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Pink is the new black:

An investigation of antibiotic resistance in wild birds through use of a novel indicator, the Greater Flamingo

A thesis submitted for the degree of Doctor of Philosophy

September 2013

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Declaration

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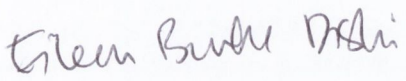


Eileen Burke Diskin

Summary

Antibiotics have revolutionized human health care. Given their importance in treating infectious disease and the explosion in their use worldwide, the latter half of the 20th century is often described, within a medical context, as the era of antibiotics. In addition to their use in human healthcare, antibiotics are used in a variety of other contexts; for example, they have been widely used in an agricultural context to treat and prevent disease and promote growth in livestock. Resulting from the increased use of antibiotics is the emergence of antibiotic resistance – the ability of bacteria to survive exposure to antibiotics. Antibiotic resistance has been described by the World Heath Organization as one of the three greatest threats to human health. Initially recognised as a problem in urban environments, antibiotic resistance has now also been identified in a variety of wildlife species in environment systems worldwide. Wild birds are particularly important to consider as reservoirs (i.e. stores) of antibiotic resistant bacteria given their ability to transfer pathogens from one location to another through both short- and long- distance movements. This thesis uses an integrated approach to investigate wild birds as reservoirs of antibiotic resistant bacteria at several different scales. Through use of a desk-based study, patterns of antibiotic resistant bacteria were evaluated on a global scale, while a fieldwork-based approach was used to investigate patterns of antibiotic resistant bacteria in the Greater Flamingo within individual sites and at a regional scale.

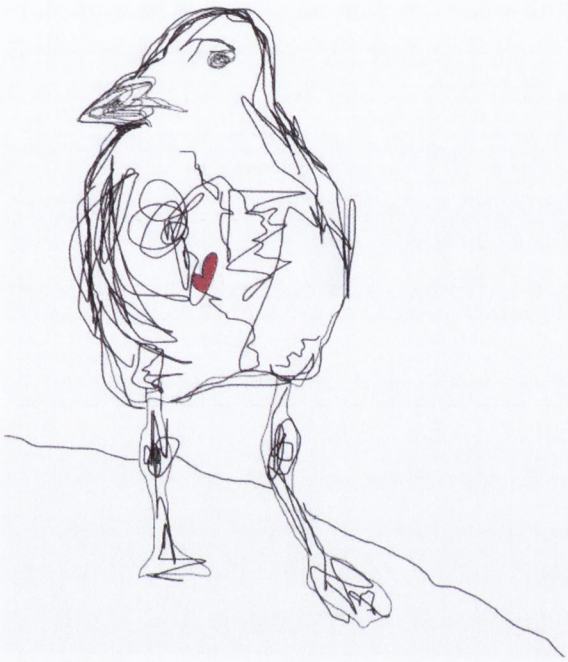
In the first chapter, a systematic review and meta-analysis was undertaken to evaluate antibiotic resistance in wild birds on a global scale, with reference to papers published between 2002 and 2012. To select papers for the meta-analysis, a list of twelve eligibility criteria was developed, over 5000 papers were returned in the initial search; these evaluated for inclusion in the study. After title/abstract and full text reviews, 37 papers were found to be eligible and were included in the meta-analysis. Data were extracted from the selected papers to compile characteristics of each of the studies, and to use in analyzing patterns of resistance. The included papers investigated antibiotic resistance in wild birds at sites worldwide, including Antarctica, Australia, Europe, North America and South America, and used a total of 74 different bird species. Overall, resistance to over twenty antibiotics was identified, typically in *E. coli*, *Salmonella* and/or *Enterococcus* isolates. Resistance to ampicillin, chloramphenicol, and tetracycline were amongst the most frequently observed, and multidrug resistance was widespread. As the first meta-analysis of antibiotic resistant bacteria in wild birds, this investigation offers insights into patterns of antibiotic resistance on a global scale that should be considered in the development of policies that target antibiotic use.

In the following chapter, a fieldwork-based investigation was used to evaluate geographic variation in antibiotic resistance on a regional scale, using the Greater Flamingo as a sentinel.

In addition to assessing the robustness of the Greater Flamingo as a sentinel of antibiotic resistant bacteria, this also provided a preliminary screening of antibiotic resistant bacteria. Cloacal swabs from 350 Greater Flamingos were collected at five of its breeding sites in Spain, France, and Italy during ringing operations in July and August 2010. A subset of these bacterial samples was screened for resistance to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamycin, kanamycin, tetracycline, and sulphamethoxazole/trimethoprim using Kirby-Bauer disc diffusion. Resistance to the eight antibiotics was found in nearly all of the samples, and variation between sites was apparent. This variation was likely due to the different land uses surrounding each of the sites, as determined in similar studies using other wild bird species.

Having validated the Greater Flamingo as a robust sentinel of antibiotic resistance, laboratory analyses were carried out to provide detailed information about the species of bacteria demonstrating resistance. Specific isolates of interest were selected from a subset of the samples used in the preliminary screening. Using broth dilution, the minimum inhibitory concentration (MIC) of these isolates was determined to provide detailed information about the extent of each of the resistances with reference to human health. Resistant isolates were then identified using 16s rDNA sequencing. Resistant isolates were found to include *E. coli* and species within the *Pseudomonas* and *Enterococcus* genera; many of the isolates were resistant at levels that are significant in a human health context. This is in agreement with previous studies, many of which found bacterial resistances to a range of commonly used antibiotics in a variety of wild bird species.

This comprehensive investigation of wild birds as reservoirs of antibiotic resistance at a variety of scales contributes to the growing body of research on antibiotic resistance in environmental systems. A systematic review and meta-analysis has provided the first analysis of patterns of antibiotic resistance in wild birds on a global scale, with consideration of the factors suggested to be driving this resistance. This desk-based analysis was complemented with a field-based study of the Greater Flamingo, which for the first time was used as a sentinel of antibiotic resistance across multiple sites. This provided evidence of a high prevalence of antibiotic resistance at five of its breeding sites in the Mediterranean region that was found to vary geographically. As migratory animals, wild birds – including the Greater Flamingo – are important vectors of infectious disease and need to be considered in epidemiological models of antibiotic resistance. This evidence is particularly relevant today given the recent focus, on both national and international levels, on the development of policies for responsible antibiotic use.



To my family.

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Cheers,
Eileen

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*In addition to photos taken by the author of this thesis, images used in plates 9-14 are from a selection of website sites, including: www.flamingoatlas.org/galeriephoto2010.php; www.picampall.org; amslaurea.unibo.ie/1953/1/Borghesi_ Fabrizio_ tesi.pdf;

1 General introduction

1.1 Chapter overview

This first chapter serves as a general introduction to my thesis. Within each of the subsequent chapters, focused introductory sections provide information specifically relevant to that chapter's particular topic, including: antibiotics and antibiotic resistance in Chapter Two; the use of wild birds as sentinels of antibiotic resistance in Chapter Three; the ecology and life history of the Greater Flamingo in Chapter Four; the bacterial species commonly found in wild birds in Chapter Five; while the introduction to Chapter Six provides a framework within which the general discussion is set. What follows in this chapter is intended to serve as a brief introduction to each of the chapters in order to provide an overview of the context within which this project was undertaken.

1.2 Humans, environment, and infectious disease

> "Not all the winds, and storms, and earthquakes, and seas, and seasons of the world, have done so much to revolutionize the earth as Man, the power of an endless life, has done since the day he came forth upon it, and received dominion over it." (H. Bushnell, quoted in Marsh, 1846)

The interactions between human societies and the environment have been a topic of scientific investigation for well over a century (Marsh, 1846; Vitousek *et al.*, 1997; Grove and Damodaran, 2006). Human modification of Earth's ecosystems is wide-ranging, and the factors governing its impacts are complex (Morse, 1995; McMichael, 2004). Included in the effects of human interactions with the environment are emerging infectious diseases. Human history has been affected, to varying extents, by the occurrence of infectious disease including cholera, tuberculosis, typhoid and influenza, and their links to the environment have long been recognised (Burnet and White, 1972; McMichael, 2004; Morens *et al.*, 2004; Wolfe *et al.*, 2007). An emerging paradigm over the past few decades is the role of anthropogenically-induced environmental change (including, for example, land-use change), which has been implicated as a driving force behind the emergence of various infectious diseases in environmental systems (Burnet and White, 1972; Daszak *et al.*, 2001; Patz *et al.*, 2004; Rhyan and Spraker, 2010; Murray and Daszak, 2013). Where the disease is one that has the potential to be transferred between humans and wildlife (refered to as 'zoonotic'), a threat exists for the disease to adversely affect human health (Daszak *et al.*, 2000; Taylor *et al.*, 2001; Jones *et al.*, 2008). Such zoonotic diseases represent a critical area for research, given estimates that over 60% of emerging diseases affecting human health are zoonotic (Wilcox and Gubler, 2005; Kahn, 2006; Jones *et al.*, 2008).

The transmission of infectious disease between humans and wildlife (in both directions) is often referred to as 'spill over' and 'spill back', these terms describe the phenomenon whereby an infectious disease is transferred from one species (i.e. 'reservoir' or 'maintenance' host) to another (Daszak *et al.*, 2000; Power and Mitchell, 2004; Nugent, 2011; see Quammen, 2013 for an extensive review of historical occurences of spill over). The extent to which this is facilitated by anthropogenic activity is widely acknowledged; in the recently published popular science book 'Spillover', David Quammen asserted, 'Human-caused ecological pressures and disruptions are bringing animal pathogens ever more into contact with human populations, while human technology and behaviour are spreading those pathogens ever more widely and quickly.' (Rhyan and Spraker, 2010; Palmer *et al.*, 2012; Quammen, 2013).

Although the links between human health and the environment have been recognised for centuries (as evidenced by Hippocrates writings), only recently has a framework addressing the overlap between them become widely adopted. The 'One Health' concept (which gained prominence when it was proposed as 'One Medicine' by Calvin Schwabe in 1976) is an initative that recognises these links, in offering a novel framework by which health can be approached via collaborative efforts across disciplines (Zinsstag, 2011). Whereas traditional frameworks have recognised minimal interactions between human, wildlife, and environmental health, the One Health initiataive considers the three as one and is inclusive of a variety of sectors at all geographic scales in efforts including: educational, communication, surveillance and control, research, diagnostic and therapeutic product development, and public awareness (One Health Initiative, 2014). A variety of prominent organisations and institutions have supported, promoted, and/or adopted the One Health approach, including the Centers for Disease Control and Prevention (CDC) in the United States, the Food and Agriculture Organisation (FAO) the European Union, and the World Bank (National Environmental Health Association, 2008; Zinsstag, 2011; The World Bank, 2012)

Perhaps nowhere is the dynamic between humans, animals, and the environment better exemplified than with antibiotic resistance, the prevalence of which has increased notably in the past 50-60 years, causing the World Health Organisation to cite it as one of the three greatest threats to human health (ISDA, 2010).

1.3 Antibiotic resistance

Since the discovery of penicillin in 1928, the use of antibiotics has become widespread (Lewis, 2012). When they were first discovered, antibiotics were heralded as miracle drugs, and their use was predicted to change the course of human healthcare (Alanis, 2005). Indeed, many infections that had previously proven fatal could be treated, and within two decades following the introduction of antibiotics, it was proclaimed that 'the war against infectious disease has

been won' (Spellberg and Taylor-Blake, 2013). Originally used in the treatment of infections and disease in humans, the context(s) within which antibiotics were applied expanded to include their widespread use in agriculture. For example, antibiotics became frequently used in the maintenance of health and in the promotion of growth in livestock (McEwen and Fedorka-Cray, 2002; Martinez, 2009). Their use was not closely regulated, however, and in the decades following their introduction, the use of antibiotics became widespread (Singer *et al.*, 2003; Davies and Davies, 2010). As a result, although antibiotics have allowed for positive advances in both human medicine and agriculture, their increased use has had unintended negative consequences (Gould, 2009).

One of the notable consequences of increased antibiotic usage is an increased prevalence of antibiotic resistance. Antibiotic resistance describes the ability of a microorganism to survive exposure to an antibiotic (World Health Organization, 2002). It is believed that antibiotic resistance is developed by prolonged and/or repeated exposure to a given antibiotic, although cases where resistance has evolved naturally – i.e., in the absence of antibiotics – have also been reported (Levy and Marshall, 2004; Singer *et al.*, 2006; Blanco *et al.*, 2009; Thaller *et al.*, 2010). Given that they undermine the potential success of antibiotics, antibiotic resistant bacteria are of critical concern to human health. To combat antibiotic resistant bacteria, the continued development of novel antibiotics is necessary, given that the proliferation of antibiotic resistant bacteria renders antibiotics obsolete – sometimes within years of an antibiotics' introduction to market (Cirz *et al.*, 2005; Spellberg *et al.*, 2008; Fischbach and Walsh, 2009). The problem has been exacerbated by the fact that, as resistance is increasing, very few new antibiotics have emerged from the so-called 'antibiotics pipeline', due to the coincidence of several factors (widely reviewed in the literature) that contribute to an overall lack of investment within the pharmaceutical industry in the research and development of antibiotics (Clarke, 2003; Leeb, 2004; Alanis, 2005; Morel and Mossialos, 2010; Butler and Cooper, 2011; Cooper and Shales, 2011). Although not ideal, what is expected to persist as an inevitable "cycle of resistance" (as described Christopher by Walsh, cited in Leeb, 2004) is in reality has become increasingly problematic, given that there is "unequivocal evidence that antimicrobial research is on a steep downward slope," (John Edwards, head of policy at the Infecious Diseases Society of America, cited in Clarke, 2003). Described as a 'perfect storm' by Cooper and Shlaes (2011), this has resulted in an increased prevalence of resistance. As early as 1992, the emerging problem of antibiotic resistance was termed a 'crisis' (Neu, 1992), and it is a problem that has not gone away; it has more recently been described as 'catastrophic', 'epidemic', and a 'serious threat' (Sjolund *et al.*, 2008; Allen *et al.*, 2010; Thaller *et al.*, 2010; Spellberg *et al.*, 2013; Wright, 2013).

That the increased occurrence of antibiotic resistance is the result of our use of antibiotics – which have in many ways been so beneficial to human health – is a paradox that is acknowledged within the literature as one of the greatest failings of modern medicine (Levy, 2002). Today, although they continue to save lives, the widespread use of antibiotics in urban environments (both clinical and home use), wastewater treatment processes, and further afield in agricultural contexts, has resulted in the proliferation of antibiotic resistant bacteria, resistant genes, and antibiotic residues in a variety of contexts – all of which have the potential to spread antibiotic resistance (Hirsch *et al.*, 1999; O'Brien *et al.*, 2002; Sévano *et* al., 2002; Kümmerer, 2004; Sarmah *et al.*, 2006; Martinez, 2009).

The role of domestic and production animals in the spread of antibiotic resistance in environmental systems – which serve to provide a link between humans and wildlife species – is important to consider (Daszak *et al.*, 2000; McEwen and Fedorka-Cray, 2002; Guardabassi *et al.*, 2004). Given the role of antibiotic use in driving the spread of antibiotic resistance, it is unsurprising that changes in agricultural practices as they relate to the use of antibiotics in production animals, for example, will affect the transmission of antibiotic resistance by providing robust pathways for their transfer (Karesh *et al.*, 2012; Jones *et al.*, 2013). As a result, antibiotic resistant bacteria have been identified in a variety of environmental systems that serve as 'reservoirs' (i.e. stores) of antibiotic resistance. This is important to consider given numerous pathways that are recognised to provide mechanisms by which resistant bacteria and resistance genes can be transferred to human populations.

1.4 Wildlife reservoirs of antibiotic resistant bacteria

Widely recognised in the dynamic between humans and environmental reservoirs of antibiotic resistance is the importance of wildlife species. As described above, the use of antibiotics in a variety of contexts results in the proliferation of resistant bacteria, resistance genes, and antibiotic residues into environmental systems, which wildlife are exposed to and can acquire. In addition to acting as reservoirs of antibiotic resistant bacteria, wildlife can serve as vectors, providing a mechanism by which resistance can be transferred to humans, with implications for human health. Over the past three decades, antibiotic resistant bacteria have been identified in a variety of wildlife species, including mammals (Routman *et al.*, 1985; Graves *et al.*, 1988; Caprioli *et al.*, 1991; Sherley *et al.*, 2000; Livermore *et al.*, 2000; Österblad *et al.*, 2001; Mallon *et al.*, 2002; Lillehaug *et al.*, 2005; Poeta *et al.*, 2005; Kozak *et al.*, 2008), reptiles (Al Bahry *et al.*, 2009; Foti *et al.*, 2009), amphibians (Cissell, 2006; Slaughter *et al.*, 2010; Rana *et al.*, 2011), and fish (Björklund *et al.*,1990; Miranda and Zemelman, 2001; Blackburn *et* al., 2010; Rose *et* al., 2010; Karki, 2013).

A notably large amount of the evidence of wildlife reservoirs of antibiotic resistant bacteria comes from wild birds (Livermore *et al.*, 2001, Bonnedahl, 2011; Radhouani *et al.*, 2012). This use of birds as indicators (or sentinels) of antibiotic resistance mirrors, to a certain extent, the widespread use of birds in monitoring the effects of enviornmental change in general, including in both pollution and infectious disease research (Camarda *et al.*, 2010). There are several reasons why wild birds are suitable to use in evaluating environmental health within these contexts, including their status at (or near) the top of food webs, the diversity of ecosystems within which they are found across the globe, their relatively large abundance, and the fact that they are, in general, a well-studied taxonomic group (Burger and Gochfield, 2004; Mallory *et al.*, 2010). Further to these justifications, given that most species demonstrate both short- and long- distance movements, the use of wild birds can provide interesting insights into the mechanisms by which pathogens might spread from one environment to another, or perhaps more critically, from an environmental system to human populations (Reed *et al.*, 2003).

