The Gulf of Ambracia's Common Bottlenose Dolphins, Tursiops truncatus: A Highly Dense and yet Threatened Population

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Bottled(nose) dolphins *Tursiops truncatus* in the Gulf of Ambracia, western Greece

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Abstract

The bottlenose dolphin is the only cetacean present in the semi-closed waters of the Gulf of Ambracia, western Greece. This increasingly degraded coastal ecosystem hosts one of the highest observed densities in the Mediterranean for this species. Photo-identification data and tissue samples collected through skin-swabbing and remote biopsy sampling techniques during boat-based surveys conducted between 2006-2015 in the Gulf, were used to examine bottlenose dolphin abundance, population trends, site fidelity, genetic differentiation and toxicological status. Bottlenose dolphins showed high levels of year-round site fidelity throughout the 10-year study period. Dolphin population estimates mostly fell between 130 and 170 with CVs averaging about 10%; a trend in population size over the 10 years was a decline of 1.6% per year but this was not significant. A clear genetic differentiation between the bottlenose dolphins of the Gulf and their conspecifics from neighboring populations was detected and low genetic diversity was also found. In addition, pesticides where identified as factors posing a real toxicological problem for local bottlenose dolphins. Therefore, in the Gulf of Ambracia, high dolphin density does not seem to be indicative of favorable conservation status or pristine habitat. On the contrary, Ambracian dolphins are probably facing a high risk of extinction.

Key words: Bottlenose dolphin, *Tursiops truncatus*, photo-identification, site-fidelity, abundance estimate, mark-recapture, pollution, genetics, Gulf of Ambracia, Amvrakikos.

Introduction

The common bottlenose dolphin *Tursiops truncatus*, hereafter bottlenose dolphin, is the most common cetacean on the continental shelf of the Mediterranean Sea, where its distribution appears to be scattered and fragmented into small units (Notarbartolo di Sciara, 2002). Despite a worldwide distribution and their capability of moving across long distances, bottlenose dolphins show a marked population genetic structure throughout their range (Natoli et al., 2004; Tezanos-Pinto et al., 2009). Coastal bottlenose dolphin populations in particular are often resident and prone to population fragmentation sometimes exhibiting strong population differentiation even across a small geographic scale (Natoli et al., 2005; Richards et al., 2013; Mirimin et al., 2011). Identifying those population units and assessing their boundaries is crucial to be able to implement effective conservation measures to protect small resident populations and ensure the survival of this species across its range.

In 2006, the International Union for Conservation of Nature (IUCN) Red List Authority and the Agreement on the Conservation of Cetaceans in the Black Sea, Mediterranean Sea and contiguous Atlantic area (ACCOBAMS) agreed to classify the Mediterranean sub-population of bottlenose dolphins as 'Vulnerable' according to the IUCN Red List criteria and based on a suspected population decline of at least 30% over the last 60 years (Reeves and Notarbartolo di Sciara, 2006). Intentional kills and extermination campaigns conducted until at least the early 1960s in portions of the basin and recent and on-going incidental mortality in fishing gear (i.e.,by-catch) are considered to be among the main factors contributing to such decline, including also other ongoing threats derived from overfishing and from generalized habitat degradation caused by contamination and disturbance from marine traffic (Bearzi et al., 2008b). The bottlenose dolphin is listed in the Appendix II of the Convention on the Conservation of Migratory Species of Wild Animals (CMS), in the Appendix II (Strictly Protected Fauna Species) of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention), and in the Annexes II and IV of the EU Habitats Directive (Council Directive 92/43/EEC).

Greek waters still harbor a remarkable richness of cetacean fauna compared to the rest of the Mediterranean. And yet, such richness is progressively eroding due to rapidly growing degradation of the marine environment (Notarbartolo di Sciara and Bearzi, 2010). The bottlenose dolphin is the cetacean species that is most commonly found in Greek coastal waters and the second most abundant species after the striped dolphins *Stenella coeruleoalba* (Frantzis, 2009). Cetaceans living in coastal areas, and particularly in semi-closed inshore habitats, such as the Gulf of Ambracia in western Greece, are exposed to risks deriving from a variety of anthropogenic impacts and are especially vulnerable because they often have restricted geographic ranges, disjoined distributions and limited movements (Reeves et al., 2003). In this increasingly degraded Gulf, where the bottlenose dolphin is the only cetacean present at an average density of 0.37 animals km^2, one of the highest observed densities in the Mediterranean for this species (Bearzi et al., 2008a), dolphins may be suffering significant physiological stress by anthropogenic activities (Gonzalvo et al., 2015). This study focuses in the conservation status of bottlenose dolphins in the Gulf of Ambracia (Fig. 1) by examining their abundance, population trends, site fidelity, toxicological status and genetic differentiation.

Materials and Methods

Study Area

The Gulf of Ambracia, also referred to as Amvrakikos Gulf, is a shallow, semi-closed embayment of 405 km^2 whose only communication with the open Ionian Sea is through the Preveza Channel, a narrow (minimum width of 370m) and shallow (2–12 m deep) 3 km-long corridor (Fig. 2). On average, the depth of the Gulf is approximately 30 m (maximum 60 m deep), and its bottom mostly consists of mud or sand (Ferentinos et al., 2010). It is characterized by its abundant wildlife and, in addition to providing key habitat for bottlenose dolphins (Bearzi et al., 2008a; Gonzalvo et al., 2015), it is an important foraging place for the endangered loggerhead sea turtle *Caretta caretta* (Rees et al., 2013) and a breeding site for the also critically endangered Dalmatian pelican *Pelecanus crispus* (Catsadorakis et al., 2015). Its northern side, a complex ecosystem, is composed of a double delta from the rivers Arachthos and Louros and their associated marshes and lagoons are of particular importance for bird diversity. The Gulf of Ambracia is partially included in Natura 2000 and Ramsar sites and is protected by national, European and international regulations. In 2008 it was designated as a ‘National Park’ in accordance with the Greek national legislation (11989/08 KYA).

Due to its isolated character the water quality is strongly influenced by man-made processes; input of organic matter and pollutants comes from various sources (Friligos et al., 1997; Karras et al., 2007; Kountoura and Zacharias, 2013; Tsangaris et al., 2010). Rivers Louros and Arachthos are the main pathways bringing agricultural runoff (Tsabaris et al., 2015; Tsangaris et al., 2010). The use of Dichlorodiphenyltrichloroethane group (DDTs), an organochlorine known for its insecticidal properties, as well as of other xenobiotic compounds such as polychlorinated biphenyls (PCBs) and hexachlorobenzene (HCB), was banned by the Stockholm Convention in 2001 because of their environmental impacts. Nevertheless, in the Gulf of Ambracia, high levels of these compounds have been detected in eggs of Dalmatian pelicans as well as in eels *Anguila anguila* (Albanis et al., 1995), mussels *Mytilus galloprovincialis* (Tsangaris et al., 2010) and fishes (Hatzianestis et al., 2001). Although the use of these chemicals reached its peak between the decades of the 40s and 50s, and continued until the late 70s, nowadays they can still be considered priority contaminants in toxicological studies. Hence, they are often referred as legacy contaminants, which persist in the environment, bioaccumulate through the food web, and pose a risk to human health and the environment.

Fish farms, agriculture, livestock and discharges of domestic sewage from coastal towns and villages further contribute to the nutrient enrichment of the Ambracian waters (Ferentinos et al., 2010; Gonzalvo et al., 2014), which are rather murky and highly eutrophic, with Secchi disk readings often as small as 2 m (Bearzi et al., 2008a). The western part of the Gulf is seasonally hypoxic while the eastern part is seasonally anoxic (Kountoura and Zacharias, 2013). The local active fishing fleet totals about 360 boats and is composed exclusively of small-scale fishing boats working primarily with set nets (i.e. trammel and gill nets), targeting mainly small pelagic/epipelagic fish and shrimp *Penaeus kerathurus* (Gonzalvo et al., 2014).

Boat-based survey data collection

Boat surveys were conducted between 2006 and 2015 from a 5.80 m long inflatable boat with fiberglass keel (Novamarine RH-580) powered by a 100 HP four-stroke outboard engine, based on predefined routes (i.e., transects) designed to guarantee a uniform effort coverage of the whole Gulf of Ambracia on a monthly basis. Survey conditions were considered as “positive” under daylight and good visibility, sea state ≤3 Beaufort (large wavelets, crests beginning to break and scattered whitecaps) and with, at least, two observers scanning the sea surface looking for dolphins. When spotted, dolphin groups were approached at low speed, progressively converging with their routes and avoiding sudden changes of speed and directionality to minimize potential disturbance. As proposed by Mann (2000), when observing a dolphin group both dolphin activity and distance among individuals was considered; hence, a group was defined as 'that composed of dolphins within approximately 100 m radius of each other (Irvine et al., 1981) that were moreover observed in apparent association, moving in the same direction and often, but not always, engaged in the same activity (Shane, 1990)'. Group size estimates and composition where recorded. The latter included four age categories (i.e., new-born, calf, juvenile and adult) based on visual assessment of sizes in relation to average adult size (Bearzi et al., 1997).

Photo-identification

At each dolphin sighting, photo-identification effort was dedicated to obtain as many good images as possible of every individual present in our focal group throughout the duration of the observation, using digital Canon SLR cameras equipped with Canon EF 70–200 mm f/2.8L USM zoom lens, avoiding bias toward any particular individuals. Photo-identification was consistently based on long-term natural marks such as notches and nicks in the dolphins' dorsal fins (Würsig and Würsig, 1979; Würsig and Jefferson, 1990; Wilson et al., 1997), as well as on any additional mark in other parts of the body.

Photos were then cropped around the dorsal fin and visible part of the body. Selection of photographs followed recommendations provided by Read et al. (2003); therefore, it was exclusively based on photographic quality using consistent criteria (i.e. entire dorsal fin visible, fin perpendicular to camera, high sharpness and resolution, no water spray or other interference masking fin profile). The best images of every dolphin during each sighting were selected and compared with a catalogue of identified individuals, where dolphins were classified, depending on their degree of distinctiveness, into one of the following four categories: D1 - highly marked (i.e. very distinctive); D2 - marked; D3 - moderately marked; and D4 - slightly marked (poorly distinguishable). When a match was not found, the individual was given a unique identification code and added to the catalogue. The matching procedure was done twice by two different experienced researchers, working independently and using exactly the same protocol to minimize the number of matching errors. Identifications and details relating to dolphin group/sighting membership were recorded on a database to construct individual sighting histories. In most cases, calves were recognized in the field primarily based on their regular association with an identifiable adult dolphin (i.e. mother); therefore, only calves and juveniles with distinctive marks were included in the analysis.

