water.

Moreover, these results demonstrate that anammox bacteria are abundant and are important in the nitrogen cycle of the Black Sea. In fact, the widespread occurrence of ammonium consumption in suboxic marine waters as well as in sediments[7] suggests that anammox bacteria could have an important but as yet neglected role in the oceanic loss of fixed nitrogen. □

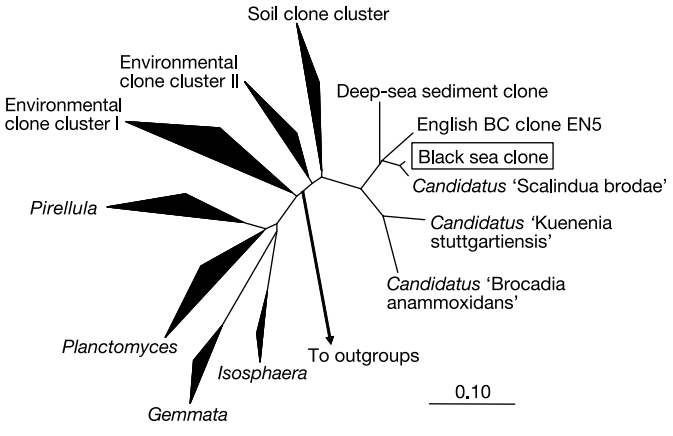

Figure 3 Phylogenetic tree of 16S rRNA gene sequences showing the order Planctomycetales and the position of the anammox-affiliated organisms from the Black Sea (indicated by a rectangle). The black triangles indicate phylogenetic groups. The bar represents 10% estimated sequence divergence. Deep-sea sediment clone is from ref. 26; English BC clone EN5 and *Candidatus* 'Scalindua brodae' are from M. Schmid, K. Walsh, R. Webb, W. I. Rijpstra, K. T. van de Pas Schoonen, T. C. J. Hill, B. F. Moffett, J. A. Fuerst, J.S.S.D., J. A. Harris, P. J. Shaw, M.S.M.J. and M. Strauss (unpublished observations).

Methods

Nutrient analyses

Water samples for nutrient analyses were obtained by a pumpcast conductivity–temperature–depth (CTD) system equipped with an oxygen sensor. Before analyses, $ZnCl_2$ was added to the samples from the anoxic part of the water column to precipitate sulphide. Nitrate, nitrite and ammonium concentrations (detection limits 0.1, 0.01 and 0.5 μM, respectively) were determined on board with an autoanalyser, immediately after sampling.

^{15}N incubations and analysis

Black Sea water collected from specific water depths was flushed for 1 h with argon and, after the addition of 500 μM $^{15}NH_4Cl$ and 100 μM $Na^{14}NO_2^-$, incubated for 4 days at *in situ* temperatures (~8 °C). Subsequently, the samples were stored at 4 °C until analysis. $^{14}N^{15}N$:$^{14}N^{14}N$ ratios were determined by gas chromatography–isotope ratio mass spectrometry and expressed as $\delta^{14}N^{15}N$ values ($\delta^{14}N^{15}N = [(^{14}N^{15}N{:}^{14}N^{14}N)_{sample}]$: $[(^{14}N^{15}N{:}^{14}N^{14}N)_{standard}] - 1$; air was used as the standard).

Lipid analysis

Particulate organic matter for lipid analyses was collected from specific water depths by *in situ* filtration of large volumes (~1,000 l) of water through 292-mm diameter precombusted (at 450 °C) glass fibre filters (GFF; nominal pore size 0.7 μm) with *in situ* pumps. Because filtration through 0.7-μm pore-size filters could lead to an undersampling of anammox cells, the calculated ladderane lipid concentrations are minimum values. The GFF were extracted for 24 h in a Soxhlet apparatus to obtain the total lipid extracts. Aliquots of the total extracts were saponified after addition of an internal standard and separated into fatty acid and neutral lipid fractions. The fatty acid fractions were methylated and the neutral fractions were silylated and analysed by gas chromatography–mass spectrometry for the identification and quantification of ladderane lipids. Repeated concentration measurements were within ±10%.