1.5 The Greater Flamingo: a candidate sentinel of antibiotic resistance

There are six species of flamingo that comprise the genus *Phoenicopterus*, and of these, the Greater Flamingo (*Phoenicopterus roseus*) is the most widespread, found throughout Southern Europe, Western Asia, and Southern, Eastern, and Northern Africa (BirdLife International, 2012; Geraci *et al.* 2012). Across its range, the Greater Flamingo exists as several metapopulations, one of which – comprising an estimated 300,000 individuals – is located in the Mediterranean region (Balkız *et al.*, 2007; Geraci *et al.*, 2012). Within this region, there is a great diversity in the ecology and the landscape characteristics of areas at which the Greater Flamingo is found (Borghesi *et al.*, 2011). The exact locations and number of breeding sites are driven by climate, and as a result they vary from year to year; in addition to natural wetlands, Greater Flamingos inhabit both active and abandoned saltpans across the Mediterranean region (Paracuellos *et al.*, 2002; Bechet *et al.*, 2012). As a result of the diversity of sites at which it is found, there exists a wide range of factors that affect the conservation status of the Greater Flamingo. Consequently, although it is described by the International Union for Conservation of Nature (IUCN) as a species of 'least concern', the Greater Flamingo remains protected under several conventions; it is listed in Appendix II of the Convention on Trade in Endangered Species (CITES) and in Appendix A of Commission Regulation (EU) No. 709/2010 (on the 'protection of species of wild fauna and flora by regulating trade therein'). As a charismatic species, many aspects of the Greater Flamingo are well studied, ranging from the often posed questions like, 'why does a flamingo stand on one leg' and 'why are flamingos pink' to details on the connectivity of the population that exist within its range. In addition to gathering data on the population dynamics of the species via the unique identification rings (which vary in their colour and/or alphanumeric code between sites and years), annual ringing

operations at many of the sites across its range provide an opportunity for researchers to collect biological samples.

1.6 Justification for research and research objectives

The increased prevalence of antibiotic resistance is widely recognised as a threat to human health, and the existing literature provides a good basis of information on reservoirs of antibiotic resistant bacteria in a variety of environmental systems worldwide. It is widely agreed that wildlife are particularly important to consider in regards to environmental reservoirs of antibiotic resistance, given the potential for zoonotic transmission to occur – and that birds, in particular, can offer a robust taxonomic group for investigation (Wolfe *et al.*, 2007). However, there exist several gaps within our understanding of antibiotic resistance in wild birds. In particular, information on spatial patterns of antibiotic resistant bacteria in wild birds – both within and between species – is lacking. This provokes several questions about the factors that might be driving resistance at a variety of scales – ranging from variation in resistances that might exist within a single site, to large-scale variation across the globe. The dynamics and patterns of antibiotic resistant bacteria in wild birds are of critical research interest, and the use of a multi-faceted approach is required to adequately address these questions.

This research project examines antibiotic resistance in wild birds on several scales – within individual sites, regionally, and globally. Through use of a multifaceted approach involving both desk- and field- based research, this thesis aims to increase our understanding of the prevalence of antibiotic resistant bacteria in wild birds. Chapter Two sets the historical context for this study, addressing the introduction of antibiotics and the subsequent development and spread of resistance. In Chapter Three, patterns of antibiotic resistance are examined through use of a meta-analysis approach, in which previous research on antibiotic resistance in wild birds is synthesised and analysed on a global scale. In the following chapter, a preliminary screening of bacterial isolates is presented to evaluate the Greater Flamingo as a sentinel (indicator) species of antibiotic resistance. This is followed in Chapter Five by a focused investigation of isolates that demonstrated resistance in pre-screening, to identify bacterial species and to determine their antibiotic resistance with reference to established standards. These four chapters will be drawn together in Chapter Six, which provides a general discussion and overall conclusions, including consideration of the underlying economic and social factors driving use of antibiotics, which to date, has been largely absent from the scientific literature. Overall, this thesis will provide insight into the extent to which wild birds serve as reservoirs of antibiotic resistant bacteria at a variety of spatial scales, will consider the implications for human health, and offer suggestions for the development of appropriate policy.

1.7 References

Alanis, A.J. Resistance to antibiotics: are we in the post-antibiotic era? Archives of Medical Research **36,** 697-705.

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J. and Handelsman, J. 2010. Call of the wild: antibiotic resistance genes in natural environments. Nature Reviews Microbiology **8,** 251-259. doi:10.1038/nrmicro2312.

Al-Bahrya, S., Mahmouda, I., Elshafiea, A., Al-Harthya, A., Al-Ghafria, S., Al-Amrib, I., Alkindia, A. 2009. Bacterial flora and antibiotic resistance from eggs of green turtles Chelonia mydas: an indication of polluted effluents. Marine Pollution Bulletin **58**, 720–725.

Balkız, Ö., Özesmi, U., Pradel, R., Germain, C., Sıkı, M., Amat, J. A., ... & Béchet, A. (2007). Range of the Greater Flamingo, Phoenicopterus roseus, metapopulation in the Mediterranean: new insights from Turkey. *Journal of Ornithology*, *148*(3), 347-355.

Béchet, A., Rendón-Martos, M., Rendón, M. A., Amat, J.A., Johnson, A.R. and Gauthier-Clerc, M. 2012. Global economy interacts with climate change to jeopardize species conservation: a case study in the Greater flamingo in the Mediterranean and West Africa. Environmental Conservation **39**, 1-3.

Björklund, H., Bondestam, J. and Bylund, G. 1990. Residues of oxytetracycline in wild fish and sediments from fish farms. Aquaculture **86,** 359-367.

Blackburn, J.K., Mitchell, M.A., Blackburn, M.-C.H., Curtis, A. and Thompson, B.A. 2010. Evidence of antibiotic resistance in free-swimming, top-level marine predatory fishes. Journal of Zoo and Wildlife Medicine **41**, 7-16.

Blanco, G., Lemus, J.A. and Grande, J. 2009. Microbial pollution in wildlife: linking agricultural manuring and bacterial antibiotic resistance in red-billed choughs. Environmental Research **109,** 405-412. doi: 10.1016/j.envres.2009.01.007.

Borghesi, F., Andreotti, A., Baccetti, N., Bianchi, N., Birke, M., Migani, F. and Dinelli, E. 2011. Flamingo feathers to monitor metal contamination of coastal wetlands: methods and initial results concerning the presence of mercury at six Mediterranean sites. Chemistry and Ecology **27**, 137-151.

Burger, J. and Gochfeld, M. 2004. Marine birds as sentinels of environmental pollution. EcoHealth **1,** 263-274.

Burnet, F.M. and White, D. 1972. Natural History of Infectious Disease. Press Syndicate of the University of Cambridge, Cambridge, UK, 278 pp.

Butler, M.S. and Cooper, M.A. 2011. Antibiotics in the clinical pipeline in 2011. The Journal of Antibiotics **64,** 413-425.

Camarda, A., Circella, E., Pennelli, D., Madio, A., Bruni, G., Lagrasta, V., ...& Campagnari, E. (2010). Wild Birds as biological indicators of environmental pollution: biotyping and antimicrobial resistance patterns of Escherichia coli isolated from Audouin's gulls (Larus Audouinii) living in the Bay of Gallipoli (Italy). Italian Journal of Animal Science, **5**(3), 287-290.

Caprioli, A., Donelli, G., Falbo, V., Passi, C., Pagano, A. and Mantovani, A. 1991. Antimicrobial resistance and production of toxins in Escherichia coli strains from wild ruminants and the alpine marmot. Journal of Wildlife Disease **27**, 324-327.

Cirz, R.T., Chin, J.K., Andes, D.R., de Crécy-Lagard, V., Craig, W.A. and Romesberg, F.E. 2005. Inhibition of mutation and combating the evolution of antibiotic resistance. PLoS Biology **3,** doi:10.1371/journal.pbio.0030176

Cissell, R.L. 2006. Occurrence of Antibiotic Resistance in Environmental and Amphibian E. coli Isolates Associated with Cattle and Aquatic Environments. Master's Thesis, University of Tennessee, 2006, http://trace.tennessee.edu/utk_gradthes/1526.

Clarke, T. 2003. Drug companies snub antibiotics as pipeline threatens to run dry. Nature **425,** 225.

Cooper, M.A. and Shlaes, D. 2011. Fix the antibiotics pipeline. Nature **472,** 32.

Daszak, P., Cunningham, A.A. and Hyatt, A.D. 2000. Emerging infectious diseases of wildlife – threats to biodiversity and human health. Science **287,** 443-449.

Daszak, P., Cunningham, A.A. and Hyatt, A.D. 2001. Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Tropica **78,** 103-116.

Davies, J. and Davies, D. 2010. Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews **74,** 417-433.

Fischbach, M.A. and Walsh, C.T. 2009. Antibiotics for emerging pathogens. Science **325,** 1089-1093.

Fotia, M., Giacopelloa, C., Bottarib, T., Fisichellaa, V., Rinaldoa, D. and Mamminac, C. 2009. Antibiotic resistance of gram negative isolates from loggerhead sea turtles (Carettacaretta) in the central Mediterranean Sea. Marine Pollution Bulletin **58**, 1363–1366.

Geraci, J., Béchet, A., Cézilly, F., Ficheuz, S., Baccetti, N., Samraoui, B. and Wattier, R. 2012. Greater flamingo colonies around the Mediterranean form a single interbreeding population and share a common history. Journal of Avian Biology **43,** 341-354.

Gould, I.M. 2009. Controversies in infection: infection control or antibiotic stewardship to control healthcare-acquired infection? Journal of Hospital Infection **73,** 386-391.

Graves, S.R., Kennelly-Merrit, S.A., Tidemann, C.R., Rawlinson, P.A., Harvey, K.J. and Thornton, I.W.B. 1988. Antibiotic-Resistance Patterns of Enteric Bacteria of Wild Mammals on the Krakatau Islands and West Java, Indonesia. Philosophical Transactions of the Royal Society London B **322**, 339-353.

Grove, R. and Damodaran, V. 2006. Imperialism, intellectual networks, and environmental change: origins and evolution of global environmental history, 1676-2000: part 1. Economic and Political Weekly **41,** 4345-4354.

Hirsh, T., Ternes, T., Haberer, K. and Kratz, K.-L. 1999. Occurrence of antibiotics in the aquatic environment. Science of The Total Environment **225**, 109-118. http://dx.doi.org/10.1016/S0048-9697(98)00337-4.

Infectious Diseases Society of America (ISDA). 2010. The 10 × '20 Initiative: Pursuing a Global Commitment to Develop 10 New Antibacterial Drugs by 2020. Clinical Infectious Diseases **50,** 1080-1083.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L. and Daszak, P. 2008. Global trends in emerging infectious diseases. Nature **451,** 990-993.

Kahn, L.H. 2006. Confronting zoonoses, linking human and veterinary medicine. Emerging Infectious Diseases **12,** 556-561.

Karki, H. 2013. Survey of antibiotic resistant bacteria in fish gut collected from selected sites. MSc Dissertation, Indiana University-Purdue University Fort Wayne.

Kozak, G.K., Boerlin, P., Janecko, N., Reid-Smith, R.J. and Jardine, C. 2009. Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. Applied Environmental Microbiolology **75,** 3 559-566.

Kümmerer, K. 2004. Resistance in the environment. Journal of Antimicrobial Chemotherapy **54,** 311-320.

Leeb, M. 2004. Antibiotics: a shot in the arm. Nature **431,** 892-893.

Levy, S.B. 2002. The Antibiotic Paradox: How the Misuse of Antibiotics Destroys Their Curative Powers. Perseus Publishing, New York, USA. 304pp.

Levy, S. B., & Marshall, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nature medicine **10**, S122-S129.

Lewis, K. 2012. Antibiotics: Recover the lost art of drug discovery. Nature **485**, 439-440.

Lillehaug, A., Bergsjø, B., Schau, J., Bruheim, T., Vikøren, V. and Handeland, K. 2005. *Campylobacter spp.*, *Salmonella* spp., Verocytotoxic *Escherichia coli*, and antibiotic resistance in indicator organisms in wild cervids. Acta Veterinaria Scandinavica **46**: 23-32. doi:10.1186/1751-0147-46-23

Livermore, D.M., Warner, M., Hall, Enne, L.C.M., Projan, S.J., Dunman, P.M.,. Wooster, S.L. and Harrison, G. 2001. Antibiotic resistance in bacteria from magpies (*Pica pica*) and rabbits (*Oryctolagus cuniculus*) from west Wales. Environmental Microbiology **3,** 658–661. DOI: 10.1046/j.1462-2920.2001.00239.x

Marsh, G.P. 1864. Man and Nature; Or, Physical Geography as Modified by Human Action. Charles Scribner and Company, New York, New York, USA.

Mallon, D.J.P., Corkill, J.E., Hazel, S.M., Wilson, J.S., French, N.P., Bennett, M. and Hart, C.A. 2002. Excretion of vancomycin-resistant *Enterococci* by wild mammals. Emerging Infectious Diseases **8**, 636–638. doi: 10.3201/eid0806.010247

Mallory, M.L., Robinson, S.A., Herbert, C.E. and Forbes, M.R. 2010. Seabirds as indicators of aquatic ecosystem conditions: a case for gathering multiple proxies of seabird health. Marine Pollution Bulletin **60,** 7-12.

Martinez, J.L. 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environmental Pollution **157,** 2893-2902.

McEwen, S.A. and Fedorka-Cray, P.J. 2002. Antimicrobial use and resistance in animals. Clinical infectious Diseases **34,** S93-S106.

McMichael, A.J. 2004. Environmental and social influences on emerging infectious diseases: past, present and future. Proceedings of the Royal Society Published 29 July 2004 doi: 10.1098/rstb.2004.1480

Miranda, C.D. and Zemelman, R. 2001. Antibiotic resistant bacteria in fish from the Concepción Bay, Chile. Marine Pollution Bulletin **42,** 1096-1102.

Morel, C.M. and Mossialos, E. 2010. Stoking the antibiotic pipeline. BMJ **340,** doi: http://dx.doi.org/10.1136/bmj.c2115

Morens, D.M., Folkers, G.K. and Fauci, A.S. 2004. The challenge of emerging and re-emerging infectious diseases. Nature **430**, 242-249. doi:10.1038/nature02759

Murray, K.A. and Daszak, P. 2013. Human ecology in pathogenic landscapes: two hypotheses on how land use change drives viral emergence. Current Opinion in Virology **3,** 79-83.

National Environmental Health Association. 2008. Position Paper on the "One Health Initaitive" Available online at: www.neha.org/pdf/position_one_world.pdf. Accessed 8 April 2014.

Neu, H.C. 1992. The crisis in antibiotic resistance. Science **21,** 1064-1073.

Nugent, G. 2011. Maintenance, spillover and spillback transmission of bovine tuberculosis in multi-host wildlife complexes: A New Zealand case study. Veterinary Microbiology **151,** 34-42.

O'Brien, T. F. 2002. Emergence, Spread, and Environmental Effect of Antimicrobial Resistance: How Use of an Antimicrobial Anywhere Can Increase Resistance to Any Antimicrobial Anywhere else. Clinical Infectious Diseases **34,** S78-S84. doi: 10.1086/340244

One Health Initiative. 2014. One Health Initiative Mission Statement. Available online at: www.onehealthinitaitive.com/mission.php. Accessed 8 April 2014.

Österblad, M., Norrdahl, K., Korpimäki, E. and Huovinen, P. 1999. Antibiotic resistance: How wild are wild mammals? Nature **409**, 37-38. doi:10.1038/35051173

Palmer, M.V., Thacker, T.C. Waters, W.R., Gortázar, C. and Corner, L.A.L. 2012. Mycobacterium bovis: a model pathogen at the interface of livestock, wildlife, and humans. Veterinary Medicine International. http://dx.doi.org/10.1155/2012/236205=

Paracuellos, M., Castro, H., Nevado, J.C., Oña, J.A., Matamala, J.J., García, G. and Salas, G. 2002. Repercussions of the abandonment of Mediterranean saltpans on waterbird communities. Waterbirds **25,** 492-498.

Patz, J.A., Daszak, P, and Members of the Working Group on Land Use Change Disease Emergence. 2004. Unhealthy Landscapes: Policy Recommendations on Land Use Change and Infectious Disease Emergence. Environmental Health Perspectives **112,** 1092-1098.

Poeta, P., Costa, D, Sáenz, Y., Klibi, N., Ruiz-Larrea, F., Rodrigues, J. and Torres, C. 2005. Characterization of antibiotic resistance genes and virulence factors in faecal Enterococci of wild animals in Portugal. Journal of Veterinary Medicine, Series B **52**, 396–402. DOI: 10.1111/j.1439-0450.2005.00881.x

Power, A.G. and Mitchell, C.E. 2004. Pathogen spillover in disease epidemics. The American Naturalist **164,** S79-S89.

Quammen, D. 2013. Spillover: Animal Infections and the Next Human Pandemic. W.W. Norton and Company, New York, New York. 592pp.

Radhouani, H., Poeta, P., Gonçalves, A., Pacheco, R., Sargo, R., & Igrejas, G. 2012. Wild birds as biological indicators of environmental pollution: antimicrobial resistance patterns of

Escherichia coli and enterococci isolated from common buzzards (Buteo buteo). Journal of medical microbiology **61**(Pt 6), 837-843.

Rana, S.W., Kumar, A., Walia, S.K., Berven, K., Cumper, K. and Walia, S.K. 2011. Isolation of Tn*1546*-like elements in vancomycin-resistant *Enterococcus faecium* isolated from wood frogs: an emerging risk for zoonotic bacterial infections to humans. Journal of Applied Microbiology **110,** 35-43. DOI: 10.1111/j.1365-2672.2010.04860.x

Reed, K.D., Meece, J.K. Henkel, J.S. and Shuka, S.K. 2003. Birds, migration and emerging zoonoses: West Nile virus, Lyme disease, influenza A and enteropathogens. Clinical Medicine Research **1,** 5-12.

Rhyan, J.C. and Spraker, T.R. 2010. Emergence of disease from wildlife reservoirs. Veterinary Pathology **47**, 34-39.

Rose, J.M., Gast, R.J., Bogomolni, A., Ellis, J.C., Lentell, B.J., Touhey, K. and Moore, M. 2010. Occurrence and patterns of antibiotic resistance in vertebrates off the Northeastern United States coast. FEMS Microbiology Ecology **67**, 421–431. DOI: 10.1111/j.1574-6941.2009.00648.x

Routman, E., Miller, R.D., Phillips-Conroy, J. and Hartl, D.L. 1985. Antibiotic resistance and population structure in Escherichia coli from free-ranging African yellow baboons. Applied Environmental Microbiology **50,** 749-754.

Sarmah, A.K., Meyer, M.T. and Boxall, A.B.A. 2005. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere **65,** 725–759.

Séveno, N. A., Kallifidas, D., Smalla, K.; van Elsas, J. D., Collard, J.-M.; Karagouni, A. D. and Wellington, E. M. H. 2002. Occurrence and reservoirs of antibiotic resistance genes in the environment. Reviews in Medical Microbiology **13,** 15-27

Sherley, M., Gordon, D.M. and Collignon, P.J. 2000. Variations in antibiotic resistance profile in Enterobacteriaceae isolated from wild Australian mammals. Environmental Microbiology **2,** 620–631.

Singer, R.S., Finch, R., Wegener, H.C., Bywater, R., Walters, J., Lipsitch, M. 2003. Antibiotic resistance—the interplay between antibiotic use in animals and human beings. The Lancet Infectious Diseases **3,** 47-51.