Estimation of population size

Population size was estimated within each year separately using the multi-sample closed population models available in CAPTURE, run within software MARK (Otis, et al., 1978; http://www.phidot.org/software/mark/). The assumptions of closed models are well known and discussed in, for example, Hammond (2009). All closed population models assume that no births, death, permanent immigration or emigration occur within the study period. In addition, there are three fundamental assumptions related to the data: marks are unique; marks cannot be lost; and all marks are correctly recorded and reported. Other assumptions that relate to variation in capture probability depend on the particular mark-recapture model used. Failure to meet these assumptions can lead to bias in estimates of population size (Hammond, 2009, 2010). In this case, the assumptions were mostly addressed as described by Bearzi et al. (2008), who produced the first population estimate for bottlenose dolphins living in the Gulf of Ambracia based on photo-identification effort conducted between 2002 and 2005.

Capture histories for each individual bottlenose dolphin identified in the Gulf of Ambracia were constructed for each year using a calendar month as a sampling occasion. A time frame of one month was considered a good compromise between three factors: having a reasonable number of sampling occasions per year; maximising capture probability within each capture occasion; and ensuring that the population mixes between sampling occasions (Genov et al., 2008; Hammond, 2010). For the years 2006-2014, five sampling occasions (i.e., May-September) were chosen for each year. In 2015, the season was shorter resulting in only four sampling occasions (i.e., June-September).

The models available in program CAPTURE are: M_0, no variation in capture probability; M_t, variation in capture probability among sampling occasions (Darroch, 1958); M_h, variation in capture probability among individuals (Burnham and Overton, 1978); and M_b, variation in capture probability as a behavioral response to first capture (Otis et al., 1978). Models M_{th} (Chao et al. 1992), M_{tb} (Pollock, 1975) and M_{bh} (Norris and Pollock, 1996) combine these effects. In analysis, program CAPTURE was first used to determine the most appropriate of these models for each data subset D1+D2 and D1+D2+D3 in each year according to the model section criteria based on the goodness of fit tests conducted. Behavioral response models are not generally considered appropriate in a photo-identification study because there is no reason why dolphins should react differently following first capture. Nevertheless, they were included here to investigate whether they were selected as the most appropriate in any year. In addition, a consistent set of models was run on all years (M_t, M_h, M_{th}) with the aim of constructing consistent time series to investigate trends in population size over time.

Mark–recapture estimates refer to the population of animals that have appropriate marks, as defined above. To derive an estimate of the total population, the estimated number of marked individuals must be divided by the proportion of marked animals in the population (e.g., Wilson et al., 1999).

The proportion of marked individuals (θ) was estimated for each year as the mean of the proportion of "marked" and "unmarked" individuals in each encounter for the D1+D2 and D1+D2+D3 data subsets. The variance of estimated total population size was estimated as:

$$var(\widehat{N}_{total}) = \widehat{N}_{total}^{2}\left(\frac{var(\widehat{N})}{\widehat{N}^{2}} + \frac{var(\theta)}{\theta^{2}}\right)$$

where N is the number of marked individuals in the population and N_{total} is total population size (Urian et al., 2015) .

In analysis, two data subsets were considered. The first was restricted to the most distinctively marked individuals (D1+D2). The second also included moderately marked individuals (D1+D2+D3). Including moderately distinct animals maximizes sample size but may cause positive bias because of missed matches between individuals. Using only the most distinct animals minimizes the possibility of bias resulting from missed matches but the reduced sample size will result in poorer precision. Slightly marked individuals (D4) were not considered at all in analysis. The estimate of the number of marked animals in the D1+D2 data subset is expected to be smaller than in the D1+D2+D3 data subset. However, the proportion of marked animals in the population is will also be smaller for D1+D2 subset than the D1+D2+D3 subsets and, theoretically, estimates of the total population from both analyses should be the same. Consideration of both sets of estimates allowed the trade-off between bias and precision to be investigated.

Tissue sampling (skin swabbing for genetics and biopsy sampling for toxicology)

Remote biopsy sampling of free-ranging cetaceans is an inexpensive tool used to collect valuable data for conservation and management. Analyses of skin and blubber samples have provided information on population genetics, hormone levels, and contaminant loads (Gorgone et al., 2008). Between 2001 and 2007, 20 skin samples were collected from bow-riding dolphins with the use of a sterilized nylon scrub pad affixed to the round end of a wooden pole through "skin swabbing"; a method, adapted from Harlin et al. (1999), that provides samples suitable for amplification and sequencing of mitochondrial DNA. Other skin samples were also opportunistically obtained from four dead individuals found stranded or floating adrift.

In July 2013 a 1-week biopsy sampling campaign was conducted using standard procedures and equipment (Gorgone et al., 2008; Kiszka et al., 2010). Skin and blubber tissue samples were collected using a crossbow (Barnett Panzer V, 68 kg draw weight) and custom made bolts and tips (Ceta Dart, Copenhagen, Denmark). Tips were 25 mm in length and 7 mm in internal diameter. A high-pressure molded stopper prevented the bolt from penetrating more than about 20 mm forcing it to bounce back. The dorso-lateral area below the dorsal fin was the body region aimed at. Sampling was only attempted on adult dolphins. No individuals accompanied by offspring (or in groups in which offspring is present) were sampled. In case of doubt a precautionary approach was applied (i.e., no sampling attempted). Continuous photo-identification effort allowed identifying 15 of the 16 sampled animals.

Genetic analysis

A total of 74 bottlenose dolphin samples from 5 different populations were included in this study (Fig. 2). From the 24 skin samples of bottlenose dolphins from the Gulf of Ambracia (GA), only 19 were viable (Table 1). Those nineteen dolphins were sequenced for the first time, and compared with previously analyzed samples from the Western Mediterranean Sea (WM, n = 13), Adriatic Sea (ADR, n=20), Inner Ionian Sea Archipelago (IISA, n=9) and the Aegean Sea (AEG, n=13) (Natoli et al., 2005; Viaud-Martinez et al., 2008). The methodology followed for tissue preservation, DNA isolation and the sequencing of the first 442 base pairs (bp) of the mitochondrial DNA (mtDNA) control region was followed as described in Viaud-Martinez et al. 2008.

Sequence alignment was performed using 650 bp of mtDNA obtained from previously published studies collected from dolphins in the ADR (n =16), IISA (n = 9) (Natoli et al. 2005), and 442 bp of mtDNA from dolphins in the ADR (n = 4) and AEG (n = 13) (Viaud-Martinez et al. 2008). These sequences were aligned with new dolphin sequences obtained from the Gulf of Ambracia (n = 19). Sequence alignment was performed using Sequencher 3.1 (Gene Codes Corporation). Final haplotypes were reported for the 442 bp shared between these populations.

Genetic diversity within dolphin populations was measured using haplotype diversity (H_e), which is equivalent to expected heterozygosity after adjustment to population size, and nucleotide diversity (π; Nei, 1987) using Arlequin v.3.5.1.2 (Excoffier and Lischer, 2010). Analysis of molecular variance (AMOVA) was used to estimate the degree of subdivision among the 5 dolphin populations. The null hypothesis of no genetic divergence between the 5 populations was tested using Contingency or Exact Test in Arlequin v.3.5.1.2 (Excoffier and Lischer, 2010); this test has previously been demonstrated to be a robust measurement for identifying population boundaries (Waples and Gaggiotti, 2006).

Genetic differentiation among pairwise population was inferred as F_{ST} (Weir and Cockerham, 1984), Tajima's D and Fu's Fs estimates for each population were calculated with Arlequin v.3.5.1.2 (Excoffier and Lischer, 2010). Haplotype genealogy was reconstructed using Haplotype Viewer (Ewing, 2010) using maximum parsimony (Phylip v.3.69; Felsenstein, 1989), where the size of each node (circle) represents the frequency of a haplotype and the length of the links (or number of black nodes) represents the genetic divergence among haplotypes.

Toxicology analysis

HCB, DDTs and PCBs were measured in subcutaneous blubber of 14 bottlenose dolphins according to the EPA (Environmental Protection Agency) method 8081/8082 with modifications (Marsili and Focardi, 1997). The samples (about 0.1 g) were lyophilized in an Edwards freeze drier for 2 days and extracted with n-hexane for gas chromatography in a Soxhlet apparatus (Marsili et al., 2014). Capillary gas-chromatography revealed HCB, op'- and pp'- isomers of DDT and its derivatives DDD and DDE, and 30 PCB congeners. Total PCBs were quantified as the sum of individual congeners (IUPAC no. 95, 101, 99,151, 144, 135, 149, 118, 146, 153, 141, 138, 178, 187, 183, 128, 174, 177, 156, 171, 202, 172, 180, 199, 170, 196, 201, 195, 194, 206; Ballschmiter and Zell, 1980), while total DDTs were calculated as the sum of pp'DDT, op'DDT, pp'DDE, op'DDE, pp'DDD and op'DDD. The detection limit was 0.1 ng/kg (ppt) for all the OC analysed. The extracted organic matter (EOM%) from freeze-dried samples has been evaluated in all samples.

Data were processed using Statistica 5.0 (Statsoft). Precise information on the distribution of values was obtained by the Shapiro–Wilks W (significance level: $p<0.05$), a normality test. Most of the variables were not normally distributed; so non-parametric tests should be used. However, do to the small number of samples no subsequent statistical elaboration was possible to evaluate differences between groups.

Results

Boat-based survey

Across 10 years of research in the Gulf of Ambracia, with 74 months spent in the field and a total of 770 daily surveys, more than 13000 km of survey effort were conducted under positive conditions, resulting in 631 bottlenose dolphin sightings and almost a thousand hours spent with dolphins (table 2). In years 2006 and 2007 monthly surveys were also conducted in the Ionian Sea open waters to look for possible sightings of bottlenose dolphins in the vicinities of the mouth of the Gulf, where a total of 667 km of survey effort under favorable conditions produced only one sighting in which 15 bottlenose dolphins were photo-identified; none of them had (or has been) ever found inside the Gulf.

Photo-identification

A total of 185 dolphins were identified. The rate at which new individuals were photo-identified during the 10-year study period is shown in Figure 3. This discovery curve rose sharply in 2006, coinciding with the start of the photo-identification work, and then increased more slowly followed by an asymptotic pattern from 2007 onwards. The progressive flattening of the discovery curve and the high site-fidelity shown by the dolphins (Fig. 4), indicate that the population was effectively geographically closed (i.e. confined to the Gulf of Ambracia) during breeding (sampling) seasons during the period of the study.