Molecular cloning and phylogeny

DNA extraction, isolation and cloning were performed as described previously[19]. Phylogenetic analysis was performed with the ARB software package[15]. The phylogenetic tree is based on a maximum-likelihood analysis of different data sets.

FISH and microscopy

Filter material was stained with an oligonucleotide probe specific for Planctomycetes (Pla46, S-P-Planc-0046-a-A-18)[20], a newly designed Anammox probe (AmxBS820, S-*-BS-820-a-A-22 (5′-TAATTCCCTCTACTTAGTGCCC-3′)), a eubacterial probe (EUB338, S-D-Bact-0338-a-A-18)[21] and DAPI to determine the abundance of anammox and total bacteria. FISH and DAPI staining were performed as described[22] and the average number of anammox bacteria was determined by analysing 20 different slides.

Flux calculations

Nitrate and ammonium fluxes and ammonium oxidation rates were calculated from the concentration profiles and a vertical diffusion coefficient (K_z) with the program Profile[23]. Published estimates of the vertical diffusion coefficient for the suboxic zone vary over an order of magnitude (0.02–0.7 $cm^2\ s^{-1}$)[8,24,25]. However, most calculations of chemical fluxes[24,25] have used a K_z value close to the lower end of the range. Accordingly, a K_z of 0.04 $cm^2\ s^{-1}$ was used here. The model predicted zones of net ammonium and nitrate consumption at 106–93 and 88–94 m, respectively.

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Correspondence and requests for materials should be addressed to M.M.M.K. (e-mail: mkuypers@mpi-bremen.de).

Catastrophic ape decline in western equatorial Africa

Peter D. Walsh*, Kate A. Abernethy†‡, Magdalena Bermejo§, Rene Beyers‖, Pauwel De Wachter¶, Marc Ella Akou¶, Bas Huijbregts¶, Daniel Idiata Mambounga#, Andre Kamdem Toham¶, Annelisa M. Kilbourn‖, Sally A. Lahm☆, Stefanie Latour‖, Fiona Maisels‖, Christian Mbina‖, Yves Mihindou‖, Sosthène Ndong Obiang#, Ernestine Ntsame Effa#, Malcolm P. Starkey‖††, Paul Telfer†‡‡, Marc Thibault¶, Caroline E. G. Tutin†‡, Lee J. T. White‖ & David S. Wilkie‖**

** Department of Ecology and Evolutionary Biology, Guyot Hall, Princeton, New Jersey 08540, USA*
† Centre International de Recherches Médicales, BP 769, Franceville, Gabon
‡ Department of Biological and Molecular Sciences, University of Stirling, Stirling FK9 4LA, UK
§ Departamento Biología Animal (Vertebrados), Facultad de Biología, Universidad de Barcelona, Avda. Diagonal 645, 08028 Barcelona, Spain
‖ Wildlife Conservation Society, Bronx, New York, New York 10460-1099, USA
¶ WWF Central Africa Regional Program Office, BP 9144, Libreville, Gabon
 # Ministère de l'Economie Forestière, des Eaux, de la Pêche chargé de l'Environnement et de la Protection de la Nature, Direction de la Faune et de la Chasse, BP 1128, Libreville, Gabon
☆ Institut de Recherche en Ecologie Tropicale, BP 13354, Libreville, Gabon
*** Institute of Cell, Animal and Population Biology, Edinburgh University, Edinburgh EH9 3JT, UK*
†† Department of Geography, University of Cambridge, Downing Place, Cambridge CB2 3EN, UK
‡‡ New York University, Department of Anthropology, 25 Waverly Place, New York, New York 10003, USA

Because rapidly expanding human populations have devastated gorilla (*Gorilla gorilla*) and common chimpanzee (*Pan troglodytes*) habitats in East and West Africa, the relatively intact forests of western equatorial Africa have been viewed as the last stronghold of African apes[1]. Gabon and the Republic of Congo alone are thought to hold roughly 80% of the world's gorillas[2] and most of the common chimpanzees[1]. Here we present survey results conservatively indicating that ape populations in Gabon declined by more than half between 1983 and 2000. The primary cause of the decline in ape numbers during this period was commercial hunting, facilitated by the rapid expansion of