Sjölund, M., Bonnedahl, J., Hernandez, J., Bengtsson, S., Cederbrant, G., Pinhassi, J., Kahlmeter, G. And Olsen, B. 2008. Dissemination of multidrug-resistant bacteria into the Arctic. Emerging Infectious Diseases **14,** 70-72.

Slaughter, D.M., Patton , T.G., Sievert G., Sobieski, R.J. and Crupper, S.S. 2001. Antibiotic resistance in coagulase-negative staphylococci isolated from Cope's gray treefrogs (*Hyla chrysoscelis*). FEMS Microbiolgy Letters **205,** 265-270.

Spellberg, B., Guidos, R., Gilbert, D., Bradley, J., Boucher, H.W., Scheld, W.M., Bartlett, J.G., Edwards Jr., J. and the Infectious Diseases Society of America. 2008. The Epidemic of Antibiotic-Resistant Infections: A Call to Action for the Medical Community from the Infectious Diseases Society of America. Clinical Infectious Diseases 46, 155-164.

Spellberg, B., Bartlett, J.G. and Gilbert, D.N. 2013. The future of antibiotics and resistance. The New England Journal of Medicine **368,** 299-302.

Spellberg, B. and Taylor-Blake, B. 2013. On the exoneration of Dr. William H. Stewart: debunking an urban legend. Infectious Diseases of Poverty **2,** http://www.idpjournal.com/content/2/1/3

Taylor, L.H., Latham, S.M., Woolhouse, M.E.J. 2001. Risk factors for human disease emergence. Philosophical Transactions of the Royal Society London B **356,** 983-989.

Thaller MC, Migliore L, Marquez C, Tapia W, Cedeño V, et al. 2010. Tracking acquired antibiotic resistance in commensal bacteria of Galápagos land iguanas: no man, no resistance. PLoS ONE 5(2): e8989. doi:10.1371/journal.pone.0008989

The World Bank. 2012. People, Pathogens, and our Planet, Volume 2: The Economics of One Health. The World Bank, Washington DC, USA. 50pp. Available online at: www.worldbank.org. Accessed 8 April 2014.

Vitousek, P.M., Mooney, H.A., Lubchenco, J. and Melillo, J.M. 1997. Human Domination of Earth's Ecosystems. Science **25,** 494-499. DOI: 10.1126/science.277.5325.494

Wilcox, B.A. and Gubler, D.J. 2005. Disease ecology and the global emergence of zoonotic pathogens. Environmental Health and Preventive Medicine **10,** 263-272.

Wolfe, N.D., Dunavan, C. P. And Diamond, J. 2007. Origins of major human infectious diseases. Nature **447**, 279-283. doi:10.1038/nature05775.

World Health Organisation. 2002. Surveillance standards for antimicrobial resistance. A background document for the WHO global strategy for containment of antimicrobial resistance. Available online: http://whqlibdoc.who.int/hq/2002/WHO_CDS_CSR_DRS_2001.5.pdf

Wright, G.D. 2013. Q&A: antibiotic resistance: what more do we know and what more can we do? BMC Biology **11,** doi:10.1186/1741-7007-11-51.

Zinsstag, J., Schelling, E., Waltner-Toews, D. and Tanner, M. 2011. From "one medicine" to "one health" and systemic approaches to health and well-being. Preventative Veterinary Medicine **101,** 148-156.

2 From the 'mould juice' miracle to one of the greatest threats to human health: an overview of the discovery of antibiotics and the emergence of antibiotic resistance

2.1 Chapter overview

This chapter serves to give an overview of the historical context within which this research project was undertaken. By introducing concepts relating to the historical dynamics between human societies, their environments, and infectious disease; the inception and development of the antibiotic industry; and the emergence of antibiotic resistance, the reader will have an understanding of the concepts underlying the entirety of this work.

2.2 Introduction

Throughout history, human societies have been affected, to varying extents, by the occurrence of infectious disease (Satcher, 1995; Weiss and McMichael, 2004; Moren *et al.*, 2004). A society's ability to effectively respond to an emerging disease outbreak, or control the factors underling its emergence to prevent it in the first place, has long served as a challenge (Morse, 1995; Satcher, 1995). Pandemics of infectious disease such as cholera, tuberculosis, typhoid and influenza have served as a major cause of mortality; the inability of humans to combat these explains why historically, the average life expectancy was much lower than it is now (Burnet and White, 1972; Schlipköter and Flahault, 2010; Vaupel, 2010). In his Pulitzer Prize-winning book *Guns, Germs, and Steel,* Jared Diamond (1997) examines this dynamic in human society. Diamond's book focuses primarily on the interaction between human society, technology, and disease, using examples from throughout history to describe the factors that have regulated a given society's dominance. What he, and others, have concluded, is that infectious disease has played an instrumental role in shaping history, and that to a large extent, it is mediated by the geographical and environmental context within which humans live (Jones *et al.*, 2008; Steffen *et al.*, 2011).

Though the specific diseases have changed over the course of history, the fact remains that human societies are indelibly linked with their environments – and that the interactions between the two play a key role in the emergence and spread of infectious disease (Tenover, 2006). These links were recognized as far back as 400 B.C.E. by Hippocrates, whose writings on human health and infectious disease are still influential today (Pappas *et al.*, 2008). In *Airs, Waters, and Places*, Hippocrates writes about the effect of the environment on human health. Here, he describes how variation in water quality can affect human health,

> "And I wish to give an account of the other kinds of waters, namely, of such as are wholesome and such are unwholesome, and what bad and what good effects may be derived from water, for water contributes much towards health...The best are those which flow from elevated grounds, and hills of earth; these are sweet, clear, and can bear a little wine..."

Nearly 2,500 years later, we are still enjoying the occasional glass of wine, as made possible by the waters of our Earth. But along with such benefits and the 'good effects' we continue to reap, we also continue to experience the bad effects from water and other aspects of our environment. Today, we are faced with an emerging threat to human health that demonstrates, once again, how the dynamics of interactions between humans and our environment can serve to drive the spread of disease – and the extent to which we are responsible for finding a solution. This threat is antibiotic resistance, and it has been described by the World Health Organization as one of the three greatest threats to human health (ISDA, 2010).

Paradoxically, this great threat to human health is the result of the success of the antibiotics that revolutionized healthcare in the twentieth century, allowing for the prevention and treatment of bacterial infections that had previously proven fatal (Carlet *et al.*, 2011). Our use of antibiotics is so crucial to how healthcare has evolved that they have been said to 'underpin modern medicine' (Piddock, 2012). As such, the latter half of the 20th century is often described, within a medical context, as the era of antibiotics (Waldvogel, 2004; Hancock, 2007; Gould, 2012). The list of treatments and procedures that have been made possible by the discovery of antibiotics is astounding. Without them, surgeries, organ transplants, successful delivery and care of premature infants, and chemotherapy treatment regimes for cancer would not be possible (Hancock, 2007; Cars *et al.*, 2011). Given this context, it is notable that these so-called 'miracle drugs' are now the focus of campaigns calling to reduce their use, in response to the emergence of this new threat, antibiotic resistance. In early March of 2013, Dame Sally Davies, the Chief Medical Officer for the United Kingdom, spoke at a press conference that coincided with the release of the publication of her annual report. Calling on global leaders to address antibiotic resistance, she asserted that, "antimicrobial resistance poses a catastrophic threat." The news media jumped on the story and in the days that followed the press conference, social media took notice; on Twitter, for example, Davies' statement was re-tweeted by hundreds of users, while on Google, the 12-month peak in web search interest for the search term 'antibiotic resistance' occurred following the press conference (Figure 2.1).

Figure 2.1 Google Trends chart showing the web search interest for the query 'antibiotic resistance' between September 2012 and September 2013. Notable is the 12-month peak (in box) occurring in the week 10th-16th March, coinciding with the press conference held by Dame Sally Davies at which she referred to antibiotic resistance as a 'catastrophic threat'.

The statement by Davies was one of many made by experts in the past several years that have increased society's awareness of the threat of antibiotic resistance. The news media frequently features articles about 'superbugs'. Maryn McKenna, an author and journalist, has a blog *Superbug* that is featured on Wired magazine's website, at which she posts the latest updates on antibiotic resistance, and Bill Bryson wrote of antibiotic resistant bacteria in his best-selling popular science book *A Short History of Nearly Everything* (2003). It is clear that antibiotic resistance is a problem, and that people have taken notice of it. What has been lacking, however, is an effective governmental response to the problem, in countries across the globe and despite the fact that there has been an increased amount of research, providing a growing amount of evidence of the extent of the problem, many agree that the response has been insufficient (Cars *et al.*, 2008; Spellberg *et al.*, 2008; Bush *et al.*, 2011; Lee and Wakabayashi, 2013). This research project aims to contribute to the growing body of evidence of the widespread problem of antibiotic resistance, and in particular, that which examines environmental systems as reservoirs of antibiotic resistant bacteria. It involves experimental work in which wildlife living in the waters that comprise Southern Europe's wetlands were visited, to evaluate the extent to which (in the words of Aristotle) bad or good effects were derived from them. This project also examines the phenomena of natural environments as reservoirs of antibiotic resistance on a larger scale, through use of a systematic literature review and meta-analysis that synthesizes the results of previous research efforts that have investigated antibiotic resistance in wild birds across the globe. But first, this chapter serves as an introduction to antibiotics and antibiotic resistance, to provide a context within which this research was conducted.

2.3 Antibiotics

2.3.1 Historical context & the discovery of 'mould juice'

The terms "antibiotics" and "antimicrobials" are often used interchangeably. Here, the term antibiotics is used to describe a class of drugs that kill or otherwise inhibit the growth of bacteria (antibiotics refer to substances produced by a micro-organism); the broader term antimicrobials applies to compounds that act on fungi and viruses, in addition to bacteria (ETAG, 2006). The history of antibiotic discovery, development, and use offers an interesting tale that describes much of how human healthcare has evolved in the last century. At present, the research, development, and marketing of antibiotics is a multi-billion euro a year industry, and one that is not without controversy (Demain, 2013). The dynamics that shape how (and why) this industry operates as it does raises important questions, both scientific and ethical in nature, the answers and solutions to which have the potential to radically alter how human healthcare will evolve in the next one hundred years and beyond (Aminov, 2010; Hunter, 2010; Bergström, 2011; Bush *et al.*, 2011; Spellberg *et al.*, 2013).

Antibiotics and human health were first linked following the serendipitous discovery of penicillin in 1928 by Alexander Fleming, who originally referred to it as 'mould juice'. The story of its discovery is an oft-repeated tale of a laboratory mishap gone well, and one for which – most likely due to numerous retellings – several versions exist (Diggins, 2000; Goldsworthy and McFarlane, 2002; Bentley, 2005). Perhaps it is Fleming himself who can provide the most accurate version, in his appropriately (if not somewhat uninspiring) titled article 'The Discovery of Penicillin', published in 1944 – though it should be noted that he prefaces his paper by noting, "I have been asked to say how I came to discover penicillin. After a lapse of fifteen years it is very difficult to say what processes of thought were involved..." As he recounts, during a study that involved the examination of bacterial growth on culture plates, Fleming – having just returned from a holiday – noticed that one had been contaminated with mould (Plate 1). This was an occurrence that was not altogether unexpected, given the frequent uncovering and re-covering of the plates. What was unexpected was that growth of the *Staphylococcal* colonies in the vicinity of the mould was inhibited. As Fleming writes, a previous research interest in substances that inhibited bacterial growth caused him to take a special interest and thus, he conducted further tests using the mould to investigate fully its antibacterial properties.

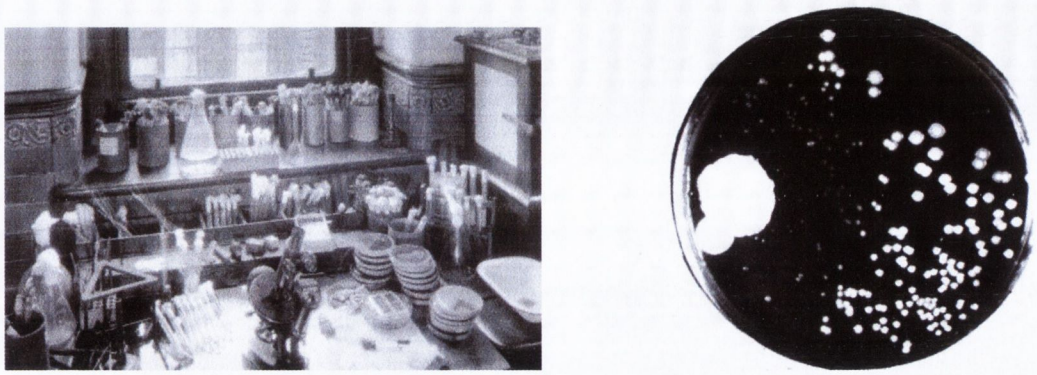


Plate 1. Fleming's (very crowded!) lab bench (left), and the famous culture plate (right). The mould is on the left of the plate; note that the growth of bacteria is diminished around the mould compared to the right, where there is substantially more bacterial growth. Images from Diggins, 2003.

Due to the coincidence of several factors (including, amongst others, the necessity of conducting experiments to fully determine its appropriate dosage, a reluctance to prescribe it, and sourcing of equipment and funding to produce it on a large scale), over a decade would pass before the use of penicillin in treatment regimes was implemented (Henderson, 1997; Lerner, 2004). This ultimately did occur by the early 1940s, at which time the use of antibiotics was hailed as a new era in human healthcare given their potential use in the treatment of infections and conditions that had often proven fatal (Wenzel, 2004). In these early years, antibiotics were not generally released for large-scale public use due to costly and inefficient manufacturing processes; instead, they were primarily used within the context of the treatment of soldiers during World War II (Learner, 2004). It wasn't until technological advances allowed manufacturing processes to be streamlined that antibiotics were released for widespread use, in the mid-1940s (Alanis, 2005). The transformation from small- to large-scale was swift: within a two-year period, monthly production of penicillin rose from an approximate 100 million units in the early months of 1943 to over 650 *billion* units in August 1945 (Grossman, 2008). During this time, laboratories focused their efforts on the production of antibiotics, proclaiming their benefits as the magazine excerpt in Plate 2 shows.

Plate 2. Advertisement for penicillin by Schenley Laboratories, Inc. (Available online at: http://www.digitaldeliftp.com/DigitalDeliToo/Images/The-Doctor-Fights-Schenley-Ad-1944)

The accompanying text describes the 'miraculous substance' of penicillin, and asserts that,

> "When the thunderous battles of this war have subsided to pages of silent print in a history book, the greatest news event of World War II may well be the discovery and development – not of some vicious secret weapon that destroys – but of a weapon that saves lives. That weapon, of course, is penicillin."

The advertisement, by Schenley Laboratories, Inc. notes that, "A year ago, production of penicillin was difficult, costly. Today, due to specially devised methods of mass-production, in use by Schenley Laboratories, Inc. and the 20 other firms designated by the government to make penicillin, it is available in ever-increasing quantity, at progressively lower cost." The production of penicillin – and its use in treating a variety of infections (as evidenced in Plate 3) – meant that it became widely available. The impact on society cannot be overstated. In 1962, just one year after winning the Nobel Prize in Medicine, Sir Frank MacFarlane Burnet wrote, "One can think of the middle of the twentieth century as the end of one of the most important social revolutions in history, the virtual elimination of the infectious diseases as a significant factor in social life" (Pier, 2008).

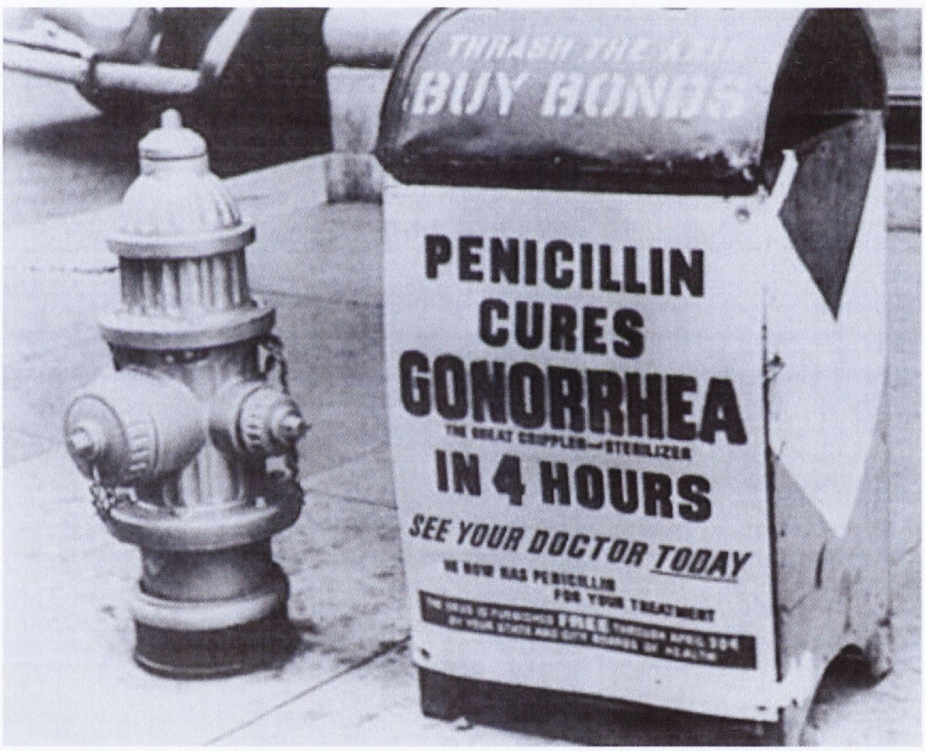


Plate 3. Originally used to treat infections in wounded soldiers, the use of penicillin soon widened. The impact of penicillin on society is evidenced in this photo from the 1940s, in which an advertisement for penicillin appears on a public waste bin. (http://i0.wp.com/listverse.com/wp-content/uploads/2009/08/penicillinpsaedit.jpg)

Given the overwhelmingly positive response to penicillin (and the profits that it brought), it didn't take long for the search for new antibiotics to commence. In the decades that followed, the rate at which new antibiotics were developed and introduced was remarkable; the use of antibiotics has become so widespread that their use has been said to 'underpin modern medicine' (Piddock, 2012). The 1950's and 1960s have been described as the age of discovery, or 'golden era' of the era of antibiotics, as a large amount of research was devoted

to finding new antibiotics (Davies, 2006; Aminov, 2010). In the 1960s, the success of antibiotics in treating infections prompted the US Surgeon General William H. Stewart to reportedly declare, "The war against infectious diseases has been won" (though recent evidence has questioned the veracity of this statement) (Spellberg and Taylor-Blake, 2013). Similarly, the aforementioned MacFarlane Burnett mused that, "at times one feels that to write about infectious disease is almost to write of something that has passed into history" (MacFarlane Burnett, 1962 as cited in Lashley and Durham, 2007).