Population size and trend

The data subsets D1+D2 and D1+D2+D3 included 108 and 148 individuals, respectively. The population estimates produced for both subsets are shown in Table 3. The model selected as most appropriate varied from year to year. Model M_{tb} was selected in 2009 for both data subsets but the estimates were considerably larger than any other and were very imprecise; they were therefore considered unreliable and excluded from further consideration. Estimates for the D1+D2+D3 data subset were larger and slightly more precise than for the D1+D2 data subset in each year, as expected because they include additional less distinct animals and therefore larger sample sizes. The D1+D2+D3 data subset estimates were, however, more variable among years.

Estimates for each year did not vary greatly among models. Model M_0 was selected as the most appropriate in three of the 10 years for each data subset; in each case the estimates were the same as for model Mt. Models including heterogeneity of capture probabilities, M_h and M_{th}, were selected as the most appropriate in four of the 10 years for each data subset. Estimates from these models were almost always larger than from model M_t, which is indicative of heterogeneity of capture probabilities in the data causing a negative bias if not accounted for. Estimates from model M_{th} were usually the least precise but CVs were generally less than 0.1.

All things considered, the series of estimates from model M_{th} are identified as the most robust. These estimates from the same model are much more consistent over the 10 year study period than estimates chosen as most appropriate by CAPTURE. They are also likely to be the least biased estimates because they allow capture probability to vary by both time and individual.

Table 4 shows these estimates from model M_{th} corrected for the estimated proportion of marked animals in the population for each year and each data subset. The estimated proportion of marked animals in the population is larger in each year for data subset D1+D2+D3 than for data subset D1+D2, as expected. The estimated proportions are quite consistent for data subset D1+D2 (0.432-0.645) and highly consistent for data subset D1+D2+D3 (0.636-0.772). Estimates of total population size for each data subsets are very consistent in most years, differing by less than 10% except in 2007 (D1+D2 higher) and 2013 (D1+D2+D3 higher). Thus, there is no evidence of any bias caused by including moderately distinctive animals in analysis. In addition, estimates of total population size for data subset D1+D2+D3 are consistently more precise than those from the D1+D2 data subset (mean CV of 0.096 for D1+D2, compared to 0.118 for D1+D2+D3).

The best series of population estimates is therefore considered to be the one using the M_{th} model applied to the D1+D2+D3 data subset (Fig. 5). A regression of log total population size generated an annual decline of 1.64% (SE=1.54%; 95% CI = -5.04% - 1.89%) but this was not significant (p=0.31).

Genetics

The analysis of 74 sequences for 442 bp identified 16 unique haplotypes characterized by 22 polymorphic sites (Table 5; Genbank accession in progress). The Gulf of Ambracia population (GA) was characterized by only two haplotypes and one of those was observed in 18/19 individuals. Both haplotypes were unique to the GA population. GA dolphins did not share any haplotype with the other 4 populations. The haplotype network identifies a highly represented haplotype among all the populations except for the GA and IISA populations (Fig. 6). The two unique haplotypes that characterize the GA population were one and five mutation steps away from a haplotype observed in the ADR and AEG populations, respectively.

Both haplotype and nucleotide diversities were lower for the GA population ($H_e = 0.11$, $\pi = 0.003$) when compared to all the other populations ($0.74 \leq H_e \leq 0.92$, and $0.009 \leq \pi \leq 0.0016$). Tajima's D were not significant for all the populations except for GA (Tajima's D = -2.273, p value = 0.001), whereas Fu's values were all not significant including for the GA population.

The analysis of population differentiation resulted highly significant (AMOVA F_{ST} = 0.375, df = 73, overall $p < 0.001$). Exact Tests of haplotype distributions supported significant differences between the 5 populations. The null hypothesis of no genetic divergence was rejected ($p < 0.001$). Genetic differentiation among pairwise populations is reported in Table 6. Significant genetic differentiation was observed among all populations, except between the ADR and AEG population. The GA population showed the highest F_{ST} values when compared with all the other populations analyzed.

Toxicology

The OCs levels found in the subcutaneous blubber of the 14 sampled bottlenose dolphins, reported as dry weight and in lipid weight values (d.w. and l.w., respectively), together with the lipid percent (EOM%), water percent (H_2O%) and information on their sex and sexual maturity, are shown in Table 7. The sex of the individuals was established genetically. Males of at least 10 years of age and 5-10 years old females were considered sexually mature (Wells and Scott, 1999). The age of the dolphins was estimated based on their photo-identification stories. For instance, a dolphin recorded as a new-born in September 2006, when first identified, was considered to be 7 years old when sampled in July 2013. The individual AMV12 was genetically recognized as a male but, since it was not photo-identified during the sampling, his age could not be determined.

To make comparisons between the different xenobiotic levels detected in each single individual and within the various interest groups (gender and/or sexual maturity), the results were considered on the basis of lipid content. From the analyzed OCs, HCB was the compound with the lowest levels and with relatively similar values among individuals (Table 7). Large differences were detected in the levels of DDTs and PCBs between individual blubber samples, which ranged from 7.6 µg/g l.w. to 399.7 µg/g l.w. and from 3.0 µg/g l.w. to 108.7 µg/g l.w., respectively. DDTs values were higher for all the analyzed animals, except for dolphin AMV04, who had almost the same levels of the two xenobiotics, and for AMV11, who had three times more PCBs than the DDTs (Fig. 7).

The ratio between DDTs and PCBs ($\Sigma DDT/\Sigma PCB$) has been used for characterizing the magnitude of the contributions from agricultural and industrial sources to marine mammal contamination (Aguilar et al., 1999), because generally it is higher in water masses closer to agricultural areas and lower in waters closer to industrialized areas. In the Ambracian dolphins, the ΣDDT/ΣPCB ratio varied between 0.352 and 7.463, with a mean value of 2.43. A typical technical DDT is composed of pp'DDT (77.1%), op'DDT (14.9%), pp'DDD (0.3%), op'DDD (0.1%), pp'DDE (4.0%), op'DDE (0.1%) and unidentified compounds (3.5%) (WHO, 1979) and presents a pp'DDE/pp'DDT ratio of 0.05. Therefore, if the ratio pp'DDE/pp'DDT has high values, it can be deduced that the majority of the active substance (pp'DDT) has been degraded to pp'DDE, which means that no recent entries of insecticide occurred (Aguilar, 1984). In all bottlenose dolphins, pp'DDE/pp'DDT ratio was always higher than 10, reaching a maximum value of 147 in blubber of AMV6. pp'DDE/DDTs ratio, as well as having a similar meaning to the pp'DDE/pp'DDT ratio, can also indicate the efficiency of the metabolic processes of a population (Borrell and Aguilar, 1987). In fact, the ppDDE/DDTs ratio indicates the relative abundance of metabolized forms of DDT; with values from 0.81 to 0.96.

Sexually mature males (i.e., adults) had the higher levels of all xenobiotics, than immature dolphins of the same sex (Table 8). This difference was particularly high in the case of DDTs and was observed independently of the group in which male dolphin AMV12, whose sexual maturity was considered uncertain, was included. Higher levels of xenobiotics where also detected in females, when sexually mature. Mean value of DDTs was higher in males than in the females. Contrarily, females had higher HCB and PCBs mean values.

Discussion

Site-fidelity

Bottlenose dolphins in the Gulf of Ambracia showed high levels of year-round site fidelity throughout the 10-year study period, which is consistent with previous findings (Bearzi et al., 2008). Dolphin groups encountered in the southwest portion of the Gulf, in areas closer to the Preveza channel, were followed for periods of up to several hours, but they never entered the narrow and shallow corridor leading to open seawaters. Three individuals firstly photo-identified in the Gulf of Ambracia and regularly observed between 2003-2008, were subsequently found in the Inner Ionian Sea archipelago and in the Gulf of Corinth (Bearzi et al., 2010), not to be seen ever again in Ambracian waters (Gonzalvo, unpublished data; Fig. 4). Based on photographs of their genital area, all three animals were males. This is consistent with the hypothesis that males are more wide-ranging than females, and they may therefore be the primary vectors of genetic exchange (Wells et al. 1987; Bearzi et al. 1997). Such observations indicate some small degree of emigration, but no immigration into the Gulf has been recorded so far. The observed extent of occurrence of the population, defined as the area contained within the shortest continuous imaginary boundary which can be drawn to encompass all the known, inferred or projected sites of present occurrence of a taxon, excluding cases of vagrancy, was about three quarters of the total area of the Gulf (i.e., approx. 300 km^2, excluding enclosed marshes and lagoons), falling within the range of values required (< 5000 km^2) for classification as Endangered under criterion B1 of the IUCN Red List (IUCN, 2012).

Population size and trend

The approach taken to analyze the photo-identification data to estimate population size was to use closed population models applied to monthly (during summer) sampling occasions within years rather than open population models applied to annual sampling occasions. Open models are best suited for estimation of survival rate and tend to be less robust than closed models for estimating population size; in particular, they are unable to take account of heterogeneity of capture probabilities (Hammond 2009; 2010), which was shown to be influential in our case. An alternative could be to use the robust design analytical framework, which would combine the use of closed models based on monthly sampling occasions to estimate population size within years with open models based on annual data to estimate survival rate. Temporary emigration/immigration rates between years can also be estimated and this could be interesting to investigate here. However, robust design models are data-hungry and the small number of animals in this population may limit the utility of this approach.

Estimates of the number of marked individuals from the closed population models were variable from year to year if the model selected as the most appropriate was used in each year. There was much more consistency when a single model was used for each year. Models accounting for heterogeneity of capture probabilities among individuals consistently gave larger annual estimates than models without heterogeneity, indicating that even when these models were not chosen there was still likely some heterogeneity in the data. Thus, we feel confident that choosing the time series of estimates from a single model with heterogeneity is justified as the best approach in this study. A similar approach was taken in a study of northern Norwegian killer whales (Kuningas et al. 2014).

Analysing data subsets with and without moderately distinct individuals to explore the trade-off between bias and precision in estimates showed that including moderately distinct individuals increased precision, as expected because of larger sample size, but found no evidence that including moderately distinct individuals caused bias. Thus, our estimates based on the D1+D2+D3 data subset were the best estimates.

These population estimates over the 10-year study mostly fell between 130 and 170 with CVs averaging about 10%. Confidence that the true size of the population lies within this range is therefore high. The estimated trend in population size over the 10 years was a decline of 1.6% per year but this was not significant. A simple power analysis (Gerrodette, 1987) indicates that the power of the data to detect a rate of decline of this magnitude is only around 30%. For the power to increase to the typically accepted desirable level of 80%, there would need to be an additional five years of survey with estimates at the same precision to detect a decline of 1.6% per year.