Soon after the use of antibiotics in human healthcare commenced, their use was applied to veterinary medicine, and in particular, the rearing, maintenance, and treatment of livestock (McEwen and Fedorka-Cray, 2002; Sarmah *et al.*, 2005). It was a serendipitous research finding in 1946 by Thomas H. Jukes, working in the United States, which first indicated that antibiotics could be used to improve the growth of chicks. At the time he reported, "A unique phenomena – perhaps without precedent in the history of medicine" (Wise, 2007). This discovery was followed by a series of experiments indicated a similar result in swine and cattle, the economic implications of which were hugely significant (Gustafson and Bowen, 1997; Wise, 2007). This research occurred at a time when intensive livestock farming was emerging in the US and Europe, and coincided with the improvements in the manufacturing processes of antimicrobials that made them significantly cheaper than they had when they were first introduced (Alanis, 2005). Together, this resulted in a proliferation in the use of antibiotics, given that large numbers of livestock could be housed in small areas and today, antibiotics are integral to the agricultural industry (Sarmah *et al.*, 2006).

2.3.2 Classes of antibiotics

Throughout this so-called era of antibiotics, the research conducted in the search for new antibiotics was in large part based on the fact that there are a number of ways in which a bacterium can be prevented from 'doing its job' and proliferating (i.e. causing an infection). Antibiotics are grouped into classes with reference to their mechanism of action against bacteria – consideration is typically also given to its 'spectrum', that is, the range of bacteria it works against; they are referred to as either 'narrow spectrum' or 'broad spectrum' (Fischbach and Walsch, 2009). In its most simplistic sense, antibiotics work by either a) killing the bacterium or b) preventing it from replicating; these are referred to as 'bactericidal' and 'bacteriostatic', respectively (Hancock, 2005). For each of these two actions, however, there are myriad ways in which they can act. In part, multiple classes of antibiotics are required because of the range of infectious bacteria, and the physiological differences between them. That is to say, that for many of the classes of antibiotics, there exist specific 'target' bacterial species for which they are most effective. Even broadly speaking, and taking into consideration a relatively basic level of understanding of histology, consider, for example, the

differences between gram-positive and gram-negative bacteria. As you may remember from your school biology practicals, bacteria can be described by their cell wall: Gram-positive bacteria have a thick, peptidoglycan-rich outer layer that takes on a purple hue with Gram staining. In Gram-negative bacteria, the outer cell wall is thinner and lacks peptidoglycan; as a result, Gram-negative bacteria appear pink with Gram staining. This variation in the cell wall between Gram-positive and Gram-negative bacteria means that different mechanisms are required to effectively infiltrate the bacteria, and therefore, different antibiotics are required. For example, some classes, like the 'Beta-Lactams & Penicillins', work by inhibiting cell wall synthesis, while others like the 'Tetracyclines' and the 'Fluoroquinolones' classes work by inhibiting protein and DNA synthesis, respectively (Levy and Marhsall, 2004). A variety of classification systems exist; the one used here is the frequently-updated Anatomical Therapeutic Chemical (ATC) classification system offered by the WHO Collaborating Centre for Drug Statistics Methodology (2013). Within each of the antibiotic classes, a number of different antibiotics have been discovered (Figure 2.2). What has been termed a 'discovery void' (or alternatively, 'innovation gap') is evident in the latter three to four decades of the 20th century; the implications of which will be discussed in the following section (Powers, 2004; Fischbach and Walsh, 2009; Silver, 2011).

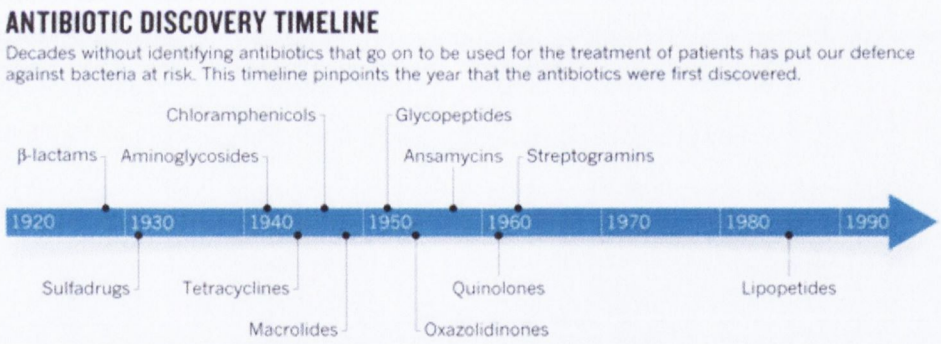


Figure 2.2. Timeline depicting the discovery of the primary classes of antibiotics. Between the mid-1960s and today, very few new classes of antibiotics have been discovered; this has been termed the 'discovery void'. (Image from Lewis, 2012).

2.4 Antibiotic resistance

2.4.1 Antibiotic resistance defined

While the benefits of antibiotics changed the way that human healthcare and agriculture have evolved, their excessive use – often termed 'misuse' or described as 'irrational' within the scientific literature – in more recent years has resulted in significant problems (Wenzel, 2004; Blanco *et al.*, 2009; Allen *et al.*, 2010). In particular, the development of antibiotic resistance

has been cited as a major problem linked to our use of antibiotics; it is believed to be such a severe problem that it has served to provide a basis for the suggestion that the use of antibiotics should be minimized and restricted (ETAG, 2006). Antibiotic resistance describes the ability of a microorganism to survive exposure to an antibiotic (World Health Organization, 2013). As early as 1992, the emerging problem of antibiotic resistance was termed a 'crisis' (Neu, 1992), and it is a problem that has not gone away. It has more recently been described as 'catastrophic', 'epidemic', and a 'serious threat' (Sjölund *et al.*, 2008; Allen *et al.*, 2010; Thaller *et al.*, 2010).

Although the emergence of antibiotic resistance has only recently become a leading news item, it is in reality one that was first noted very early on, following on not long after the introduction of antibiotics to the consumer market in the early 1940s. In 1945 – the same year that he was awarded a Nobel Prize for his discovery of penicillin – Alexander Fleming warned about antibiotic resistance in an article in the New York Times, asserting that the consequence of misusing antibiotics is that "instead of clearing up infection the microbes are educated to resist penicillin" (Rosenblatt-Farrell, 2009). After Fleming cited it as a problem in 1945, evidence of antibiotic resistance began to emerge in a clinical context. In comparing the effectiveness of penicillin over a two-year period in a nationwide clinical study, Pillsbury (1946) noted a greatly reduced efficacy of the antibiotic in the treatment of syphilis. Since then, the problem of antibiotic resistant bacteria has continued, making antibiotic resistance an issue of increasing concern – and as first noted with penicillin, resistances to many antibiotics have developed within several years of their introduction to the market, as indicated in Table 2.1 (Palumbi, 2001; Lubelchek and Weinstein, 2008; Högberg *et al.*, 2010). In the following section, factors that have contributed to the proliferation of antibiotic resistant bacteria in the past several decades are discussed.

Table 2.1. Introduction of antibiotics and the development of resistance

Antibiotic	Year introduced	Resistance observed
Sulfonamides	1930s	1940s
Penicillin	1943	1946
Streptomycin	1943	1959
Chloramphenicol	1947	1959
Tetracycline	1948	1953
Erythromycin	1952	1988
Vancomycin	1956	1988
Methicillin	1960	1961
Ampicillin	1961	1973
Cephalosporins	1960s	Late 1960s

The year in which antibiotics were introduced, and the year in which resistance was first noted (adapted from Palumbi, 2001)

2.4.2 Factors contributing to the proliferation of antibiotic resistance

A review of the literature reveals that many factors have contributed to the problem of antibiotic resistance, and that in large part, they continue to do so. By reviewing these factors it becomes clear that the problem of antibiotic resistance is multifaceted and complex. There are two primary 'players' involved in the development and spread of antibiotic resistance in bacteria – the bacteria itself, and the antibiotic. It is widely acknowledged that the dynamics between the two is complex and that although significant advances in the field have been made, much remains to be discovered; the role of human societies in the development and spread of antibiotic resistance has also long been recognised such that in reality, it should be considered the third 'player' in the dynamics which govern the proliferation of antibiotic resistance (Cohen, 1992; Levy, 2001; Kümmerer, 2004).

There are several known mechanisms by which bacteria can develop or acquire resistance to an antibiotic (Hancock, 2005; Tenover, 2006). The ability of bacteria to develop resistance to antibiotics was aptly put by Jean-Jacques Giraud, the director of clinical research at a pharmaceutical research company, in an interview for an article in the New York Times (Stolberg, 1998). In the article, Giraud describes how, following the introduction of vancomycin, efforts to develop further antibiotics were abandoned because it was thought there was no need, as no bacteria could possibly be resistant to vancomycin; he states that, "that was pretty arrogant thinking. The bacteria were more creative". Such so-called 'creative' mechanisms include mutations and selections within a population of bacteria; resistance can spread via the transfer of resistance-coding genes from other populations of bacteria (Sévano,

2002; Tenover, 2006). In 1958, Joshua Lederberg won the Nobel Prize for his groundbreaking investigations into the exchange of drug-resistant genes between bacteria. In his public lecture following receipt of the award, Lederberg (1959) described the emergence of this new area of research, "the development of resistance apparently induced by drugs revived illusions that bacterial genes might be alterable...no wonder that the mechanism of drug resistance has excited so much controversy!" Today, many mechanisms by which bacteria develop and exhibit antibiotic resistance are recognized, and of these, many are inherently linked to our use of antibiotics (Davies, 1997; Sévano, 2002; Alanis, 2005; Chan, 2011).

Where antibiotics are concerned, the factors that contribute to the development of antibiotic resistance can be broadly divided into two classes: (1) an increased use of antibiotics, and (2) the lack of new antibiotic drug development. Because antibiotic resistance can develop as the result of repeated exposure to an antibiotic, the increased use of antibiotics serves to drive resistance. The link between the increased use of antibiotics and antibiotic resistance in a clinical context has long been recognized (Cohen, 1992). The variety of terms used in the recent scientific literature to describe the level at which antibiotics are currently used is alarmist in tone. Amongst the many terms used, the following offer an indication of the general attitude towards the level at which we are using antibiotics: 'irrational', 'misuse', 'abuse', and 'excessive', (Alanis, 2005; Levy and Marshall, 2005; Okeke *et al.*, 2005; Carlet *et al.*, 2011). It has been estimated that up to 50% of antibiotics prescribed to patients might not be necessary (Hicks *et al.*, 2013). This is not only true for antibiotics prescribed in a clinical context; in an agricultural context, the majority of antimicrobial use in relation to health is preventative, and thus not necessarily required. In 1998, Wise *et al.* concluded that between 40% and 80% of the use of antibiotics in agricultural contexts was 'highly questionable'. The link between agricultural use of antibiotics and antibiotic resistance in humans has also been recognized for decades (Smith, 1968; Austin and Kristinsson, 1999). Together, the overuse of antibiotics in both clinical and veterinary contexts has served to drive the prevalence of antibiotic resistance, and the continuous development of new antibiotics is required to provide alternatives when an antibiotic fails. However, this has not occurred at the necessary rate. As alluded to previously in reference to the aforementioned 'discovery void' that is evident in Figure 4, most classes of antibiotics that we are aware of now were discovered in an initial phase of antibiotic research in the decades immediately following the discovery of penicillin – a period of exponential growth of research and development which began to decline in the 1960s. Of the current group of twenty-two antibiotic classes, eighteen were discovered within the first twenty years of antibiotic discovery – and in the four decades since, only four new classes of antibiotic have been discovered (Coates *et al.*, 2011; Cooper and Shlaes, 2011). Both small and large pharmaceutical companies have invested significantly less in the research and development of antimicrobials over the past few decades and as a result, there has been a decline in the number of new antibiotics approved (Spellberg *et al.*, 2004; Projan

and Shlaes, 2004). In 2004, of the drugs that the world's biggest pharmaceutical companies were working to develop, only 1.6% were antibiotics (Spellberg *et al.*, 2004). This lack of research and development into new antibiotics is due to a number of factors that can be broadly classed into two categories that describe their implications: 1) increased costs for research and development, and 2) decreased revenues (Norrby *et al.*, 2005). Factors included in these categories are summarized in Table 2.2.

Table 2.2 Factors contributing to the 'antibiotic void'

Factor	Implication	Reference(s)
Antibiotics have a short shelf-life	Decreased revenue	Walsh, 2003
Low profit margin for antibiotics	Decreased revenue	Nathan, 2004; Katz *et al.*, 2006
Patents expire quickly allowing other companies to enter the market	Decreased revenue	Norrby *et al.*, 2005; Outterson *et al.*, 2007,
Strict regulatory requirements	Increased R&D costs	Projan, 2003; Norrby *et al.*, 2005; Coates *et al.*, 2011; Jabes, 2011; Kuehn, 2011
Antibiotics typically prescribed for short-term use for once off infections	Decreased revenue	Eiland and Gatlin, 2008; Kuehn, 2011

Thus, it is evident that through both an increased cost of developing new antibiotics and decreased revenues for those that exist, it has become less financially viable for pharmaceutical companies to initiate new research and development programmes for antibiotics (Walsh, 2003; Nathan, 2004; Katz *et al.*, 2006). Essentially, the development of new antibiotics has become a numbers game for the pharmaceutical companies, with few incentives encouraging the development of new drugs (Norrby *et al.*, 2005). Coupled with increased regulations that serve to discourage new endeavors, the development of new antibiotics has stalled amongst the major drug companies, and prevented new companies from entering the market (Norrby *et al.*, 2005; Coates *et al.*, 2011; Jabes, 2011; Kuehn, 2011). Overall, this has resulted in very little progression over the past several decades and such is the decline in antibiotic research and development that it has been described as a 'lost art' (Lewis, 2012).

2.4.3 Consequences of antibiotic resistance

The consequence of the lack of development of new antibiotics, coupled with their overuse, is severe in that it has created a 'perfect storm' by which antibiotic resistance has become increasingly abundant (Spellberg *et al.*, 2004). Given that they undermine the potential success of antibiotics, antibiotic resistant bacteria are of critical concern with regard to human health; many studies have examined the consequences including increased health care costs and in some cases, mortality (Cohen, 1992; Carmeli *et* al., 1999; Levy, 2001; Eliopolous *et* al., 2003; Cosgrove *et al.*, 2005, Cosgrove, 2006; WHO, 2013). Recent work by de Kraker *et al.* (2011) attempted to quantify the impacts through use of a standardized metric in public health research, the Burden of Disease (BoD). Using empirical data from 31 European countries, the authors determined that in 2007, two clinically important antibiotic resistances – methicillin-resistant *Staphylococcus aureus* (MRSA) and third-generation cephalosporin resistant *E. coli* (G3SEC)] – were associated with over 8,000 deaths and excess costs of over 60 million Euro as a result of blood stream infections (de Kraker *et al.*, 2011). In 2009, the European Centre for Disease Control (ECDC) carried out a much broader study (which included consideration of more resistant bacteria and infection types), which found a burden of infection of over 900 million Euro in 2007. The problem is not limited to Europe or the developed world; the issue of antibiotic resistance and its associated effects are particularly profound in the developing world, where antibiotics are often of poor quality, access to alternative antibiotics is limited, and antibiotic use is not always well-regulated (Levy, 1998; Witte, 1998; Okeke *et al.* 2005). Though the consequences of it may vary between geographic regions, the problem of antibiotic resistant bacteria is a global one, and the environments within which antibiotic resistance is found are changing. The spread of antibiotic resistance into natural environments from the clinical environments in which they were originally found has occurred over the past few decades (Levy and Marshall, 2004). It is believed that resistances were largely limited to hospitals until the mid-1960s (Alanis, 2005). After this, evidence suggesting the spread of antibiotic resistant bacteria into natural environments emerged.

Antibiotic resistance can arise and/or spread into environmental systems via a variety of mechanisms – through the spread of resistant bacteria, transfer of resistance genes, or through prolonged and/or repeated exposure to a given antibiotic compound (or its residues) that can persist in the environment (Hirsch *et al.*, 1999; O'Brien *et al.*, 2002; Sévano *et* al., 2002; Kümmerer, 2004; Sarmah *et al.*, 2006; Martinez, 2009). Beyond home and hospital use, antibiotics are widely used in a variety of arenas, including in agriculture, fish farming, and industry; one decade ago, it was estimated that 50% of antimicrobials were used in veterinary medicine (Teuber, 2001). Transport through waterways can facilitate the spread (or development) of antibiotic resistant bacteria into new environments (Björkland *et al.*, 1990; Chelossi, 2003; Kümmerer, 2003; Cabello, 2006; McEwen, 2006; Schaefer *et al.*, 2009; Shah,

2012). The extent to which natural environments have come to serve as reservoirs of antibiotic resistance is widely recognized as an important area for research, given the potential for wildlife to serve as reservoirs (stores) of antibiotic resistant bacteria (McEwen and Fedorka-Cray, 2002; Singer *et al.*, 2006; Literak *et al.*, 2007). The use of monitoring programmes to evaluate environmental reservoirs of antibiotic resistance therefore often involves the use of wildlife as indicators and to date, antibiotic resistant bacteria have been found in a variety of wildlife species including mammals, fish, birds, reptiles and amphibians worldwide (Allen, 2010; Blackburn, 2010; Al Bahry *et* al., 2011; Hacioglu and Tosunoglu, 2013). This is worrying given the potential for transmission of antibiotic resistance to occur through these wildlife vectors at human-livestock-wildlife interfaces (Blanco *et al.* 2007; da Costa *et al.*, 2013). Diseases and pathogens that have the potential to be transmitted between humans and non-human animals are termed *zoonotic,* and the potential for such transmission cannot be ignored, given estimates that over 60% of emerging diseases affecting humans are zoonotic (Jones *et al.*, 2008). Although a significant body of research now provides us with case-study evidence of the presence of ARB in natural environments, many of these studies have been limited because they have largely been restricted to single-site investigations, limiting the potential for comparative analyses across a large geographic range, and the need for investigating patterns of antibiotic resistance (both spatial and temporal), is widely recognized (Aminov, 2010). Proximity to human activity has been cited as a factor that serves to increase the incidence of antibiotic resistant bacteria, though this has been the subject of debate within the literature (Cole *et* al., 2003; Allen *et al.*, 2010). Cases where resistance has evolved naturally in environmental systems – that is, in the absence of antibiotics, or where transfer of resistant bacteria through the environment is unlikely – have been described as well (Levy and Marshall, 2004; Singer *et al.*, 2006; Blanco *et al.*, 2009; Thaller *et al.*, 2010; Bhullar *et al.*, 2012). It is clear that much remains to be understood regarding the extent to which environmental systems serve as reservoirs of antibiotic resistant bacteria, and the use of wildlife can provide important insights, and wild birds have been identified as useful indicators of environmental health and in particular, infectious disease (Bildstein, 2001; Blackburn *et al.*, 2010; Amat and Green, 2010).