A population size estimated to number fewer than 250 mature individuals can be classified as Endangered under criterion D (IUCN, 2012). Our most robust estimates of the total number of bottlenose dolphins in the Gulf of Ambracia never exceeded 170 individuals and even the widest 95% confidence interval did not exceed 250.

Genetics

Despite our relatively small sample sizes, our results indicate that the dolphins of the Gulf of Ambracia are genetically differentiated from the surrounding Mediterranean populations. Furthermore, the genetic diversity observed for the GA population was the lowest when compared with the rest of populations analyzed in this study and with previous studies found in the literature (Curry, 1997; Natoli et al., 2005; Viaud-Martinez et al., 2008) which suggests that the GA population is highly isolated.

Samples used in this study were obtained by different agencies through a variety of means and over the course of several years. In the case of GA samples (Table 1), whenever a sample was taken, the research boat abandoned that particular dolphin group and headed to a different side of the Gulf in search for other specimens, to minimize the possibility of samples taken the same day representing closely related individuals. Bottlenose dolphins from the neighboring IISA area, which belong to an even smaller resident population, despite their smaller sample size, exhibited a higher genetic diversity. Nevertheless, relatedness among GA samples cannot be ruled out.

The genetic uniqueness of the dolphins of the Gulf of Ambracia, as shown by low mtDNA diversity and the presence of unique haplotypes, demonstrates low connectivity with neighboring Adriatic Sea and Ionian Sea and could be explained by historical events, and/or low gene flow among populations. Additionally, the fact that the Tajima’s D estimate was negative and significant for the GA population indicates that the population may have undergone a recent reduction, however Fu’s Fs was not in support of this scenario. To estimate both historical and contemporary connectivity between the GA and the neighboring populations, as well as understanding the ancestry of the GA population, the analysis of a larger sample set and the use of nuclear marker would be required.

Toxicology

Free-ranging bottlenose dolphins sampled in the Gulf of Ambracia, were considered to be in good nutritional status (no signs of emaciation) and were frequently engaged in surface-feeding. Nevertheless, lipid content in their subcutaneous blubber (represented by EOM%) resulted in 28.6%. This value is much lower than what was reported in bottlenose dolphins of the Sea of Cortez (n = 11; 43.5%), the Gibraltar Strait (N = 3; 48.9%), the Strait of Sicily (n = 4; 60.8%), and a single specimen from the Ionian Sea with 74.0%, but it was similar to EOM% from specimens of the Croatian Adriatic Sea (n = 14; 24.5%) (Marsili, unpublished data; Marsili et al., 1996). Variables such as nutritional status, reproductive status, ontogeny, environmental temperature changes and pathologies have been observed to affect blubber composition (Wells and Scott, 1999; Struntz et al., 2004; Durkin et al., 2005). Although some of those variables may have contributed to the variation in lipid content (range 14.0-54.7), a mean value as low as that detected in the present study is indication of a generalized poor health condition.

With respect to the dolphin’s toxicological status, it must be also considered that when lipids mobilize from the blubber, lipophylic contaminants redistribute leading to higher tissue concentrations. Consequently, specimens with a low EOM%, may have a higher risk of suffering exposure-related adverse health effects (Yordy et al., 2010). Generally in waters near industrialized areas ΣDDT/ΣPCB ratio is <1 and in waters close to agricultural areas is >1. Hence, our results indicate that contamination in the Gulf has predominantly an agricultural origin. This is in accordance with other studies, reporting a predominance of DDTs rather than PCBs, conducted in this part of the Mediterranean (Marsili and Focardi, 1996; Marsili, 2000; Fossi et al., 2004; Marsili et al., 2014). Values of pp’DDE/pp’DDT ratio >0.05 for all sampled animals showed that DDT contamination was not recent. Moreover, based on the values obtained of the pp'DDE/DDTs ratio, varying from 0.81 to 0.96, bottlenose dolphins of the Gulf of Ambracia manifested a very high metabolism of this pesticide.

Gender and sexual maturity are important variables when assessing dolphins’ toxicological status. Females lose up to 90% of their total body burden of OCs during pregnancy and lactation (Borrell and Aguilar, 2005; Tanabe et al., 1982).

In addition, the milk of these marine mammals contains very high levels of fats, mostly triglycerides; the milk of striped dolphin, for instance, contains 258 mg/g of triglycerides in 280 mg/g total fats (Kawai and Fukushima, 1981). Consequently, males accumulate persistent organic pollutants for their entire life, increasing in contamination levels with age, while females do not. The mean value of DDTs found in bottlenose dolphins of the Gulf of Ambracia was higher in males than in females, but contrarily to what we expected, the exact opposite was found for HCB and PCBs. Three sexually mature females had levels of average HCB, DDTs and PCBs higher than younger immature females. However, when looking at their personal history (i.e., health, nutritional status, pregnancies/lactation), only female AMV15 (firstly identified in July 2003 as an adult) had been observed with offspring; one in 2005 and another one in 2008 (both currently alive according to our photo-identification records). In fact this female had very low OC levels, contrarily to the other two, which were never observed in association with offspring and are unlikely to have reproduced.

Anthropogenic pollution may have important consequences in dolphin population dynamics (Garcia-Alvarez et al., 2014). Most studies on the toxicological status from OCs in bottlenose dolphins in the Mediterranean Sea were exclusively based on stranded animals (Marsili and Focardi, 1997; Romanic et al., 2014; Shoham-Frider et al., 2009; Storelli et al., 2007; Storelli and Marcotrigiano, 2003; Wafo et al., 2005). A recent review paper by Jepson et al. (2016), using samples from both stranded and free-living biopsied animals, reported high mean ∑PCB concentrations (> 100.0 mg/kg l.w.) in bottlenose dolphins from western Mediterranean and northern Adriatic Sea. The present study is one of the few works conducted in the Mediterranean Sea based uniquely in free-ranging bottlenose dolphins and provides unprecedented information on this matter for the Gulf. OCs levels found in bottlenose dolphins resulted very similar to those reported by Fossi et al. (2003) for the same species in the neighboring waters of the Ionian Sea for HCB and PCBs while for DDTs were 4 times higher, which indicates that the latter legacy pesticide poses a real toxicological problem for the dolphins of the Gulf of Ambracia.

Conclusions

Our findings indicate that bottlenose dolphins in the Gulf Ambracia constitute a geographically and otherwise distinct group with little demographic exchange that are exposed to high levels of pollution, mostly derived from local agriculture (i.e., pesticides). Based on photographic mark-recapture estimates, 134 animals (CV = 0.11) resided in the

Gulf in 2015. By applying standard criteria provided by the IUCN Red List of Threatened Species we conclude that this ‘subpopulation’ would qualify as Endangered according to Red List criteria. While local density of dolphins is among the highest recorded anywhere in the Mediterranean Sea, this is not indicative of favorable conservation status or pristine habitat. On the contrary, these dolphins face a high risk of extinction due to their likely reproductive isolation, small population size and small extent of occurrence, as well as to acute and growing anthropogenic impacts in their semi-closed shallow habitat. Management of human pressures is an obvious way of reducing such a risk, consistent with national and regional commitments to protect this coastal area and cetaceans generally.

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Table 1. Detail on skin samples of bottlenose dolphins taken in the Gulf of Ambracia used for genetic analysis; 20 obtained by skin swabbing from bow-riding specimens and 4 from dead animals.

Sample code	Date D-M-YYYY	Time hh:mm	Sampling Method	Notes	Haplotype
1	4-8-2004	11:26	skin swabbing	No Identified	IV
2	4-8-2004	11:40	skin swabbing	Identified as 3022	IV
3	4-8-2004	11:50	skin swabbing	No Identified	Failed sequencing
4	6-8-2004	10:42	skin swabbing	No Identified	Failed sequencing
5	6-8-2004	11:21	skin swabbing	No Identified	XXXXII
6	6-8-2004	13:09	skin swabbing	No Identified	Failed sequencing
7	9-8-2004	11:32	skin swabbing	Identified as 3036	Failed sequencing
8	15-8-2004	9:40	skin swabbing	No Identified	Failed sequencing
9	14-8-2006	9:40	skin swabbing	No Identified	IV
10	14-8-2006	11:50	skin swabbing	No Identified	IV
11	8-9-2006	10:33	skin swabbing	No Identified	IV
12	8-9-2006	11:11	skin swabbing	No Identified	IV
13	8-9-2006	11:22	skin swabbing	No Identified	IV
14	15-9-2006	10:40	skin swabbing	No Identified	IV
15	28-9-2006	19:05	dead stranded	No Identified (Juvenile, male, length=210 cm)	IV
16	20-6-2007	19:15	dead stranded	No Identified	IV
17	26-6-2007	8:52	skin swabbing	No Identified	IV
18	26-6-2007	9:04	skin swabbing	No Identified	IV
19	27-6-2007	11:38	skin swabbing	No Identified	IV
20	4-7-2007	11:47	dead floating adrift	Male new born; length about 100 cm (mother identified as 03046)	IV
21	19-8-2007	11:01	skin swabbing	No Identified	IV
22	19-8-2007	11:19	skin swabbing	No Identified	IV
23	27-8-2007	10:45	skin swabbing	No Identified	IV
24	8-9-2009	14:40	dead stranded	No Identified (Adult, male, length=270 cm)	IV

Table 2: Summary of research effort conducted in the Gulf of Ambracia (2006-2015). Framed dataset refers exclusively to the number of sightings and photo-Id effort used for the population estimates.

Year	Research calendar (Months at sea)	Days at sea	Km survey "positive"	Total Sightings	Time with dolphins	**Dataset used for Mark-Recapture Population estimates** (Sightings on transect between May-September)		
						Total Sightings	**Photos taken**	**Photos selected**
2006	Apr-Dec *(9)*	107	1630	70	99 h 32 min	38	5872	2770
2007	Year-round *(12)*	136	2776	101	139 h 45 min	25	4674	2656
2008	Year-round *(12)*	105	1648	77	110 h 52 min	25	2466	1561
2009	May-Oct *(6)*	82	1310	76	105 h 50 min	25	5478	2241
2010	Apr-Oct *(7)*	88	1234	80	97 h 36 min	29	5478	2241
2011	Apr-Sep *(6)*	43	785	35	46 h 56 min	23	4765	2045
2012	Apr-Sep *(6)*	55	656	50	75 h 19 min	37	8873	3296
2013	Apr-Sep *(6)*	60	667	55	85 h 17 min	37	8216	2992
2014	Apr-Sep *(6)*	56	707	50	77 h 30 min	33	9695	3287
2015	Jun-Sep *(4)*	38	412	37	54 h 10min	30	5462	2674
Total	74 months	770	13303	631	992 h 47 min	**302**	**60979**	**25763**

Table 3: Mark-recapture population estimates of the number of marked individuals generated by different models for data subsets D1+D2 (top) and D1+D2+D3 (bottom). Estimates from models identified by CAPTURE as the most appropriate are shown in bold. The model identified as most appropriate in 2008 for data subset D1+D2+D3 was undefined. Shaded columns correspond to the results from M_t, M_h, and M_{th} models run for each year.