2.5 References

Alanis, A.J. 2005. Resistance to antibiotics: are we in the post-antibiotic era? Archives of Medical Research **36,** 697-705.

Al-Bahry, S.N., Mahmoud, I.Y., Al-Zadjali, M., Elshafie, A., Al-Harthy, A. and Al-Alawi, W. 2011. Antibiotic resistant bacteria as bio-indicator of polluted effluent in the green turtles, Chelonia mydas in Oman. Marine Environmental Research **71**,139-144 http://dx.doi.org/10.1016/j.marenvres.2010.12.005.

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J. and Handelsman, J. 2010. Call of the wild: antibiotic resistance genes in natural environments. Nature Reviews Microbiology **8,** 251-259. doi:10.1038/nrmicro2312.

Amat, J.A. and Green, A.J. 2010. Waterbirds as bioindicators of environmental conditions. In: (C. Hurford *et al.*, eds.) Conservation Monitoring in Freshwater Habitats, Springer, 415pp.

Aminov, R.I. 2009. The role of antibiotics and antibiotic resistance in nature. Environmental Microbiology **11,** 2970-2988.

Aminov, R.I. 2010. A brief history of the antibiotic era: lessons learned and challenges for the future. Frontiers in Microbiology **134.** doi: 10.3389/fmicb.2010.00134

Austin D.J., Kristinsson, K.G. and Anderson, R.M. 1999. The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. Proceedings of the National Academy of Sciences USA **96,** 1152-1156.

Bentley, R. 2005. The development of penicillin: genesis of a famous antibiotic. Perspectives in Biology and Medicine **48,** 444-452.

Bergström, R. 2011. The role of the pharmaceutical industry in meeting the public health threat of antibacterial resistance. Drug Resistance Updates **14,** 77-78.

Bhullar, K., Waglechner, N., Pawlowski, A. *et al.* 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS ONE 7(4): e34953. doi:10.1371/journal.pone.0034953

Bildstein, K.L. 2001. Why migratory birds of prey make great biological indicator. pp 169-179 *in* K.L. Bildstein and D. Klem, Jr. (eds.). Hawkwatching in the Americas. Hawk Migration Association of America, North Wales, PA.

Björklund, H., Bondestam, J. and Bylund, G. 1990. Residues of oxytetracycline in wild fish and sediments from fish farms. Aquaculture **86,** 359-367.

Blackburn, J.K., Mitchell, M.A., Curtis, A. and Thompson, B.A. 2010. Evidence of antibiotic resistance in free-swimming, top-level marine predatory fishes. Journal of Zoo and Wildlife Medicine 41, 7-16.

Blanco, G., Lemus, J.A., Grande, J., Gangoso, L., Grande, J.M., Donázar, J.A., Arroyo, B., Frías, O. and Hiraldo, F. 2007. Geographical variation in cloacal microflora and bacterial antibiotic resistance in a threatened avian scavenger in relation to diet and livestock farming practices. Environmental Microbiology **9,** 1738-1749.

Blanco, G., Lemus, J.A. and Grande, J. 2009. Microbial pollution in wildlife: linking agricultural manuring and bacterial antibiotic resistance in red-billed choughs. Environmental Research **109,** 405-412. doi: 10.1016/j.envres.2009.01.007.

Bryson, B. 2003. A Short History of Nearly Everything. Broadway Books, New York, New York, 554 pp.

Burnet, F.M. and White, D. 1972. Natural History of Infectious Disease. Press Syndicate of the

University of Cambridge, Cambridge, UK, 278 pp.

Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., Jacoby, G.A., Kishony, R. *et al.* 2011. Tackling antibiotic resistance. Nature Reviews Microbiology **9**, 894-896. doi:10.1038/nrmicro2693

Cabello, F.C. 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environmental Microbiology **8,** 1137-1144.

Carlet, J., Collignon, P., Goldmann, D., Goossens, H., Gyssens, I.C., Harbarth, S., Jarlier, V., Levy, S.B., N'Doye, B, Pittet, D., Richtmann, R., Seto, W.H., van der Meer, J.W.M. and Voss, A. 2011. Society's failure to protect a precious resource: antibiotics. The Lancet **378,** 369-371.

Carmeli, Y., Troillet,N., Karchmer, A.W. and Samore, M.H. 1999. Health and economic outcomes of antibiotic resistance in *Pseudomonas aeruginosa.* Archives of Internal Medicine **159**,1127-1132. doi:10.1001/archinte.159.10.1127.

Cars, O., Hedin, A. and Heddini, A. 2011. The global need for effective antibiotics—moving towards concerted action. Drug Resistance Updates **14,** 68-69.

Chan, M. 2011. Combat drug resistance: no action today means no cure tomorrow. Statement for World Health Day 2011 on 6 April 2011. Available online at: http://www.who.int/mediacentre/news/statements/2011/whd_20110407/en/index.html. Accessed 31 August 2013.

Chelossi, E., Vezzulli, L., Milano, A., Branzoni, M., Fabioano, M., Riccardi, G. And Banat, I.M. 2003. Antibiotic reisistance of benthic bacteria in fish-farm and control sediments of the Western Mediterranean. Aquaculture **219,** 83-97.

Coates, A.R., Halls, G. and Hu, Y. 2011. Novel classes of antibiotics or more of the same? British Journal of Pharmacology **163,** 184-194. doi: 10.1111/j.1476-5381.2011.01250.x.

Cohen, M.L. 1992. Epidemiology of drug resistance: implications for a post-antimicrobial era. Science **257,** 1050-1055. DOI: 10.1126/science.257.5073.1050

Cole, E.C., Addison, R.M., Rubino, J.R. *et al.* 2003. Investigation of antibiotic and antibacterial agent cross-resistance in target bacteria from homes of antibacterial product users and nonusers. Journal of Applied Microbiology **95,** 664-676.

Cooper, M.A. and Shlaes, D. 2011. Fix the antibiotics pipeline. Nature **472**, 32. doi:10.1038/472032a

Cosgrove, S.E., Qi, Y., Kaye, K.S., Harbarth, S., Karchmer, A.W. and Carmeli, Y. 2005. The impact of methicillin resistance in *Staphylococcus aureus* bacteremia on patient outcomes: mortality, length of stay, and hospital charges. Infection Control and Hospital Epidemiology **26**, 166-174.

Cosgrove, S.E. 2006. The Relationship between Antimicrobial Resistance and Patient Outcomes: Mortality, Length of Hospital Stay, and Health Care Costs. Clinical Infectious Diseases **42,** S82-S89. doi: 10.1086/499406

da Costa, P.M., Loureiro, L. and Matos, A.J.F. 2013. Transfer of Multidrug-Resistant Bacteria between Intermingled Ecological Niches: The Interface between Humans, Animals and the Environment. International Journal of Environmental Research and Public Health **10,** 278-284.

Davies, J. 2006. Where have all the antibiotics gone? Canadian Journal of Infectious Diseases and Medical Microbiology **17,** 287-290.

Davies, J.E. Origins, acquisition and dissemination of antibiotic resistance determinants. In: Antibiotic Resistance: Origins, Evolution, Selection, and Spread. Wiley, Chichester (Ciba Foundation Symposium 207), pp. 15-35.

de Kraker, M.E.A., Davey, P.G. and Grundmann, H. 2011. Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteremia: estimating the burden of antibiotic resistance in Europe. PLoS Medicine available online at: http://www.plosmedicine.org/article/info%3Adoi%2F10.1371%2Fjournal. pmed.1001104. Accessed 20 August 2013.

Demain, A.L. 2013. Importance of microbial natural products and the need to revitalize their discovery. Journal of Industrial Microbiology & Biotechnology. DOI: 10.1007/s10295-013-1325-z

Diamond, J. 1997. Guns, Germs, and Steel: The Fates of Human Societies. W.W. Norton & Company.

Diggins, F.W. 2000. The discovery of penicillin: so many get it wrong. Biologist (London) **47,** 115-119.

European Centre for Disease Control (ECDC). 2009. The bacterial challenge: time to react. Technical Report. Available online at: http://www.ecdc.europa.eu/en/publications/Publications/0909_TER_The_Bacterial_Challenge_Time_to_React.pdf.

Eiland, E.H. and Gatlin, D. Forecast of antibiotic development in an era of increasing bacterial resistance. Journal of Pharmacy Practice **21,** 313-318.

Eliopoulos, G.M., Cosgrove, S.E. and Carmeli, Y. 2003. The Impact of Antimicrobial Resistance on Health and Economic Outcomes. Clinical Infectious Diseases **36,** 1433-1437.

European Technology Assessment Group (ETAG). 2006. Antibiotic resistance. European Parliament. Policy Department. Economic and Scientific Policy. IP/A/STOA/ST/2006-4. Brussels, Belgium.

Fischbach, M.A. and Walsh, C.T. 2009. Antibiotics for emerging pathogens. Science **325,** 1089-1093.

Fleming. A. 1944. The discovery of penicillin. British Medical Bulletin **2**, 4-5.

Goldsworthy, P.D. and McFarlane, A.C. Howard Flurey, 2002. Alexander Fleming and the fairy tale of penicillin. The Medical Journal of Australia **176,** 176-178.

Gould, I.M. 2012. The Antibiotic Paradox. In: Gould, I.M. and van der Meer, J.W.M. (eds). Antibiotic Policies, pp. 15-25.

Grossman, C.M. 2008. The First Use of Penicillin in the United States. Annals of Internal Medicine **149**, 135-136.

Gustafson, R.H. and Bowen, R.E. 1997. Antibiotic use in animal agriculture. Journal of Applied Microbiology **83,** 531-541.

Hacioglu, N. and Tosunoglu, M. 2013. Determination of antimicrobial and heavy metal resistance profiles of some bacteria isolated from aquatic amphibian and reptile species. Environmental Monitoring and Assessment, DOI 10.1007/s10661-013-3385-y

Hancock, R.E.W. 2005. Mechanisms of action of newer antibiotics for Gram-positive pathogens. Lancet Infectious Disease **5,** 209-218.

Hancock, R.E.W. 2007. The end of an era? Nature Reviews Drug Discovery **6**, doi:10.1038/nrd2223.

Henderson, J.W. 1997. The yellow brick road to penicillin: a story of serendipity. Mayo Clinic Proceedings **72**, 683-687.

Hicks, L.A., Taylor, T.H., and Hunkler, R.J. 2013. U.S. Outpatient Antibiotic Prescribing, 2010. New England Journal of Medicine **368,** 1461-1462. DOI: 10.1056/NEJMc1212055

Hirsh, T., Ternes, T., Haberer, K. and Kratz, K.-L. 1999. Occurrence of antibiotics in the aquatic environment. Science of The Total Environment **225**, 109-118. http://dx.doi.org/10.1016/S0048-9697(98)00337-4.

Högberg, L.D., Heddini, A. and Cars, O. The global need for effective antibiotics: challenges and recent advances. Trends in Pharmacological Sciences **31**, 509-515. http://dx.doi.org/10.1016/j.tips.2010.08.002.

Hunter, J. 2011. Challenges for pharmaceutical industry: new partnerships for sustainable human health. Philosophical Transactions of the Royal Society A **369,**1817-1825. doi: 10.1098/rsta.2010.0377

Infectious Diseases Society of America (ISDA). 2010. The 10 × '20 Initiative: Pursuing a Global Commitment to Develop 10 New Antibacterial Drugs by 2020. Clinical Infectious Diseases **50,** 1080-1083.

Jabes, D. 2011. The antibiotic R&D pipeline: an update. Current opinion in microbiology **14**, 564-569.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L. and Daszak, P. 2008. Global trends in emerging infectious diseases. Nature **451,** 990-993.

Katz, M.L., Mueller, L.V., Polyakov, M. and Weinstock, S.F. 2006. Where have all the antibiotics gone? Nature Biotechnology **24,** 1529-1531.

Kümmerer, K. 2003. Significance of antibiotics in the environment. Journal of Antimicrobial Chemotherapy **52,** 5-7.

Kümmerer, K. 2004. Resistance in the environment. Journal of Antimicrobial Chemotherapy **54,** 311-320.

Lashley, F.R. and Durham, J.D. 2007. in: Lashley, F.R. and Durham, J.D. (Eds) Emerging Infectious Diseases: Patterns and Trends, Springer Publishing Company.

Lederberg, J. 1959. A View of Genetics. Nobel Lecture, presented on 29 May 1959. Available online at: http://www.nobelprize.org/nobel_prizes/medicine/laureates/ 1958/lederberg-lecture.pdf Accessed 29 August 2013.

Kuehn, B. M. 2011. Proposals seek to reduce resistance, boost development of new antibiotics. JAMA **305**, 1845-1846.

Lee, Y. and Wakabayashi, M. 2013. Key informant interview on antimicrobial resistance (AMR) in some countries in the western pacific region. Globalization and Health, **9**. doi:10.1186/1744-8603-9-34

Lerner, P.I. 2004. Producing penicillin. New England Journal of Medicine **5,** 524.

Levy, S.B. 1998. The challenge of antibiotic resistance. Scientific American, March 1998. Available online at: http://www.micro.utexas.edu/courses/kalthoff/bio346/PDF/ Readings/08Levy%20%281998%29.pdf

Levy, S.B. 2001. Antibiotic resistance: consequences of inaction. Clinical Infectious Diseases **33,** S124-S129. doi: 10.1086/321837.

Levy, S.B. and Marshall, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nature Medicine **10,** S122-129.

Lewis, K. 2012. Antibiotics: Recover the lost art of drug discovery. Nature **485,** 439-440. doi: 10.1038/485439a.

Literak, I., Vanko, R., Dolejská, M., Čížek, A., and Karpíšková, R. 2007. Antibiotic resistant *Escherichia coli* and *Salmonella* in Russian rooks (*Corvus frugilegus*) wintering in the Czech Republic Letters in Applied Microbiology **45,** 616-621. DOI: 10.1111/j.1472-765X.2007.02236.x

Lubelchek, R.J. and Weinstein, R.A. 2008. Antibiotic resistance and nonsocomial infections. In (Meyer, K.H. and Pizer, H.F., eds) The Social Ecology of Infectious Diseases. Academic Press, London, UK. 528pp.

Martinez, J.L. 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environmental Pollution **157,** 2893-2902.

McEwen, S.A. and Fedorka-Cray, P.J. 2002. Antimicrobial use and resistance in animals. Clinical infectious Diseases **34,** S93-S106.

McEwen, S.A. 2006. Antibiotic use in animal agriculture: what have we learned and where are we going? Animal Biotechnology **17,** 239-250.

Morens, D.M., Folkers, G.K. and Fauci, A.S. 2004. The challenge of emerging and re-emerging infectious diseases. Nature **430**, 242-249. doi:10.1038/nature02759

Morse, S.S. Factors in the emergence of infectious disease. Emerging Infectious Diseases **1,** 1.

Nathan, C. 2004. Antibiotics at the crossroads. Nature, **431**, 899-902.

Neu, H.C. 1992. The crisis in antibiotic resistance. Science **21,** 1064-1073.

Norrby, S.R., Nord, C.E. and Finch, R. 2005. Lack of development of new antimicrobial drugs: a potential serious threat to public health. The Lancet Infectious Diseases **5,** 115-119.

O'Brien, T. F. 2002. Emergence, Spread, and Environmental Effect of Antimicrobial Resistance: How Use of an Antimicrobial Anywhere Can Increase Resistance to Any Antimicrobial Anywhere else. Clinical Infectious Diseases **34,** S78-S84. doi: 10.1086/340244

Okeke, I.N., Laxminarayan, R., Bhutta, Z.A., Duse, A.G., Jenkins, P., O'Brien, T.F., Pablos-Mendez, A. and Klugman, K.P. 2005. Antimicrobial resistance in developing countries. Part I: recent trends and current status. The Lancet Infectious Diseases **5,** 481-493.

Palumbi, S.R. 2001. Humans as the world's greatest evolutionary force. Science **293,** 1786-1790.

Pappas, G., Kiriaze, I.J. and Falagas, M.E. 2008. Insights into infectious disease in the era of Hippocrates. International Journal of Infectious Diseases **12,** 347-350.

Piddock, L.J. 2012. The crisis of no new antibiotics—what is the way forward? Lancet Infectious Disease **12,** 249-253. doi: 10.1016/S1473-3099(11)70316-4.

Pier, G.B. 2008. On the greatly exaggerated reports of the death of infectious diseases. Clinical Infectious Disease **27,** 1113-1114. doi: 10.1086/592123

Pillsbury, D.M. 1946. Penicillin therapy of early syphilis. Journal of venereal disease **22,** 90-91.

Powers, J.J. 2004. Antimicrobial drug development – the past, the present, and the future. Clinical Microbiology and Infection **10,** 23-31.

Projan, S.J. and Shlaes, D.M. 2004. Antibacterial drug discovery: is it all downhill from here? Clinical Microbiology and Infection **10,** 18-22.

Rosenblatt-Farrell, N. 2009. The landscape of antibiotic resistance. Environmental Health Perspectives **117,** 244-250.

Sarmah, A.K., Meyer, M.T. and Boxall, A.B.A. 2005. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere **65,** 725–759.

Satcher, D. 1995. Emerging infections: getting ahead of the curve. Emerging Infectious Diseases **1,** 1.

Schaefer, A.M., Goldstein, J.D., Reif, J.S., Fair, P.A. and Bossart, G.D. 2009. Antibiotic-Resistant Organisms Cultured from Atlantic Bottlenose Dolphins (*Tursiops truncatus*) Inhabiting Estuarine Waters of Charleston, SC and Indian River Lagoon, FL. EcoHealth **6,** 33-41.

Schlipköter, U. and Flahault, A. 2010. Communicable diseases: achievements and challenges for public health. Public Health Reviews **32**, 90-119.

Séveno, N. A., Kallifidas, D., Smalla, K.; van Elsas, J. D., Collard, J.-M.; Karagouni, A. D. and Wellington, E. M. H. 2002. Occurrence and reservoirs of antibiotic resistance genes in the environment. Reviews in Medical Microbiology **13,** 15-27

Shah, S.Q., Colquhound, D.J. and Sørum, H. 2012. Prevalence of antibiotic resistance genes in the bacterial flora of integrated fish farming environments of Pakistan and Tanzania. Environmental Science and Technology **21,** 8672-8679.

Silver, L.L. 2011. Challenges of antimicrobial discovery. Clinical Microbiolgy Revews **24,** 71-109.

Singer, R.S., Ward, M.P. and Maldonado, G. 2006. Can landscape ecology untangle the complexity of antibiotic resistance? Nature Reviews Microbiology **4,** 943-953.