								D1-D2							
Year	**M(tb)**	SE	*CV*	**M(0)**	SE	*CV*	**M(t)**	SE	*CV*	**M(h)**	SE	*CV*	**M(th)**	SE	*CV*
2006							71	2,38	*0,033*	82	5,68	*0,069*	**78**	6,28	*0,081*
2007							**82**	3,58	*0,044*	93	6,33	*0,068*	85	7,07	*0,083*
2008				**74**	3,52	*0,048*	74	3,24	*0,044*	86	6,86	*0,080*	83	8,14	*0,098*
2009	**126**	244,63	*1,942*				78	5,25	*0,067*	101	11,48	*0,114*	98	15,12	*0,154*
2010				**66**	3,40	*0,051*	66	3,14	*0,048*	76	6,13	*0,081*	73	7,27	*0,100*
2011	**66**	4,05	*0,061*				66	2,54	*0,038*	75	5,40	*0,072*	73	6,24	*0,085*
2012							72	1,34	*0,019*	**78**	4,50	*0,058*	75	3,62	*0,048*
2013							73	2,20	*0,030*	80	9,80	*0,123*	**72**	2,91	*0,040*
2014							63	2,07	*0,033*	**69**	5,03	*0,073*	63	3,79	*0,060*
2015				**66**	3,23	*0,050*	66	3,03	*0,050*	77	6,66	*0,090*	80	10,33	*0,130*
								D1-D2-D3							
Year	**M(tb)**	SE	*CV*	**M(0)**	SE	*CV*	**M(t)**	SE	*CV*	**M(h)**	SE	*CV*	**M(th)**	SE	*CV*
2006							93	2,80	*0,030*	105	6,31	*0,060*	**99**	6,62	*0,067*
2007							104	4,21	*0,040*	119	7,67	*0,064*	**111**	8,93	*0,080*
2008							86	3,22	*0,037*	99	6,53	*0,066*	94	7,67	*0,082*
2009	**174**	391,10	*2,248*				94	6,06	*0,064*	114	11,57	*0,101*	107	14,31	*0,134*
2010				**84**	3,92	*0,047*	84	4,83	*0,058*	94	6,33	*0,067*	86	6,90	*0,080*
2011	**100**	15,27	*0,153*				94	3,64	*0,039*	112	9,33	*0,083*	111	9,99	*0,090*
2012							104	3,47	*0,033*	116	5,81	*0,050*	**111**	5,18	*0,047*
2013				**74**	3,03	*0,041*	73	2,81	*0,038*	82	5,53	*0,067*	76	5,45	*0,072*
2014							93	2,443	*0,026*	**103**	5,97	*0,058*	95	4,97	*0,052*
2015				**90**	3,96	*0,044*	90	3,75	*0,040*	100	6,23	*0,060*	95	8,81	*0,090*

Table 4: Estimates of the number of marked individuals from model M_{th}, of the proportion of marked animals in the population, and of total population size for data subsets D1+D2 and D1+D2+D3.

Year	Subset	**CAPTURE estimate**	HM θ	**Corrected estimate**	CV	SE	95%CI
2006	D1+D2	78	0.552	**141**	0.094	13.30	117-170
	D1+D2+D3	99	0.702	**141**	0.086	12.25	119-167
2007	D1+D2	85	0.582	**145**	0.121	17.72	115-185
	D1+D2+D3	111	0.679	**163**	0.107	17.575	132-201
2008	D1+D2	83	0.645	**128**	0.118	15.20	102-161
	D1+D2+D3	94	0.734	**127**	0.094	12.099	106-153
2009	D1+D2	98	0.560	**174**	0.167	29.34	126-242
	D1+D2+D3	107	0.636	**168**	0.145	24.432	126-223
2010	D1+D2	73	0.535	**136**	0.117	15.95	108-171
	D1+D2+D3	86	0.652	**131**	0.102	13.451	107-160
2011	D1+D2	73	0.469	**155**	0.133	20.73	119-201
	D1+D2+D3	111	0.661	**167**	0.110	18.470	135-207
2012	D1+D2	75	0.516	**145**	0.077	11.29	124-169
	D1+D2+D3	111	0.772	**143**	0.054	7.862	129-159
2013	D1+D2	72	0.541	**133**	0.077	10.26	114-154
	D1+D2+D3	76	0.708	**107**	0.080	8.636	91-125
2014	D1+D2	63	0.432	**145**	0.119	17.42	115-184
	D1+D2+D3	95	0.685	**138**	0.067	9.394	121-158
2015	D1+D2	80	0.582	**137**	0.153	21.12	101-185
	D1+D2+D3	95	0.707	**134**	0.110	14.84	110-170

Table 5: Haplotype affiliations across the 5 putative populations. Shared haplotypes are shaded.

		Populations				
#	*Haplotype*	*WM*	*ADR*	*IISA*	*AEG*	*GA*
1	I	2	11		6	
2	IV					18
3	XIV		3			
4	XV		2		3	
5	XVI	6		1		
6	XVII	1				
7	XVIII	2				
8	XIX		2	3	1	
9	XXII			3	2	
10	XXIII	1				
11	XXXVIII				1	
12	XXXXI	1	1			
13	XXXXII					1
14	XXXXIII		1			
15	XXXXIV			1		
16	XXXXV			1		
	n =16	*n = 13*	*n = 20*	*n = 9*	*n = 13*	*n = 19*

Table 6: Genetic differentiation estimated with *F*st based on mtDNA data. Statistical significance: * *F*st > 0 ($p < 0.05$), ** *F*st > 0 ($p \leq 0.001$).

n	*Populations*	*WM*	*ADR*	*IISA*	*GA*	*AEG*
13	*WM*					
20	*ADR*	0.199**				
9	*IISA*	0.150**	0.226**			
19	*GA*	0.600**	0.600**	0.627**		
13	*AEG*	0.172**	-0.005	0.141*	0.612**	

Table 7: Sex and sexual maturity, lipid percent (EOM%), water percent (H_2O%) and OCs levels (i.e., HCB, DDT, PCB) reported as d.w. (and l.w.) for all 14 sampled dolphins.

Dolphin code	**Sex**	**Sexual maturity**	**Photo-Id info** (to estimate minimum age and sexual maturity)	**EOM** %	**H_2O** %	**HCB** ng/g d.w. (l.w.)	**DDTs** ng/g d.w. (l.w.)	**PCBs** ng/g d.w. (l.w.)
AMV01	M	No	Firstly identified in September 2009 as a calf	30.3	55.0	6.45 (21.28)	25182.4 (83110.2)	7344.5 (24239.1)
AMV03	M	Yes	Firstly identified in September 2004 as an adult	29.7	59.3	6.63 (22.32)	2830.0 (9528.7)	1312.6 (4419.6)
AMV04	M	Yes	Firstly identified in June 2010 as an adult	14.0	55.2	5.80 (41.43)	1133.2 (8094.2)	1236.9 (8834.6)
AMV05	F	No	Firstly identified in June 2012 as a juvenile	54.7	51.0	10.02 (18.33)	14249.5 (26050.3)	5628.2 (10289.2)
AMV06	M	Yes	Firstly identified in September 2002 as an adult	28.7	58.9	6.11 (21.28)	14586.3 (50823.4)	5647.4 (19677.4)
AMV07	M	No	Firstly identified in April 2011 as a calf	21.1	56.4	6.21 (29.41)	1593.3 (7551.2)	626.1 (2967.3)
AMV08	F	No	Firstly identified in May 2011 as a juvenile	17.3	54.8	7.54 (43.56)	3072.1 (17757.7)	1348.3 (7793.7)
AMV09	M	Yes	Firstly identified in January 2007 as an adult	37.8	52.5	11.79 (31.20)	11941.1 (31590.1)	7346.5 (19435.2)
AMV10	M	Yes	Firstly identified in July 2003 as an adult	21.2	53.6	16.75 (79.00)	13025.2 (61439.7)	10302.3 (48595.5)
AMV11	F	Yes	Firstly identified in July 2003 as an adult	18.6	58.6	15.12 (81.29)	7123.5 (38298.1)	20218.9 (108704.0)
AMV12	M	?	Unknown; no photo-identified	34.4	55.8	13.97 (40.62)	18066.6 (52519.1)	9859.1 (28660.1)
AMV13	F	Yes	Firstly identified in June 2004 as an adult	22.5	56.6	12.81 (56.92)	16097.3 (71543.4)	6603.3 (29347.9)
AMV14	M	Yes	Firstly identified in July 2001 as an adult	40.3	57.3	20.61 (51.14)	161091.2 (399730.0)	21584.2 (53558.9)
AMV15	F	Yes	Firstly identified in July 2003 as an adult. Two offspring recorded (in 2005 and 2008)	30.0	55.2	12.72 (42.39)	6539.6 (21798.8)	2463.9 (8212.9)

Table 8: Arithmetic mean levels and Standard Deviation (in brackets) of HCB, DDTs and PCBs in the bottlenose dolphins of the Gulf of Ambracia, divided by gender (males and females) and sexual maturity.

	n	**HCB ng/g l.w.**	**DDTs μg/g l.w.**	**PCBs μg/g l.w.**
MALES & FEMALES	14	41.44 (20.19)	62.85 (99.92)	26.77 (28.27)
MALES	9	37.52 (18.70)	78.27 (123.4)	23.38 (18.00)
FEMALES	5	48.50 (23.01)	35.09 (21.78)	32.87 (43.33)
Sexually mature males	6	41.06 (21.80)	93.53 (151.5)	25.75 (20.56)
No-sexually mature males	3	30.44 (9.71)	47.73 (38.01)	18.62 (13.74)
No-sexually mature males *(AMV12 not included)*	2	25.35 (5.75)	45.33 (53.43)	13.60 (15.04)
Sexually mature females	3	60.20 (19.66)	43.88 (25.34)	48.75 (52.98)
No-sexually mature females	2	30.94 (17.84)	21.90 (5.86)	9.04 (17.64)

Figure 1: Common bottlenose dolphins photographed in the Gulf of Ambracia showing the characteristic morphology of the species. Bottom-right image shows an adult bottlenose severely affected by a skin condition firstly reported by Gonzalvo et al. (2015). Photos by J.Gonzalvo/Tethys Research Institute.