Sjölund, M., Bonnedahl, J., Hernandez, J., Bengtsson, S., Cederbrant, G., Pinhassi, J., Kahlmeter, G. And Olsen, B. 2008. Dissemination of multidrug-resistant bacteria into the Arctic. Emerging Infectious Diseases **14,** 70-72.

Smith, H.W. 1968. Anti-microbial drugs in animal feeds. Nature **218,** 728-731.

Spellberg, B., Powers. J.H., Brass, E.P., Miller, L.G. and Edwards, J.E. 2004. Trends in antimicrobial drug development: implications for the future. Antimicrobial Research and Development **38,** 1279-1286.

Spellberg, B., Guidos, R., Gilbert, D., Bradley, J., Boucher, H.W., Scheld, W.M., Bartlett, J.G., Edwards Jr., J. and the Infectious Diseases Society of America. 2008. The epidemic of antibiotic-resistant infectious: a call to action for the medical community from the Infectious Diseases Society of America. Clinical Infectious Diseases **46,** 155-164.

Spellberg, B. and Taylor-Blake, B. 2013. On the exoneration of Dr. William H. Stewart: debunking an urban legend. Infectious Diseases of Poverty **2,** http://www.idpjournal.com/content/2/1/3

Spellberg, B., Bartlett, J.G., and Gilbert, D.N. 2013. The Future of Antibiotics and Resistance. The New England Journal of Medicine **368**, 299-302.

Steffen, W., Grinevald, J., Crutzen, P. and McNeill, J. 2011. The anthropocene: conceptual and historical perspectives. Philosophical Transactions of the Royal Society A **369,** 842-867.

Stolberg, S.G. 1998. Superbugs. New York Times, published 2 August 1998. Available online at: http://www.nytimes.com/1998/08/02/magazine/superbugs.html? pagewanted=all&src=pm. Accessed 28 August 2013.

Tenover, F.C. 2006. Mechanisms of antimicrobial resistance in bacteria. The American Journal of Medicine **119,** S3-S10.

Teuber, M. 2001. Veterinary use and antibiotic resistance. Current Opinions in Microbiology **4,** 493-499.

Thaller MC, Migliore L, Marquez C, Tapia W, Cedeño V, et al. 2010. Tracking acquired antibiotic resistance in commensal bacteria of Galápagos land iguanas: no man, no resistance. PLoS ONE 5(2): e8989. doi:10.1371/journal.pone.0008989

Vaupel, J.W. 2010. Biodemography of human aging. Nature **464,** 536-542.

Waldvogel, F.A. 2004. Infectious diseases in the 21st century: old challenges and new opportunities. International Journal of Infectious Diseases **8,** 5-12.

Walsh, C. 2003. Where will new antibiotics come from?. *Nature Reviews Microbiology*, *1*(1), 65-70.

Weiss, R.A. and McMichael, A.J. 2004. Social and environmental risk factors in the emergence of infectious diseases. Nature Medicine **10**, S70 - S76. doi:10.1038/nm1150

Wenzel, R.P. 2004. The antibiotic pipeline—challenges, costs, and values. New England Journal of Medicine **5,** 523-526.

Wise, R., Hart, T., Cars, O., Streulens, M., Helmuth, R., Huovinen, P. and Sprenger, M. 1998. Antimicrobial resistance is a major threat to public health. BMJ **317,** 609-610.

Wise, R. 2007. An overview of the Specialist Advisory Committee on Antimicrobial Resistance (SACAR). Journal of Antimicrobial Chemotherapy **60,** i5-i7.

Witte W.1998. Medical consequences of antibiotic used in agriculture. Science. **279**, 996–97.

World Health Organisation. 2005. Containing antimicrobial resistance. WHO Policy Perspectives on Medicines. April 2005. Available online at: http://apps.who.int/medicinedocs/pdf/s7920e/s7920e.pdf.

World Health Organisation. 2013. Antimicrobial resistance fact sheet N^{0}194. Available online at http://www.who.int/mediacentre/factsheets/fs194/en/index.html. Accessed 31 August 2013.

World Health Organisation Collaborating Centre for Drug Statistics Methodology. 2013. ATC/DDD Index 2013. Available online at http://www.whocc.no/atc_ddd_index/. Accessed 19 August 2013.

3 Prevalence of antibiotic resistance in wild birds: a systematic review and meta-analysis

3.1 Introduction

3.1.1 Wild birds and antibiotic resistance

A growing body of research has documented the spread of antibiotic resistance from the urban environments (most notably hospitals) where the problem was initially recognised into so-called 'natural' environments. This research has indicated that the various sediments, waters, and wildlife that comprise environmental systems can serve as reservoirs (i.e. stores) of antibiotic resistant bacteria (Esiabo *et al.*, 2002; Séveno *et al.*, 2002; Bacquero *et al.*, 2008; Allen *et al.*, 2010). The prevalence of antibiotic resistant bacteria in environmental systems is important given the implications for human health. In particular, the potential for antibiotic resistant bacteria to be transferred to humans from environmental reservoirs (both biotic and abiotic) is widely acknowledged as a major threat to human health (Levy and Marshall, 2004; Martínez, 2008; Allen *et al.*, 2010). However, despite research efforts that have provided a large body of evidence supporting the existence of wildlife reservoirs of antibiotic resistance (typically via single-species and/or single-site case studies), little is known about patterns of antibiotic resistance in environmental systems on a large scale – including factors that affect it temporally, geographically, taxonomically, or otherwise (Allen *et al.*, 2010).

A review of the scientific literature on wildlife reservoirs of antibiotic resistance reveals that to date, much of the research has used wild birds as a focal taxa. There are an estimated 10,064 species of bird known at present (IUCN, 2012). Found in terrestrial, marine, and freshwater systems, birds occupy a diverse range of habitats across the world (IUCN, 2007). Historically, birds have been recognised as important indicators for a variety of diseases and contaminants in environmental systems and there are numerous reasons that they have been used as such (Koskimies, 1989; Furness, 1993; Bibby, 1999; Mallory *et al.*, 2010). Differences in the life histories of various species (including their geographic range, preferred habitat type, and more) means that exposure to infectious disease(s) varies considerably. The use of a wild bird species as an indicator can therefore provide an interesting (if complex!) dataset facilitating analysis of factors affecting variation (Morrison, 1986; Furness, 1993; Olsen *et al.*, 2006; Smits and Fernie, 2013). In an infectious disease context, wild birds can serve as vectors in contributing to the spread of pathogens via their migratory routes, which, depending on the species, can have the potential to transport infectious disease over long distances (Olsen *et al.*, 2006; Bonnedahl, 2011; Liu *et al.*, 2011). It follows that with regards to antibiotic resistant bacteria, wild birds are particularly important to investigate because of their potential role in the transfer of antibiotic resistant bacteria and/or antibiotic resistance genes via both short- and long-distance movements; variation in their exposure to antibiotics (and their residues) in

environmental systems also serves as a factor potentially affecting the prevalence of resistance, which makes for interesting analyses (Davies, 1997; Martinez, 2009; Allen *et al.*, 2010; Hasan *et al.*, 2012).

Antibiotic resistance has been studied in many species of bird at a variety of sites worldwide, ranging from antbirds (*Rhopornis ardesiacus* and *Myrmeciza ruficauda*) in the Brazilian rainforest to Gentoo penguins (*Pygoscelis papua*) in Antarctica, and from urban pigeons (*Columba livia*) in the Czech Republic to wild cranes (*Grus monacha* and *Grus vipio*) in Japan (Nascimento *et al.*, 2003; Bonnedahl *et al.*, 2008; Radimersky *et al.*, 2010; Kitadai *et al.*, 2012). To date, many such papers have offered insights into the prevalence of antibiotic resistance typically with reference to a single species or location; many of these papers have contributed evidence to the ongoing debate regarding the extent to which human activity affects the prevalence of antibiotic resistant bacteria in environmental systems (Thaller, 2010; Bhullar, 2012). However, although papers such as these continue to be published, a comprehensive understanding of the problem on a global scale, and across multiple species, habitat types, and geographies is lacking with the result that there remain many unanswered questions regarding the extent to which such factors – including human activity – affect the prevalence of antibiotic resistance (Davies, 1997; Palumbi, 2001; Aminov, 2009; Martinez, 2009a,b; D'Costa *et* al., 2011; Finley *et al.*, 2013). In contrast to the extensive literature on antibiotic resistance in an agricultural context, which includes several review papers that evaluate the factors driving antibiotic resistance in livestock/poultry (e.g. Silbergeld *et al.*, 2008; Rosengren *et al.*, 2010), to date, no comprehensive review has been conducted to analyse patterns and trends of antibiotic resistance in wild birds. The absence of such an assessment with reference to wild birds – and the environmental systems within which they live – has meant that our ability to develop sound, evidence-based policies in regards to the use of antibiotics has been limited. Meta-analysis is a research approach that allows for the synthesis and analysis of multiple studies; and with this, a variety of factors that might affect the prevalence of antibiotic resistance in wildlife can be examined on a large scale.

3.1.2 The meta-analysis approach

Meta-analysis – "an exercise in mega-silliness"?

In the 1970s, a debate was raging in the field of clinical psychology about the legitimacy of psychotherapy, which had, over the previous decades, emerged as a popular treatment option for a variety of mental illnesses (Strupp, 1993; Russell and Orlinksy, 1996). The debate was triggered in the early 1950s, when Hans Eysenck, a prominent and highly-cited British psychologist, suggested that it was ineffective (Eysenck, 1952; Wilson and Lipsey, 2001; Haggbloom *et al.*, 2002; Rose, 2010). Eysenck's critique of psychotherapy provoked an immediate response within the scientific literature questioning the validity of his criticism (e.g.

Luborsky, 1954; Rosenzweig, 1954). In the decades that followed, a growing body of research failed to provide a definitive answer on the efficacy of psychotherapy, given the varying levels of success it achieved in different studies (Wampold, 2001; Wilson and Lipsey, 2001). A more convincing conclusion was reached following the 1977 publication of a ground-breaking paper by a pair of American statisticians, Mary Smith and Gene Glass, in which they combined the results of nearly 400 studies, standardizing and analyzing them using a method they termed 'meta-analysis' (though it should be noted that similar techniques had been applied as far back as 1904) (Cooper, 1998; Wampold, 2001; Lemeshow *et al.*, 2005). The result? That 'strong evidence' existed demonstrating the 'beneficial effects' of psychotherapy (Smith and Glass, 1977). Eysenck (1978) remained unconvinced, and made his position clear in a paper titled 'An exercise in mega-silliness', in which he disputed their methodology and refuted their conclusions, going so far as to call their work 'an abandonment of scholarship'. Despite his criticisms – which included a pointed description of their selection and use of multiple studies as 'garbage in-garbage out' – meta-analysis became a widely accepted methodology (Eysenck, 1978; Wilson and Lipsey, 2001). In addition to a well-structured response to Eysenck's critcisms by Glass and Smith (1978), its rise in popularity was perhaps largely due to the fact that other researchers were simultaneously developing and utilising similar techniques; meta-analysis therefore became a widely accepted methodology (Wilson and Lipsey, 2001). In 1990, Spector and Thomson described an 'epidemic of meta-analyses' within the scientific literature.

Today, meta-analysis is a widely used approach that provides a structured process for the identification, appraisal, synthesis, and combination of research findings from diverse studies in a statistically robust way (Stroup *et al.*, 2000). By taking into account the outcomes across all available studies, a more comprehensive analysis of a research question is possible than what can be achieved by relying on the results of a single study. Meta-analyses are frequently used in healthcare to evaluate the effectiveness of a given drug or treatment (broadly classed as 'interventions'). In a healthcare context, meta-analysis is a particularly advantageous approach given that the results of multiple clinical trials (typically randomized controlled studies) can be combined and analysed through use of well-defined statistical techniques, providing a stronger basis upon which conclusions can be drawn (Stroup *et al.*, 2000). As a result, meta-analysis has become particularly popular given the emergence of evidence-based medicine, which has become a predominant paradigm in human healthcare (Borenstein and Hedges, 2009).

Beyond the hospital – the use of meta-analysis in other fields

Although meta-analysis was originally developed as a methodology to assess the success or failure of medical interventions – and indeed, this continues to be its predominant use – in the

decades following its introduction, other fields have adopted it as a methodology (Côté and Reynolds, 2012). Meta-analysis is now routinely used in a variety of disciplines, including both ecology and evolutionary biology, which over the past decade have taken a cue from medicine and begun to recognize the importance of evidence-based approaches (Hedges *et al.*, 1999; Gurevitch *et al.*, 2001; Pullin and Knight, 2001; Sutherland *et al.*, 2004). Work by Gurevitch *et al.* (2001) and Osenberg *et al.* (1999) have provided a comprehensive overview of its use, benefits, and shortcomings. Highlighting the necessity of drawing conclusions about patterns and trends on a 'higher order', a review by Arnqvist and Wooster (1995) called on ecologists to make wider use of meta-analyses. In an ecological context, meta-analysis provides a statistically robust mechanism of combining multiple studies, allowing for the analysis of patterns with reference to variation in responses between different groups (e.g. taxonomic and/or geographic) (Hedges *et al.*, 1999; Gates, 2002; Hillebrand, 2008). As a result, it has become a commonly used research tool in ecology (Carmel *et al.*, 2013).

Given its benefits, it is unsurprising that meta-analysis has also been applied to the disciplines of infectious disease and environmental epidemiology. Given that epidemiology inherently involves the examination of patterns and trends of disease on broad geographical scales, the previous reliance on studies, which (in most cases) use single-site approaches, seems inappropriate. The use of meta-analysis in epidemiology represents a departure from its traditional use in a clinical context, where much of the literature is focused on the analysis of randomized, controlled studies. Such study designs are not always possible in epidemiology, which often relies upon observational data (e.g. case series), and which lacks the well-controlled experimental design that defines randomized, controlled trials (Stroup *et al.*, 2000). Despite this challenge, its use in epidemiology has grown as methodologies specific to observational study designs have been developed and become widely used; for example, the 'meta-analysis of observational studies in epidemiology consensus statement' (MOOSE) proposed by Stroup *et al.* in 2000 has since been cited over 2,000 times.

Advantages of the meta-analysis approach

In addition to validating the efficacy of psychotherapy in their seminal paper that introduced meta-analysis, Smith and Glass (1977) suggested that, "scholars and clinicians are in the rather embarrassing position of knowing less than has been proven, because knowledge, atomized and sprayed across a vast landscape of journals, books, and reports, has not been accessible." This has never been truer than it is now, given that in the nearly forty years since the assertion of Smith and Glass in 1977, publication of research findings in peer-reviewed journals has increased (Larsen and von Ins, 2010). Take, for example, the online database MEDLINE – which sees the addition of 10,000 – 20,000 new citations per week (Garg *et al.*, 2008). Although this increase in published literature has provided us with an amazing source

of information, it is in actuality an overwhelmingly large resource, within which (even in a very narrow field) methods may vary and the conclusions can prove conflicting. Meta-analysis offers a way to compile this continuously emerging data to facilitate a comprehensive assessment of an issue being investigated, where, following an initial scan of the literature, a large degree of uncertainty might exist. It thus offers benefits to researchers across many disciplines (Garg *et al.*, 2008). However, it is a methodology that is not without limitations, and awareness of these caveats is critical in ensuring that the resulting conclusions will be robust. In the following table (Table 3.1) the benefits and limitations of meta-analysis are given.

Table 3.1 Benefits and limitations of meta-analysis

Benefits	**Reference**
Established, standardised methodologies exist; replicable method	Stroup *et al.* 2000; Wilson and Lipsey, 2001
Allows for systematic consideration of multiple studies, reducing the risk of drawing conclusions from random sampling errors	Spector and Thompson, 1990; Ioannidis, 2005; Stewart, 2009
Identification of large-scale patterns and trends that might be too expensive to do in a single research project	Stewart, 2009
The process is easily understood	Wilson and Lipsey, 2001
Results can be used in evidence-based decision making	Borenstein and Hedges, 2009
Can be done with a very small number of studies (as few as 2 or 3)	Wilson and Lipsey, 2001
Greater statistical power as a result of taking into account sample size (i.e. magnitude of effect)	Ioannidis and Lau, 1999; Wilson and Lipsey, 2001
The quantitative approach (in both gathering studies and analyzing them) makes it easier to compare and contrast differences in study design, as well as results	Ioannidis and Lau, 1999; Wilson and Lipsey, 2001
Takes into account variability in the 'strength' of different studies, a more sensitive technique that can provide a greater statistical significance than straight-forward studies that do not take into account the magnitude of effects	Wilson and Lipsey, 2001
Limitations	
Often seen as a 'quick and easy' approach, meta-analysis is in reality complex and time-consuming to undertake	Berman and Parker, 2002
Meta-analysis requires an explicitly defined, systematic approach that can lead to incorrect conclusions when violated	Walker *et al.*, 2008
File-drawer problem (publication bias in that null results are often not reported)	Rosenthal, 1975; Cook, 1993; Berman and Parker, 2002; Hart *et al.*, 2012
Language of returned studies	Berman and Parker, 2002
Search terms can be too specific or not specific enough	Lee, 2012
Often based on numerous, small-scale heterogeneous studies. Not necessarily as robust as a larger, full-scale clinical trial	Flather *et al.*, 1997; Walker *et al.* 2008

An awareness of the potential limitations of meta-analysis is important in addressing (or ideally, preventing) any challenges that are likely to be encountered; most of the limitations as noted above can be overcome. For example, Walker *et al.* (2008) cite the importance of noting bias in the process of selecting studies for inclusion. Similarly, these authors suggest using a standardized, well-documented search strategy and selection process to ensure a repeatable and statistically robust methodology. And indeed, the methods for meta-analyses have evolved over the years to adapt to the emergence of new statistical techniques and research approaches in a variety of fields. To address some of these changes, various conferences, collaborations, and working groups (all of which are typically comprised of multi-disciplinary participants) have developed statement papers that provide specific advice on conducting and reporting a meta-analysis. These include CONSORT (Consolidated Standards of Reporting Trials), MOOSE (Meta-analysis Of Observational Studies in Epidemiology), PRISMA (Preferred Reporting Items for Systematic reviews and Meta-Analyses), and QUOROM (the Quality of Reporting of Meta-analyses conference) (Moher *et al.*, 1999; Brand, 2009; Moher *et al.*, 2009; Schultz *et al.*, 2010; Stroup *et al.*, 2010). Although the guidelines provided by these various acronymic groups vary in their specific methodologies, they all provide a systematic approach to reviewing a given body of research, and offer a mechanism for the synthesis of findings of multiple studies in a statistically robust manner to address specific research questions.