Figure 2: (Top) Map of the Gulf of Ambracia. (Bottom) Geographic origin of samples analysed for mtDNA; WM-western Mediterranean, ADR-Adriatic Sea, IISA-Inner Ionian Sea Archipelago, GA-Gulf of Ambracia and AEG-Aegean Sea (numbers indicate the sample size).

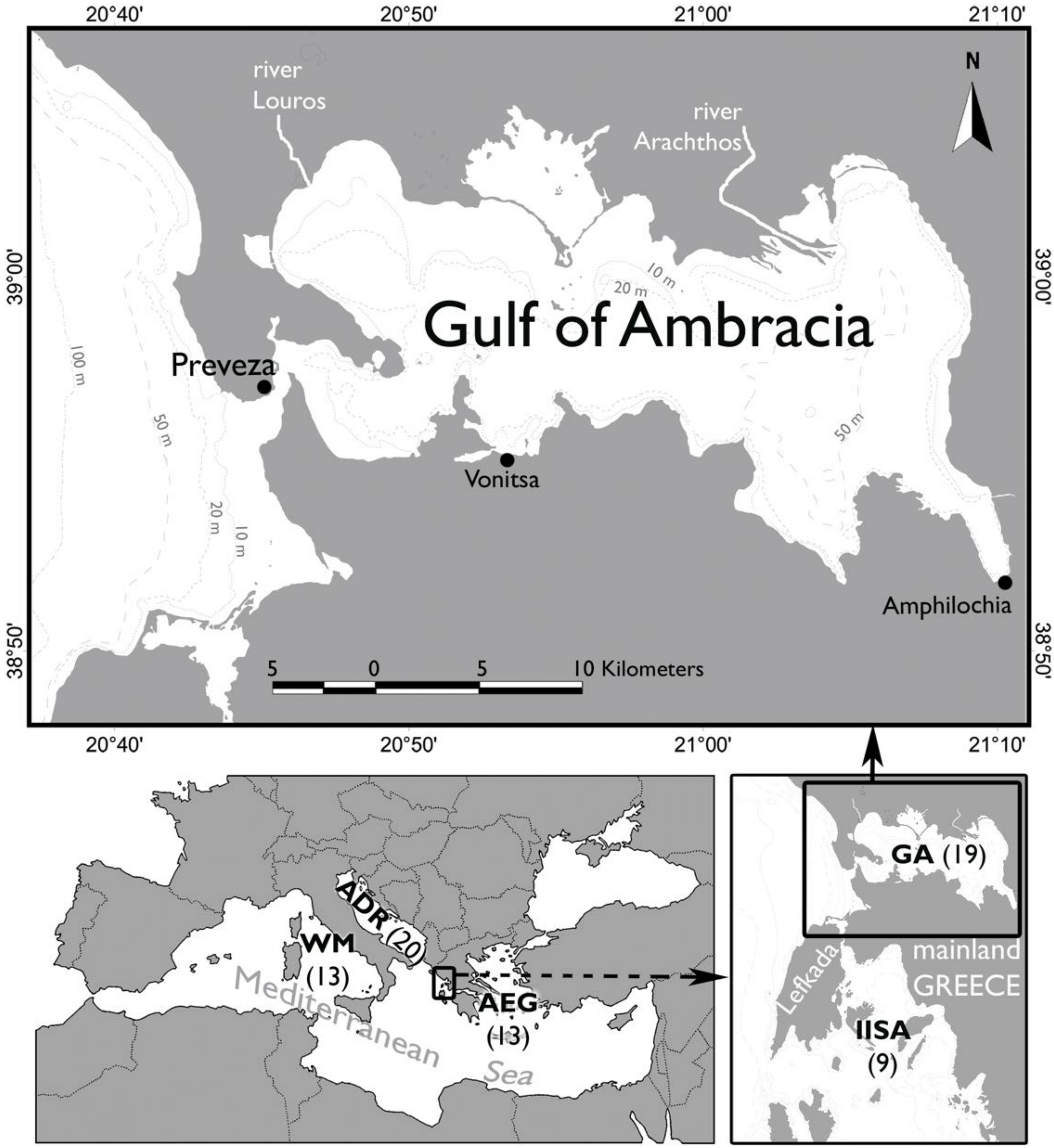
Figure 3: Discovery curves, taking into account the degree of distinctiveness (from D1-highly marked to D4-poorly marked), for individually identified bottlenose dolphins across 2006-2015 in the Gulf of Ambracia.

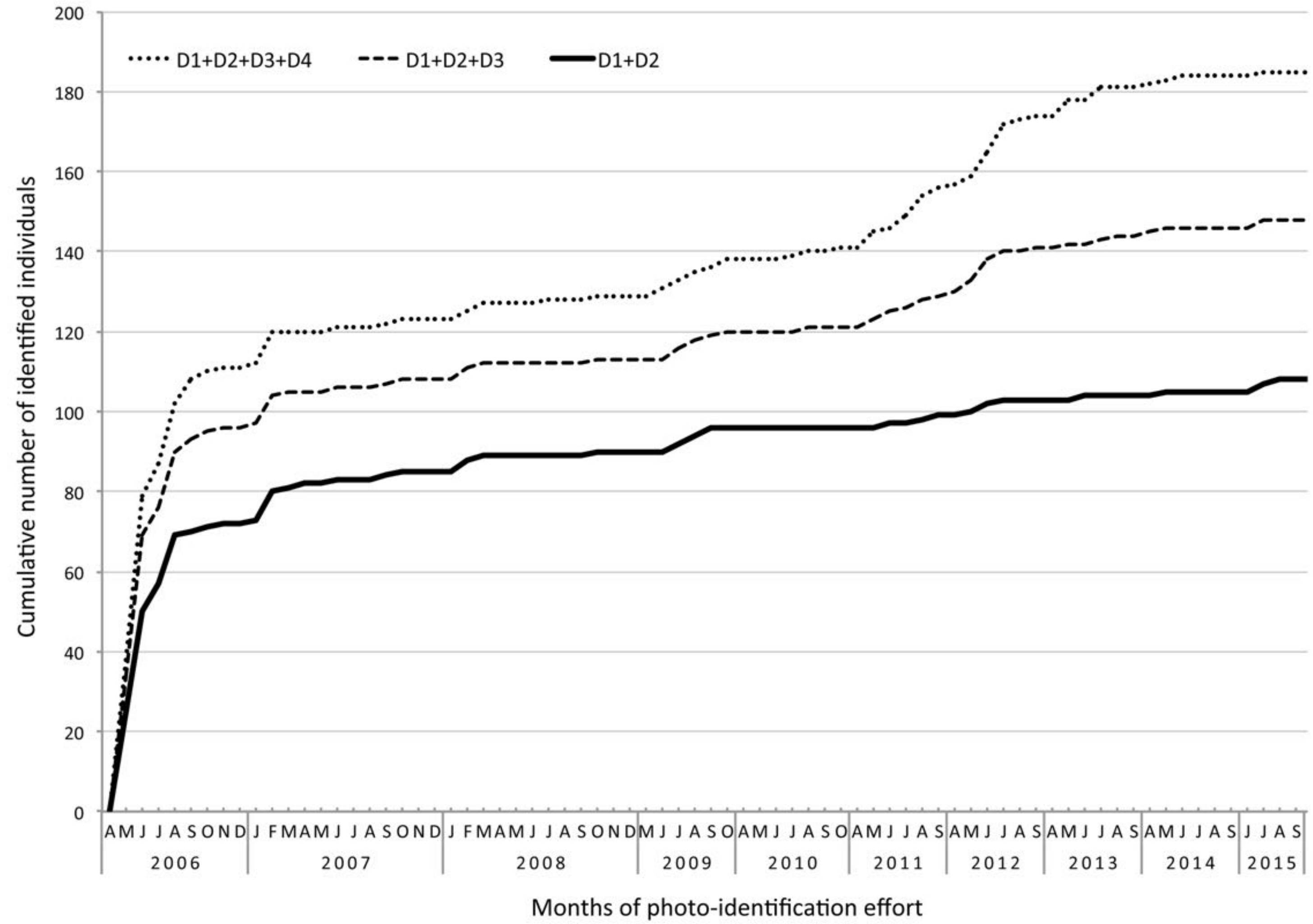
Figure 4: Residency pattern of marked individuals (D1-D2) in the Gulf of Ambracia. Grey cells indicate presence documented through digital photo-identification. Months included in the mark-recapture model are shown with white letters on black background. Three individuals with "**O**" besides their ID-Code were last seen in our study area in 2008; they were identified in June 2010 in the Inner Ionian Sea Archipelago and two of them were spotted in the Gulf of Corinth in August that same year (T1, T2 and T3 in Bearzi et al., 2010).

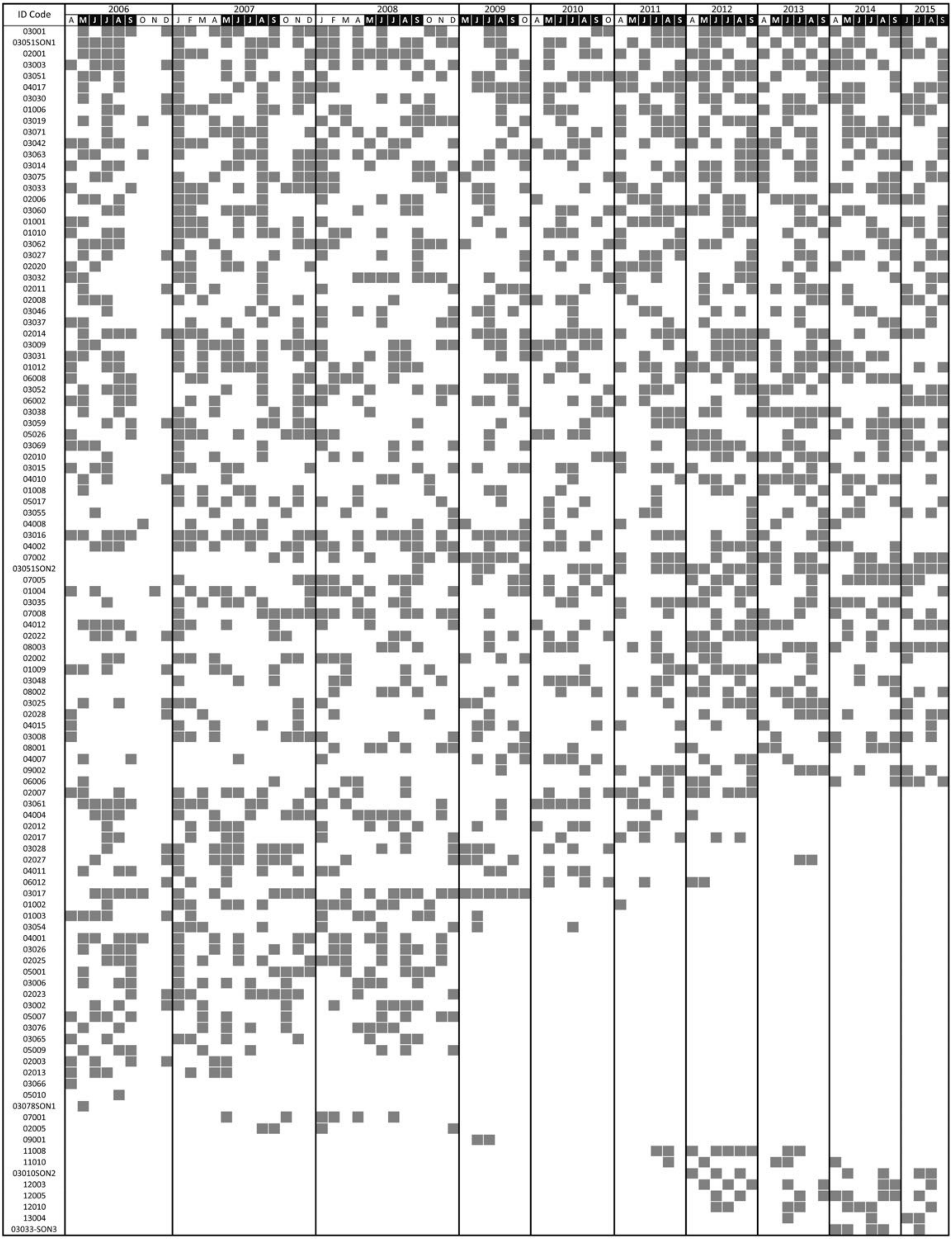
Figure 5: Estimates of the number of identifiable individuals D1+D2+D3 (grey squares) and total population size (black circles) within years using the closed model *Mth* Chao. The fitted regression line shows an exponential decline at a rate of 1.6% (SE=1.5%) per year.

Figure 6: Maximum parsimony network illustrating the relationships among the 74 bottlenose dolphin samples from WM-western Mediterranean, ADR-Adriatic Sea, IISA-Inner Ionian Sea Archipelago, GA-Gulf of Ambracia and AEG-Aegean. Each one of the 16 haplotypes is represented by a circle with a corresponding Roman numeral and number of individuals represented per population.

Figure 7: Plot of DDT and PCB levels (ng/g lipid weight) in the subcutaneous blubber of the single specimens.


20°40'
20°50'
21°00'
21°10'
39°00'
38°50'
N
river Louros
river Arachthos
Gulf of Ambracia
10 m
20 m
50 m
100 m
Preveza
Vonitsa
Amphilochia
5 0 5 10 Kilometers
ADR (20)
WM (13)
AEG (13)
Mediterranean Sea
GA (19)
IISA (9)
Lefkada
mainland GREECE


D1+D2+D3+D4
D1+D2+D3
D1+D2
Cumulative number of identified individuals
0
20
40
60
80
100
120
140
160
180
200
A M J J A S O N D
2006
J F M A M J J A S O N D
2007
J F M A M J J A S O N D
2008
M J J A S O
2009
A M J J A S O
2010
A M J J A S
2011
A M J J A S
2012
A M J J A S
2013
A M J J A S
2014
J J A S
2015
Months of photo-identification effort


ID Code
2006
A M J J A S O N D
2007
J F M A M J J A S O N D
2008
J F M A M J J A S O N D
2009
M J J A S O
2010
A M J J A S O
2011
A M J J A S
2012
A M J J A S
2013
A M J J A S
2014
A M J J A S
2015
J J A S
03001
03051SON1
02001
03003
03051
04017
03030
01006
03019
03071
03042
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03014
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02006
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02011
02008
03046
03037
02014
03009
03031
01012
06008
03052
06002
03038
03059
05026
03069
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04010
01008
05017
03055
04008
03016
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07002
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08002
03025
02028
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03008
08001
04007
09002
06006
02007
03061
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02017
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02027
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06012
03017
01002
01003
03054
04001
03026
02025
05001
03006
02023
03002
05007
03076
03065
05009
02003
02013
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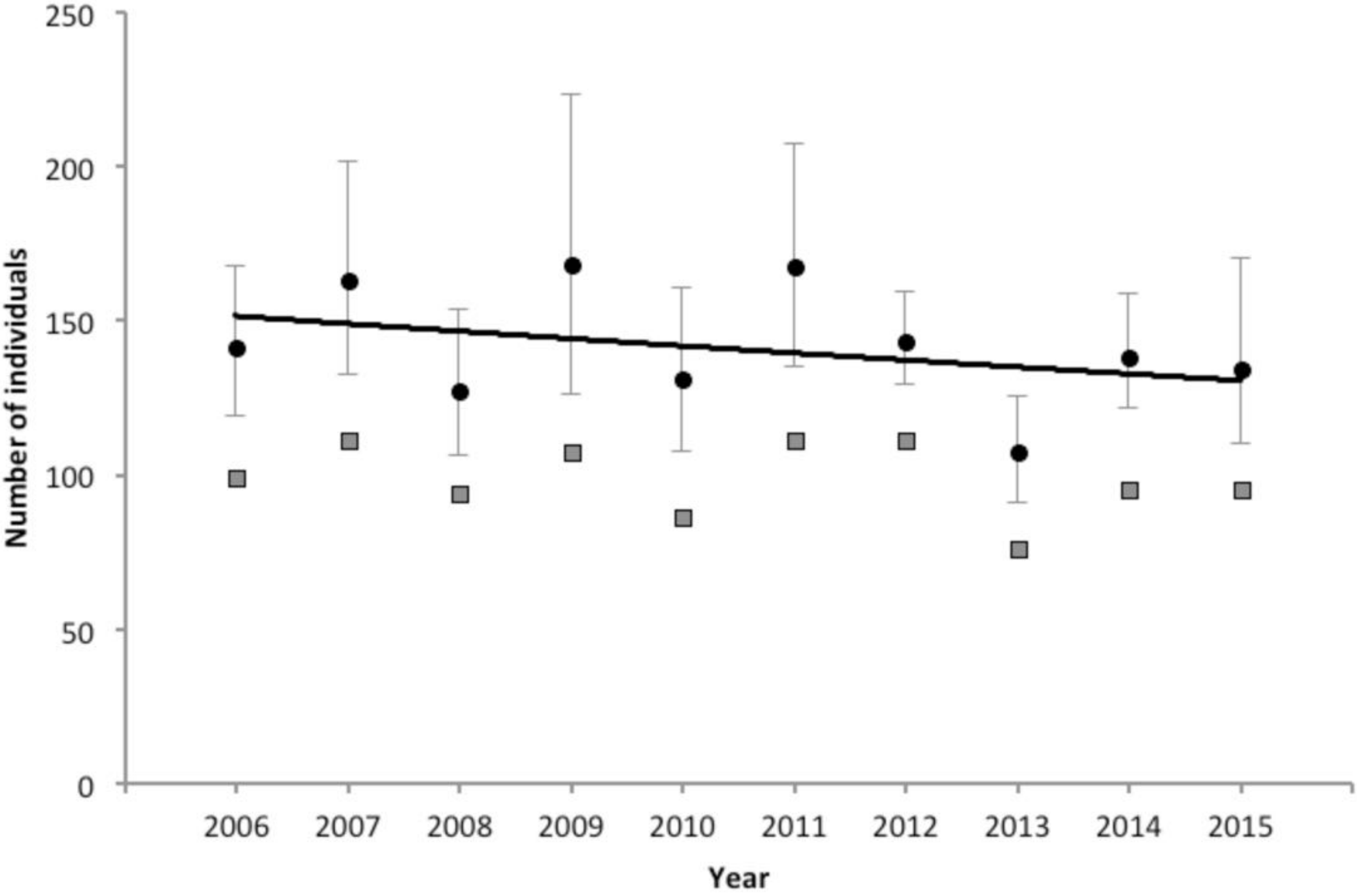
03033-SON3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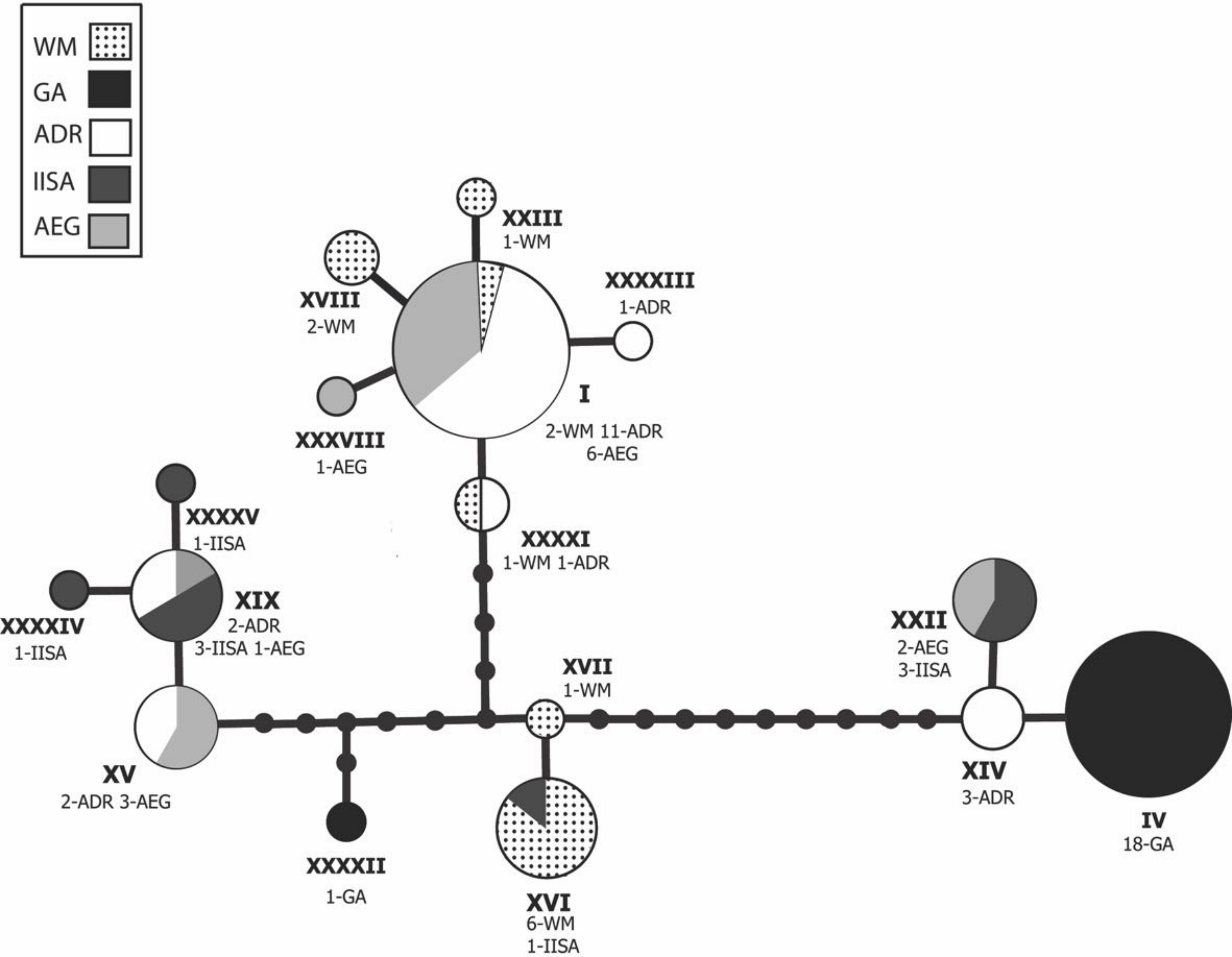
200
150
100
50
0
Number of individuals
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
Year