3.2 Aims

As described in the preceding section, meta-analysis offers numerous advantages and particularly so within the area of human health and epidemiology, where it is a well-documented approach to investigating patterns and trends on a larger scale than is typically possible within the context of a single research project (e.g. global). Given this, it seems likely that meta-analysis offers a useful tool with which patterns and trends of antibiotic resistance in wild birds can be analysed. This investigation will provide the first meta-analysis of antibiotic resistance in wild birds and as such, will offer novel insights of relevance to the study of antibiotic resistance in environmental systems on a global scale. This will be achieved by meeting the following objectives:

- Define parameters (databases to search, search terms, etc.) for a systematic literature search
- Develop eligibility criteria (inclusion/exclusion) to identify articles
- Conduct literature search, including multi-stage review of articles returned from preliminary search with reference to eligibility criteria
- Determine factors that will be investigated in statistical analysis
- Extract data from included papers and compile data on study characteristics (including location of study, species sampled, antibiotics used, etc.) and responses (number of isolates susceptible/resistant)
- Use statistical techniques to identify patterns in resistance to antibiotics, including consideration of geographic, taxonomic, and landscape characteristics

Meta-analyses offer a statistically robust mechanism by which the results of multiple studies can be combined, allowing for specific research questions to be addressed with reference to a larger body of evidence than can be achieved within the context of a single study. As a result, meta-analysis provides an ideal methodology for investigating antibiotic resistance in wild birds, because although numerous individual studies have provided case-study evidence of wild birds as reservoirs of antibiotic resistant bacteria, a comprehensive understanding of factors that might drive resistance has to date, not been achieved.

3.3 Methods

The methodology for this meta-analysis was a two-stage process that involved: 1) article identification, 2) data extraction and analysis. Details of each of these are provided below.

3.3.1 Article identification

Studies on the presence and/or absence of antibiotic resistance in wild birds were identified through a systematic search of peer-reviewed literature published over a ten-year period (2002-2012). The reason for selecting ten years as a cut-off date is to ensure that the research upon which the meta-analysis is based used up-to-date techniques, given concerns about using less recent (and therefore potentially outdated) research in meta-analysis and its resultant lack of relevance (Pastopoulous and Ioannidis, 2009). The identification of articles for inclusion in the systematic review and meta-analysis was achieved through use of a search strategy that was developed with the aim of finding all potentially relevant articles. This search strategy involved 1) selection of search terms, 2) development of eligibility criteria, 3) comprehensive literature search, and 4) article review and selection. These steps are described below.

Selection of search terms

Search terms were selected so as to ensure that all potentially relevant articles were returned from database searches. In addition to general terms such as 'bird' and 'antibiotic resistance', additional terms relevant to potential methodologies (e.g. 'disc diffusion' and 'minimum inhibitory concentration') were also included. Although the database searches returned a high proportion of articles on non-wild birds (a large amount of research relating to the poultry industry has been conducted), a decision was made not to exclude any terms such as 'broiler' or 'poultry' from the search, as these terms are commonly used in the discussions of articles. The search terms were used in combination to find relevant articles. The full list of the searches is provided in Table 3.2.

Table 3.2 Search terms used

Search terms	
"antimicrobial resistance" AND bird	("minimum inhibitory concentration" OR MIC) AND bird
"antibiotic resistance" AND bird	"disc diffusion" AND bird
"antimicrobial sensitivity" AND bird	"antimicrobial resistant" AND bird
"antibiotic sensitivity" AND bird	(isolates OR bacteria) AND (resistan*) AND (antibiotic OR antimicrobial) AND (bird OR avian)

Development of eligibility criteria

A list of inclusion/exclusion criteria was developed so that once all potentially relevant articles were located in the preliminary database searches, eligible articles for inclusion in the meta-analysis could be readily identified. The criteria that served as the basis for inclusion in the meta-analysis are provided in Table 3.3.

Table 3.3 Eligibility criteria for inclusion in meta-anaylsis

Eligibility criteria
Study must focus on antibiotic resistance
Paper must present experimental results
Only samples from healthy, live birds were included, with the exception of studies in which animals were killed for the purposes of the investigation or studies in which animals were killed for other purposes but samples were taken immediately
Samples must be from 'wild' birds – that is, birds from which samples were taken must be described or classified within the study as 'wild', 'free-living' or 'feral'
Methods must include a complete list of antibiotics used to allow for comparisons of resistance profiles
Paper must not be an editorial, letter, or review article
Bird species name(s) must be provided
Full results must be provided
Article must be written in English
Full text must be available

Comprehensive literature search

It is widely accepted that a meta-analysis should be based upon a search of multiple databases (Sampson *et* al., 2003). Lemeshow *et al.* (2005) recommended more than two databases in meta-analyses of observational studies, and as a result, three databases were searched. Using the ISI Web of Knowledge, PubMed, and Scopus databases, comprehensive literature searches were conducted to identify articles for review. These databases were selected because they include articles from diverse fields (including wildlife and medicine), and represent databases frequently used in meta-analyses (Falagas *et al.*, 2008; Via *et al.*, 2011; Jayawardena *et al.*, 2012). The literature searches were conducted in July 2012. Potentially relevant articles were found using keywords in combination as search terms as described above. The search was limited to papers written in English published in peer-reviewed journals between 2002 and 2012. The details of each of the searches were documented, including the search terms used, the number of articles returned, and the details of each of the papers.

Article review and selection

From the list of all potentially relevant articles that were returned through the database searches, a selection process was undertaken to find those that could be used in the meta-analysis. This selection process involved two stages – a title/abstract review followed by a full text review. At each of these two stages, the papers were screened to determine whether or not they met the eligibility criteria (listed above). In the first stage (i.e. title/abstract review), papers were excluded on the basis of an irrelevant title (e.g. if the title implied the article focused on poultry, as opposed to wild birds). Each combination of search terms in this preliminary search returned anywhere between several dozen and several hundred papers; specific numbers are provided in the results section. Abstracts were read if there was any level of uncertainty regarding an article's eligibility. In the full text review, all remaining papers were read to evaluate eligibility with reference to criteria that were not apparent in the title and/or abstract. Spreadsheets were used to record the entire process; at each of the two stages, where an article was excluded, a note was made describing the reason(s) for exclusion (See Appendix 1 for examples of the databases that were compiled).

3.3.2 Data extraction and analysis

Data extraction

Once all eligible articles were identified, the process of extracting relevant data was undertaken so that the data analysis and systematic review could be conducted. Each of the eligible papers was scrutinized and data was extracted for use in analyses (Table 3.4). Information abstracted related both to information about the study design itself (e.g. methodology used to determine resistance, sample type, etc.) and to the results of resistance testing (e.g. the proportion of isolates sensitive/resistant to each of the antibiotics).

Table 3.4 Data extracted from each study

Information extracted
Year(s) of study
Country(s) where the study was conducted
Bird species sampled
Sample size (number of isolates)
Sample type (cloacal, faeces, etc.)
Antibiotics used (number, name)
Bacteria investigated (*E. coli*, *Salmonella*, *Enterococcus*, etc.)
Test of antibiotic resistance used (broth microdilution, disc diffusion, etc.)
Susceptibilities/resistances determined via analysis to each of the antibiotics
Suggested source of resistance (where provided)

To allow for comparison between studies that described the above information in different ways, additional research was conducted to allow the information extracted from the papers to be transformed into common metrics, as detailed in Table 3.5.

Table 3.5. Additional details acquired for the transformation of data into common metrics.

Additional information acquired
Taxonomy of species – family & order names. Most of the papers provided the species name(s) for the birds that were sampled. To allow for comparison between different groups of birds, the Order and Family names for each of the study species was obtained from the IUCN Red List database (online) and BirdLife international websites (BirdLife International, 2012; IUCN, 2012)
Habitat(s) & systems for bird species The detail provided about the birds' habitat(s) varied from paper to paper. In order to have a standardized/consistent description, the habitats were classed with reference to the online IUCN Habitats Classification Scheme (2007), where systems (terrestrial, marine, freshwater) for all bird species worldwide are provided.
Antibiotics – class Antibiotic were grouped into classes with reference to the Anatomical Therapeutic Chemical (ATC) classification system (WHO Collaborating Centre for Drug Statistics Methodology, 2011), an online searchable database that provides detailed information on the uses, classifications, and dosage guidelines for therapeutic drugs (including antibiotics). Updated on an annual basis, the ATC classification system is primarily intended as a resource for research on drug utilization on international levels. However, by providing a classification system, the database suited the purposes of this research as it enabled each of the antibiotics used in the studies to be classed into subgroups with reference to their chemical, pharmacological, and therapeutic subgroups.

The outcome of interest in this meta-analysis was the presence or absence of antibiotic resistance of bacterial samples or isolates to each of the antibiotics used in the included studies. For the purpose of this meta-analysis, data were extracted as counts, and where results were reported using alternative metrics (e.g. proportions), the necessary calculations were made to transform data as reported into counts.

Data analysis – study characteristics

Data were coded to allow for ease of analysis by assigning numerical codes to the various study characteristics. For example, each paper was read to determine what where the research was conducted (continent and country); research conducted in the Antarctic/Arctic was coded '1', Asia was coded '2', Europe was coded '3', North America was coded '4', and South America was coded '5'. Similarly, the kind of bacterial sample taken from birds for use in laboratory analysis; were extracted, cloacal swabs coded '1', faecal samples were coded '2', etc. Coding was undertaken for most of the data listed in Table 3.4. To provide an overview of the study characteristics in general, these data were compiled (with the exception of results of susceptibility testing, the analysis of which is described in the following section). The compiled results were analysed and represented graphically, to allow for an understanding of the overall

characteristics of the included studies – including their geographic location, kinds of birds sampled, and the quantity and range of antibiotics tested.

Data analysis – subgroup analyses and patterns of antibiotic resistance

One of the key attributes of a meta-analysis approach is that it allows for the synthesis of outcomes from multiple studies. In this investigation, the primary outcome measure was resistance, with reference to a range of antibiotics. Resistance was extracted as counts from each of the included papers if reported as such, or was ascertained based on the data provided (e.g. if reported as proportions, count data was calculated with reference to total sample size). Although data characteristics were calculated for each of the 73 antibiotics used across the 37 studies (as described in Section 2.2.2), patterns of resistance and subgroup analyses were conducted for ten antibiotics. The ten antibiotics for which these further analyses were conducted included the eight that were most frequently used in the included studies (used in ~50% of studies or more), in addition to two – not included in the eight – that were used in the other investigations undertaken for this thesis.

Statistical analyses for this component of the investigation were performed in the *R* statistical software package version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria). The *metaprop* function of the *meta* package (Schwarzer, 2008) was used for the meta-analysis of proportions with reference to subgroups, as described below. The first stage of this analysis was to determine the overall prevalence of resistance amongst the bird species to each of the ten antibiotics. Using the random effects model, the mean proportion of resistance and its 95% confidence interval was calculated across all studies to each of the ten antibiotics (Halpern, 2005). The random effects model was selected (as opposed to the fixed effects model) because it allowed for resistance to be considered across the entire population; variation in the effects between studies was anticipated due to effects being measured across various taxa, geographic regions, bacterial species, etc. (Gurevitch and Hedges, 1999; Halpern *et al.*, 2005; Valkama *et al.*, 2008; Rifkin *et al.*, 2012). For this part of the analysis, in studies that used more than one species of bird, the species were considered separately as individual case studies (Pyšek *et al.*, 2012). For each of the antibiotics for which analysis was undertaken, the random effects model parameters included the prevalence of resistance and the individual study sample size, which is important to include in comparing proportions between studies (Knapp and Hartung, 2003). The model is useful in that allows for examination of treatment effect and the associated standard error, and also it allows for comparisons to be made with reference to 'predictor' variables (Schwarzer, 2014). Therefore, in addition to pooling data to explore the overall patterns of resistance for each of the ten antibiotics, data were also analysed using the *R* statistical software package with reference to a selection of subgroups that were proposed as exploratory variables, that were factors hypothesized to explain variation in outcome (i.e. antibiotic resistance) (Valkama *et al.*, 2005; Schwarzer, 2014).

Ultimately, three factors were investigated to address the original hypotheses about geographic, taxonomic, and ecosystem-driven variation in antibiotic resistance: study location (continent), the taxonomic order of the bird species being investigated, and the system within which the bird was found (as defined by the IUCN Redlist). Summary forest plots were made in SPSS (Version 20.0.0) to visualize data for the subgroups under investigation (Anzures-Cabrera and Higgins, 2010). Formal statistical analyses were not conducted with reference to the subgroups; however, the visualization of results allowed for descriptive comparisons to be made – where 95% confidence intervals did not overlap, resistance was considered to be statistically different with reference to the characteristics under investigation within each of the three subgroups (Anzures-Cabrera and Higgins, 2010; Gillen *et al.*, 2010).

3.4 Results

3.4.1 Article identification

Of 5,546 studies returned from the searches of all three databases, a total of 444 studies met the criteria for inclusion in the meta-analysis on the basis of the title/abstract review (Table 3.1). Most of the studies that were excluded were ineligible because they were not on the topic of antibiotic resistance (1,192 papers, representing 23% of the studies excluded at this stage), or did not use wild birds as a study organism (2,009 studies, or 39% percent). Many of the 2,009 studies that did not use wild birds were focused on antibiotic resistance in an agricultural context, using samples primarily from poultry to evaluate food safety. Each of the three database searches returned duplicates – 130 (PubMed), 75 (Web of Science), and 126 (Scopus), for a total of 331 duplicates, all of which were excluded. The other reasons that papers were excluded included 728 (14%) that used inappropriate study organisms (e.g. other animals or environmental samples), 616 papers (12%) that did not publish results of an experiment (i.e. review papers), and 226 (4%) that used samples from sick or dead birds.

Following the title/abstract review, the potentially eligible papers from each of the three databases were combined. These 444 articles were then subjected to a full-text review, which resulted in the exclusion of 407 articles (92%). 33% of these were excluded because they were duplicates – that is, more than one database search returned the same paper. As with the title/abstract review, the full-text review excluded many studies (107, or 26%) because they used samples from captive birds. Other reasons that studies were excluded following the full-text review included 25 (6%) that did not provide complete results, 12 (3%) that did not provide information on the species of bird from which samples were taken, and 37 studies (9%) for which the full text was not accessible.

Following the full-text review, 37 articles were determined to be eligible for the systematic review and meta-analysis. This represented 0.7% of all papers returned from the preliminary database searches.

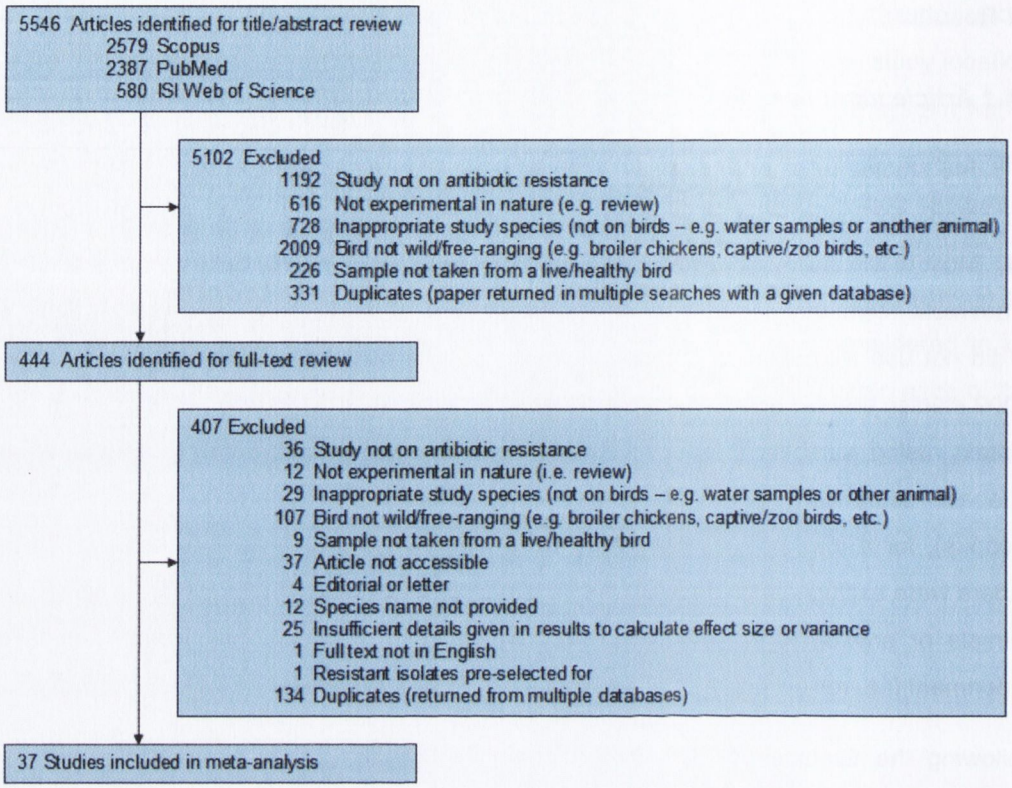


Figure 3.1 Search strategy and selection process flowchart detailing the 2-stage review process (title/abstract review and a full text review), with the number of articles returned and the reasons for article exclusion at each of the stages. From 5546 articles identified in the original search, a total of 37 were included in this meta-analysis.

3.4.2 Data analysis – study characteristics

Data extraction

The 37 included studies varied considerably with reference to the bird species used, the location where the study was conducted, and the bacterial species and antibiotics that were evaluated. Summary details of the 37 included papers are presented in Table 3.6, and an analysis of study characteristics is provided in the following section.