WM
GA
ADR
IISA
AEG
XXIII
1-WM
XVIII
2-WM
XXXXIII
1-ADR
I
2-WM 11-ADR
6-AEG
XXXVIII
1-AEG
XXXXI
1-WM 1-ADR
XXXXV
1-IISA
XIX
2-ADR
3-IISA 1-AEG
XXXXIV
1-IISA
XVII
1-WM
XXII
2-AEG
3-IISA
XV
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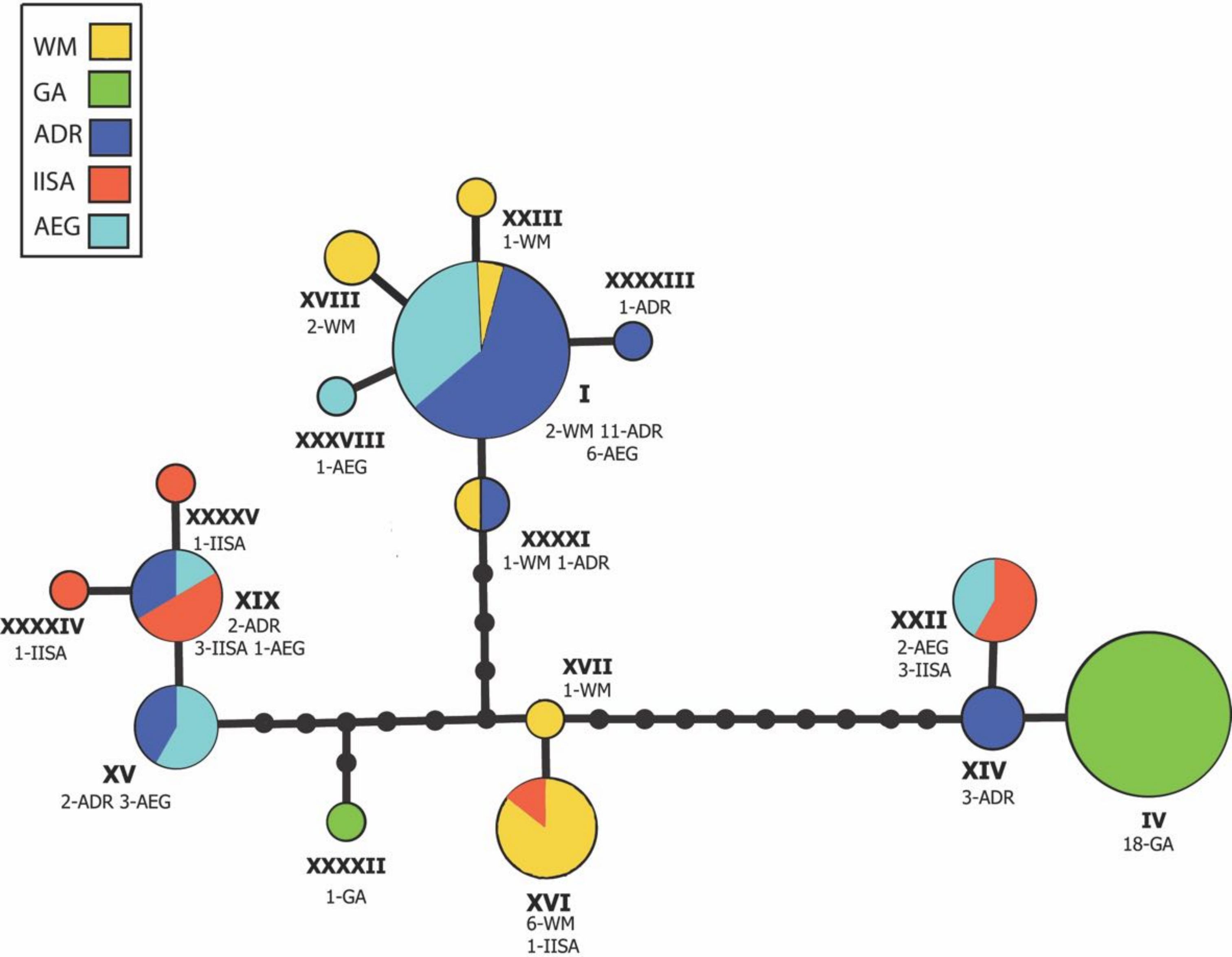
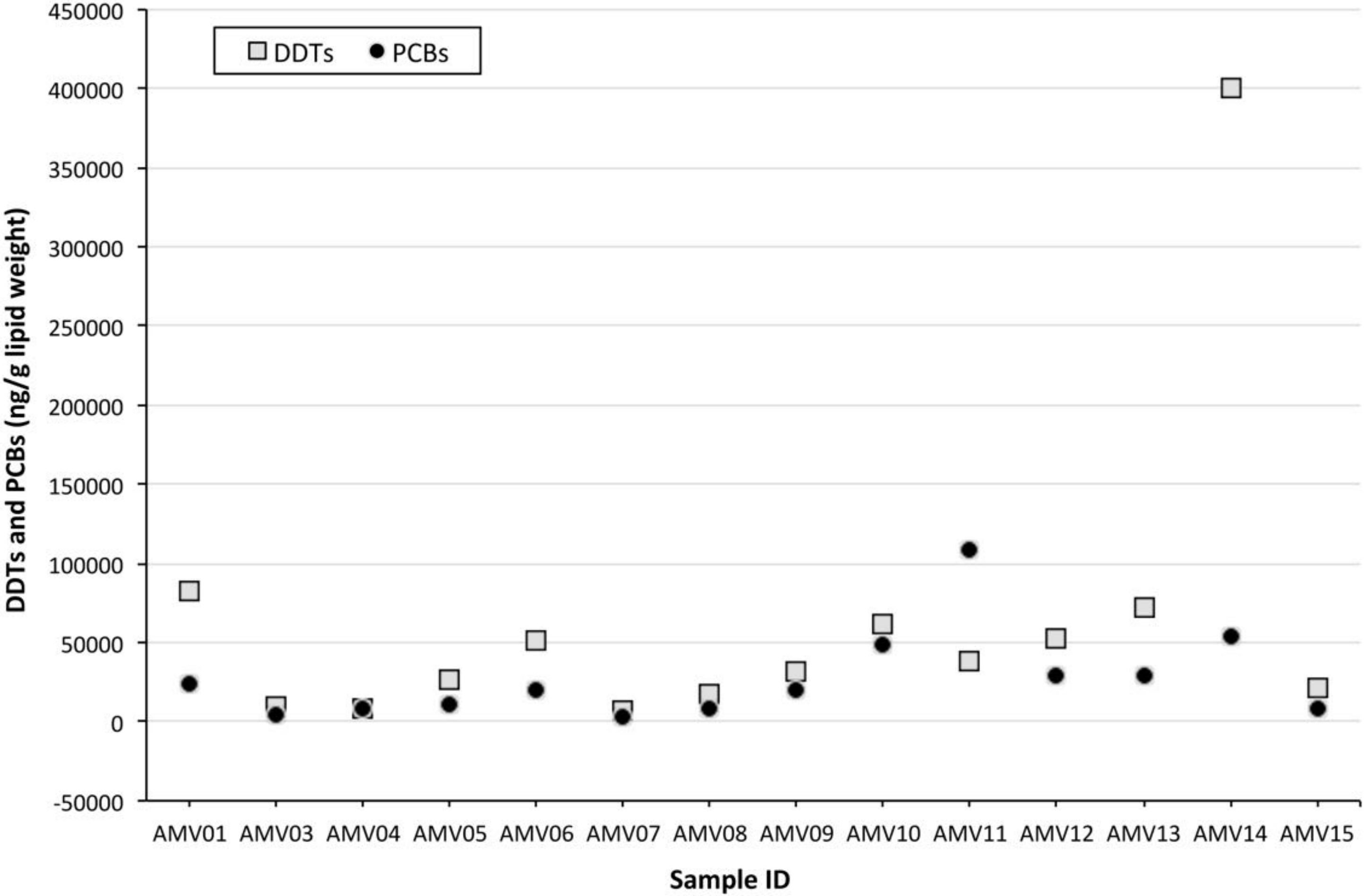
XXXXII
1-GA
XVI
6-WM
1-IISA
XIV
3-ADR
IV
18-GA


WM
GA
ADR
IISA
AEG
XXIII
1-WM
XVIII
2-WM
XXXXIII
1-ADR
I
2-WM 11-ADR
6-AEG
XXXVIII
1-AEG
XXXXV
1-IISA
XXXXI
1-WM 1-ADR
XIX
2-ADR
3-IISA 1-AEG
XXXXIV
1-IISA
XXII
2-AEG
3-IISA
XVII
1-WM
XV
2-ADR 3-AEG
XIV
3-ADR
IV
18-GA
XXXXII
1-GA
XVI
6-WM
1-IISA



☐ DDTs
● PCBs
DDTs and PCBs (ng/g lipid weight)
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400000
350000
300000
250000
200000
150000
100000
50000
0
-50000
AMV01
AMV03
AMV04
AMV05
AMV06
AMV07
AMV08
AMV09
AMV10
AMV11
AMV12
AMV13
AMV14
AMV15
Sample ID