Code	Reference	Country	Species used (common names)	No. of isols	Sample type(s)	Bacteria tested	Antibiotics tested
001	Fogarty *et al.* (2003)	USA	Gulls	23	fecal	Enterococci	GEN, STR, VAN, AMP, TET
002	Dolejská *et al.* (2009)	Czech Republic	Black-headed gulls	134	cloacal	E. coli	GEN, STR, CEF, CAZ, CHL, AMP, AMC, CIP, NAL, SAS, SXT, TET
003	Radimersky *et al.* (2010)	Czech Republic	Rock doves	346	cloacal	Enterococci	GEN, STR, CEF, CAZ, VAN, ERY, CHL, AMP, AMC, CIP, NAL, SAS, SXT, TET
004	Literák *et al.* (2007)	Czech Republic	Rooks	220	feces	E. coli	GEN, STR, CEF, CAZ, CHL, AMP, AMC, CIP, NAL, SAS, SXT, TET
005	Bonnedahl *et al.* (2008)	Antarctica	Gentoo penguins	39	cloacal	Enterobacteriaceae	GEN, STR, IPM, CEF, CXM, CTX, CAZ, ATM, CHL, TMP, AMP, AMC, TZP, CIP, NAL, SMZ, TET
006	Literák *et al.* (2010)	Poland	Herring gulls	27	cloacal	E. coli	GEN, STR, CEF, CAZ, CHL, AMP, AMC, CIP, NAL, SAS, SXT, TET
007	Nascimento *et al.* (2003)	Brazil	Rusty-breasted Nunlet, White-barred Piculet, Black-billed Scythebill, Ochre-cheeked Spinetail, Pale-legged Hornero, White-collared Foliage-gleaner, Scalopped Antbird, Slaty Antshrike, Slender Antbird, White-shouldered Fire-eye, Black-cheeked Gnateater, Ash-throated Casiornis, Yellow-breasted Flycatcher, White-bearded Manakin, Violaceous Euphonia, Creamy-bellied Thrush	191	cloacal	Gram negative	KAN, STR, CHL, AMP, TET
008	Waldenström *et al.* (2005)	Sweden	Blackbird, Broad-billed Sandpiper, Curlew Sandpiper, Dunlin, Fieldfare, Little Stint, Long-eared Owl, Mistle Thrush, Redwing, Song thrush, Black-headed Gull, Wood Sandpiper	137	cloacal	Campylobacter	GEN, NEO, STR, MET, DOX, ERY, CHL, AMP, CIP, NAL
010	Dolejská *et al.* (2008)	Czech Republic	House Sparrow	54	cloacal	E. coli	GEN, STR, CEF, CAZ, CHL, TMP, AMP, AMC, CIP, NAL, SAS, TET
012	Kitadai *et al.* (2012)	Japan	Hooded Crane and White-naped Crane	138	feces	E. coli	GEN, KAN, CFZ, OXO, ORB, ENR, FOF, CHL, AMP, CST, OXY, NAL, MIN
013	Dobbin *et al.* (2005)	Canada	Double-crested Cormorant	187	cloacal	E. coli	GEN, NEO, STR, CEF, ENR, CHL, AMX, AMP, CIP, SXT, TET
014	Hughes *et al.* (2008)	England	House Sparrow, Common Starling	3	fecal	Salmonella	AMK, FOX, CPD, CHL, AMP, AMC, OXY, CIP, NAL, SXT

015	Bonnedahl *et al.* (2010)	Sweden	Black-headed Gull	83	cloacal	E. coli	NIT, STR, MEC, CFR, FOF, CHL, TGC, TMP, AMP, NAL, SMZ, TET
016	Radhouani *et al.* (2010)	Portugal	Common Buzzard	10	fecal	E. coli	AMK, TOB, GEN, STR, IPM, FOX, CTX, CAZ, ATM, CHL, AMP, AMC, CIP, NAL, SXT, TET
017	Silva *et al.* (2009)	Brazil	Rock Dove	182	fecal	E. coli	AMK, GEN, CAZ, CRO, AMP, SAM, LVX, SXT
018	Bonnedahl *et al.* (2009)	France	Yellow-legged Gull	153	cloacal	E. colli	STR, CFR, CHL, AMP, NAL, TET
019	Sjölund *et al.* (2008)	Arctic (Russia, USA, Greenland)	Western Sandpiper, Vega/Glaucous Gull, Emperor Goose/Brent Goose, Iceland Gull/Glaucous Gull, Dunlin, Red-necked stint, Yellow wagtail, Black-legged Kittiwake, Spoon-billed sandpiper, Red-necked phalarope, Brent goose, Snow goose, Pintail	97	Fecal or cloacal	E. coli	GEN, NIT, STR, MEC, IPM, CFR, CXM, CPD, CHL, TGC, TMP, AMP, FOT, CIP, NAL, SMZ, TET
020	Middleton & Ambrose (2005)	USA	Canada Goose	2186	feces	E. coli, Enterococci	GEN, STR, PEN, CEF, CHL, AMP, CIP, STZ, TET
021	Gaukler *et al.* (2009)	USA	European Starling	206	feces or intestinal content	E. coli	AMK, GEN, KAN, STR, FOX, CTF, CRO, SSZ, CHL, AMP, AMC, CIP, NAL, SXT, TET
022	Cole *et al.* (2005)	USA	Canada Goose	48	fecal or cloacal	E. coli	AMK, APR, GEN, KAN, STR, IPM, CEF, FOX, CTF, CRO, CHL, AMP, AMC, CIP, NAL, SMZ, SXT, TET
023	Hernandez *et al.* (2010)	Russia	Glaucous-winged Gull, Tufted Puffin, Black-headed Gull, Cormorant/Shag, Red-legged Kittiwake, Red-legged Kittiwake/Black-legged Kittiwake, Rock Sandpiper, Pigeon Guillemot, Red-faced Cormorant, Black-legged Kittiwake	145	faecal or cloacal	E. coli	NIT, STR, MEC, CFR, FOF, CHL, TGC, TMP, AMP, NAL, SMZ, TET
024	Dolejská *et al.*(2007)	Czech Republic	Black-headed Gull	257	cloacal	E. coli	STR, CEF, CAZ, CHL, AMP, NAL, SAS, TET
025	Mirzaie *et al.* (2010)	Iran	House Sparrow	18	Gastro tract	Salmonella	NEO, LIN, FLU, AMP, FLO, NOR, STM, TET
026	Ghanbarpour & Daneshdoost (2012)	Iran	Rock Dove	138	fecal	E. coli	STR, LIN, ENR, FLU, CHL, AMP, OXY, TET
027	Gibbs *et al.* (2007)	USA	Yellow-headed Blackbird	33	fecal	Enterobacteriaceae	AMK, GEN, KAN, STR, CEF, CTF, SSZ, CHL, AMP, AMC, NAL, TET
028	Feng *et al.* (2009)	China	Red-crowned Crane	26	fecal	Campylobacter	AMK, GEN, KAN, STR, PEN, LEX, CEC, CFP, CTX, DOX, CLI, AZM,

							ERY, AMX, AMP, OXY, CIP, LVX, NAL, NOR, OFX, TET
029	Vigo *et al.* (2011)	Antarctica	Adelie Penguin, Skua, Kelp Gull	15	fecal	Salmonella	GEN, NIT, CTX, FOF, CHL, AMP, POL, CIP, NAL, SXT, TET
032	Radhouani *et al.* (2011)	Portugal	Caspian Gull	42	fecal	Enterococci	GEN, KAN, STR, TEC, VAN, ERY, CHL, AMP, CIP, Q-D, TET
034	Da Silva *et al.* (2011)	Brazil	Rock Dove	120	fecal	Enterococci	GEN, PEN, VAN, CHL, CIP, RIF, TET
035	Rybaříková *et al.* (2010)	Czech Republic	House Martin	309	fecal	E. coli	GEN, STR, CEF, CAZ, CHL, AMP, AMC, CIP, NAL, SAS, SXT, TET
036	Stoddard *et al.*, (2008)	USA	Western Gull	9	fecal	Salmonella	AMK, GEN, CFZ, CTF, ZOX, ENR, CHL, AMP, AMC, TIM, SXT, TET
038	Palmgren *et al.*, (2006)	Sweden	Black-headed Gull	28	cloacal	Salmonella	GEN, STR, SSZ, CHL, TMP, AMP, CIP, NAL
039	Poeta *et al.*, (2008)	Portugal	Caspian Gull	11	fecal	Enterobacteriaceae	AMK, TOB, GEN, STR, IPM, FOX, CTX, CAZ, ATM, CHL, AMP, AMC, CIP, NAL, SXT, TET
040	Timko & Kmet (2003)	Slovakia	Alpine Accentor	34	fecal	Enterobacteriaceae	AMK, GEN, CXM, CTX, CPM, CAZ, CPO, AMP, SAM, PSB, PIP, CIP, SXT
041	Han *et al.*, (2011)	South Korea	Wild Goose	155	fecal	Enterococci	GEN, KAN, STR, VAN, ERY, CHL, AMP, CIP, TET
042	Radhouani *et al.*, (2012)	Portugal	Common Buzzard	67	fecal	E. coli, Enterococci	AMK, TOB, GEN, KAN, STR, IPM, FOX, CTX, CAZ, TEC, VAN, ERY, ATM, CHL, AMP, AMC, CIP, NAL, Q-D, SXT, TET
043	Camarda *et al.*, (2006)	Italy	Audouin's Gull	48	cloacal	E. coli	AMK, APR, GEN, NEO, DIF, ENR, FLU, AMX, CST, NAL, SXT, TET

Table 3.6 Details of included studies, in order of the code that they were assigned. Abbreviations for antibiotics from the American Society for Microbiology (http://aac.asm.org) as follows: AMC (Amoxicillin-clavulanic acid), AMK (Amikacin), AMP (Ampicillin), AMX (Amoxicillin), APR (Apramycin), ATM (Aztreonam), AZM (Azithromycin), CAZ (Ceftazidime), CEC (Cefaclor), CEF (Cephalothin), CFP (Cefoperazone), CFR (Cefadroxil), CFZ (Cefazolin), CHL (Chloramphenicol), CIP (Ciprofloxacin), CLI (Clindamycin), CPD (Cefpodoxime), CPM (Cefpiramide), CPO (Cefpirome), CRO (Ceftriaxone), CST (Colistin), CTF (Ceftiofur), CTX (Cefotaxime), CXM (Cefuroxime), DIF (Difloxacin), DOX (Doxyclycline), ENR (Enrofloxacin), ERY (Erythromycin), FLO (Florfenicol), FLU (Flumequine), FOF (Fosfomycin), FOT (Fosfomycin-Trometamol), FOX (Cefoxitin), GEN (Gentamicin), IPM (Imipenem), KAN (Kanamycin), LEX (Cephalexin), LIN (Lincospectin), LVX (Levofloxacin), MEC (Mecillinam), MET (Metronidazole), MIN (Minocycline), NAL (Nalidixic Acid), NEO (Neomycin), NIT (Nitrofurantoin), NOR (Norfloxacin), OFX (Ofloxacin), ORB (Orbifloxacin), OXO (Oxolinic acid), OXY (Oxytetracycline), PEN (Penicillin G), PIP (Piperacillin), POL (Polymyxin), PSB (Piperacillin-sulbactam), Q-D (Quinupristin-dalafopristin), RIF (Rifampin), SAM (Ampicillin-sulbactam), SAS (Sulfonamides), SMZ (Sulfamethoxazole), SSZ (Sulfisoxazole), STM (Sultrim), STR (Streptomycin), STZ (Sulfathiazole), SXT (Trimethoprim-sulfamethoxazole), TEC (Teicoplanin), TET (Tetracycline), TGC (Tigecycline), TIM (Ticarcillin-clavulanic acid), TMP (Trimethoprim), TOB (Tobramycin), TZP (Piperacillin-tazobactam), VAN (Vancomycin), ZOX (Ceftizoxime).

Details from each of the papers were extracted and compiled into tables that describe the study characteristics, the results of which are provided in the following sections.

Study location

The included papers described research conducted in five continents (Table 3.7). The largest proportion of these (48.7%) represents those that were conducted in Europe, followed by North America (18.9%) and Asia (16.2%). The remaining studies were conducted in the Arctic/Antarctic and South America (both representing 8.1% of studies). None of the included studies were conducted in Africa or Australia.

Table 3.7 Continents in which studies were conducted.

Continent	n (%)
Europe	18 (48.7%)
North America	7 (18.9%)
Asia	6 (16.2%)
Arctic/Antarctic	3 (8.1%)
South America	3 (8.1%)

The research included samples collected within eighteen countries (Table 3.8). Seven of the included studies were conducted in the United States – the country in which the largest proportion of the research was conducted (18.9% of all studies). An additional six studies (16.2%) were carried out in the Czech Republic, while four (10.8%) were conducted in Portugal.

Table 3.8 Countries in which studies were conducted.

Country	n* (%)
USA	7 (18.9)
Czech Republic	6 (16.2)
Portugal	4 (10.8)
Brazil	3 (8.1)
Sweden	3 (8.1)
Antarctica	2 (5.4)
Iran	2 (5.4)
Russia	2 (5.4)
Canada	1 (2.7)
China	1 (2.7)
England	1 (2.7)
France	1 (2.7)
Greenland	1 (2.7)
Italy	1 (2.7)
Japan	1 (2.7)
Poland	1 (2.7)
Slovakia	1 (2.7)
South Korea	1 (2.7)

*n = 37 studies. The total is greater than 37 (100%) because one study (Sjolund *et al.*) used samples collected in more than one country.

Species of bird used – taxonomy and habitats

The majority of studies (83.7%) used one species of bird (Table 3.9). Only six studies (26.3%) used two or more species. Nascimento *et al.*, 2003 used the largest number of species (sixteen), while Waldenstrom *et al.* (2005) and Sjolund *et al.* (2008) used twelve and thirteen species, respectively.

Table 3.9 Number of species of bird used per study

Number of species used	Number of studies (%)
1	31 (83.8)
2	1 (2.7)
4	1 (2.7)
10	1 (2.7)
12	1 (2.7)
13	1 (2.7)
16	1 (2.7)

A total of 74 species of birds were used in the 37 studies. The 74 species belonged to a total of eleven orders (Table 3.10). The majority belonged to the Passeriformes (passerines) and Charadriiformes (gulls, button-quails, plovers, and allies), representing 36.5% and 35.1%, respectively, of the 74 species used. The other nine orders were less well represented, between them comprising a total of 28.4% of the total species used.

Table 3.10 Taxonomic order of bird species used.

Order	Number of species* (%)
Passeriformes	27 (36.5%)
Charadriiformes	26 (35.1%)
Anseriformes	7 (9.5%)
Pelecaniformes	3 (4.1%)
Falconiformes	2 (2.7%)
Gruiformes	2 (2.7%)
Piciformes	2 (2.7%)
Sphenisciformes	2 (2.7%)
Columbiformes	1 (1.4%)
Procellariiformes	1 (1.4%)
Strigiformes	1 (1.4%)

*Number of species used within each of the orders.

The 74 species used in the studies represent a total of 29 families; the most commonly used families were the Laridae (14 different species), Scolopacidae (9 different species), and Anatidae (7 different species) (Table 3.11).

Table 3.11 Taxonomic family of bird species used.

Order	Number of species* (%)
Laridae	14 (18.9%)
Scolopacidae	9 (12.2%)
Anatidae	7 (9.5%)
Turdidae	6 (8.1%)
Thamnophilidae	4 (5.4%)
Furnariidae	3 (4.1%)
Phalacrocoracidae	3 (4.1%)
Accipitridae	2 (2.7%)
Alcidae	2 (2.7%)
Gruidae	2 (2.7%)
Spheniscidae	2 (2.7%)
Sturnidae	2 (2.7%)
Tyrannidae	2 (2.7%)
Bucconidae	1 (1.4%)
Columbidae	1 (1.4%)
Conopophagidae	1 (1.4%)
Corvidae	1 (1.4%)
Dendrocolaptidae	1 (1.4%)
Hirundinidae	1 (1.4%)
Icteridae	1 (1.4%)
Motacillidae	1 (1.4%)
Passeridae	1 (1.4%)
Picidae	1 (1.4%)
Pipridae	1 (1.4%)
Procellariidae	1 (1.4%)
Prunellidae	1 (1.4%)
Stercorariidae	1 (1.4%)
Strigidae	1 (1.4%)
Thraupidae	1 (1.4%)

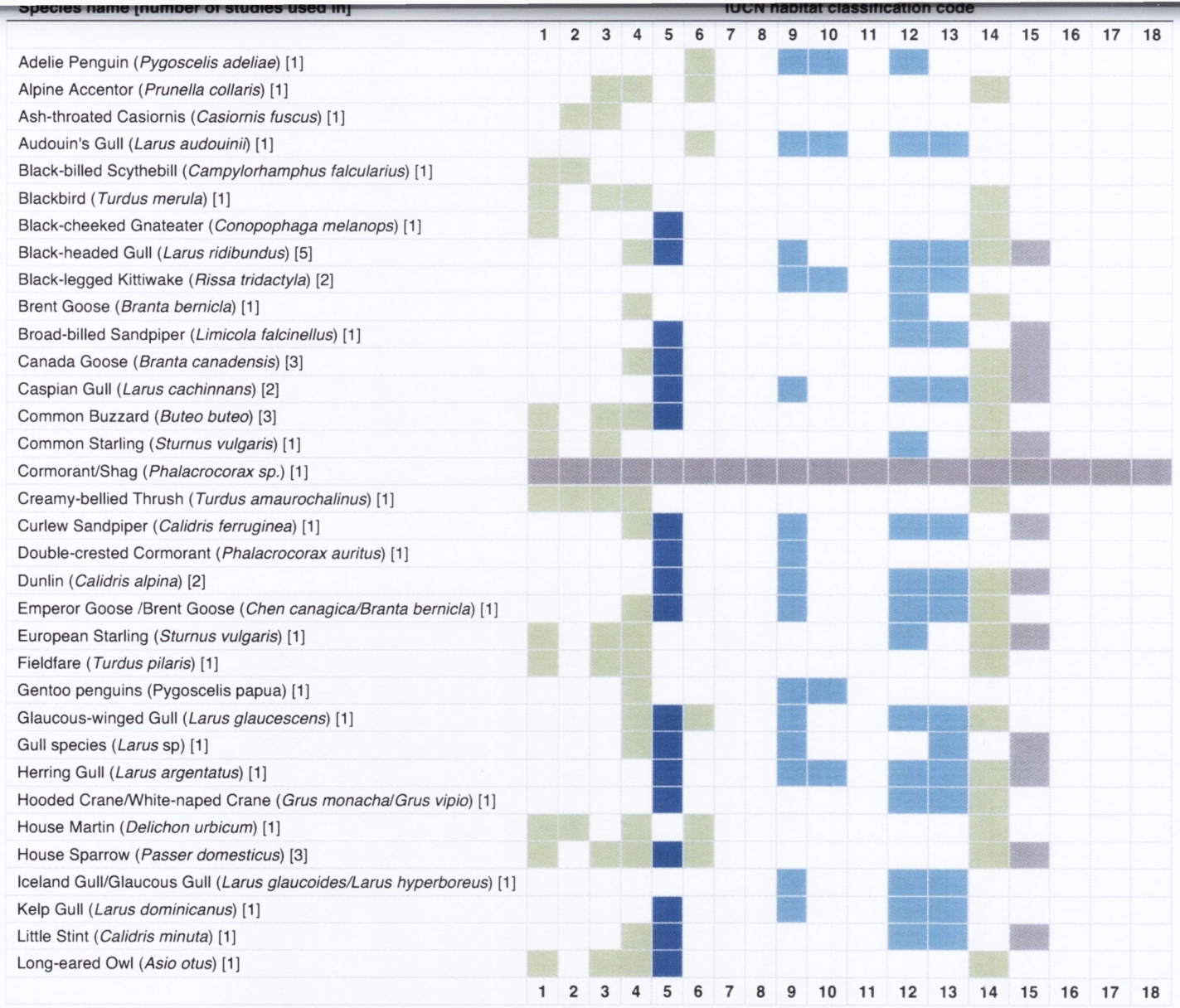
*Number of species used within each of the families.

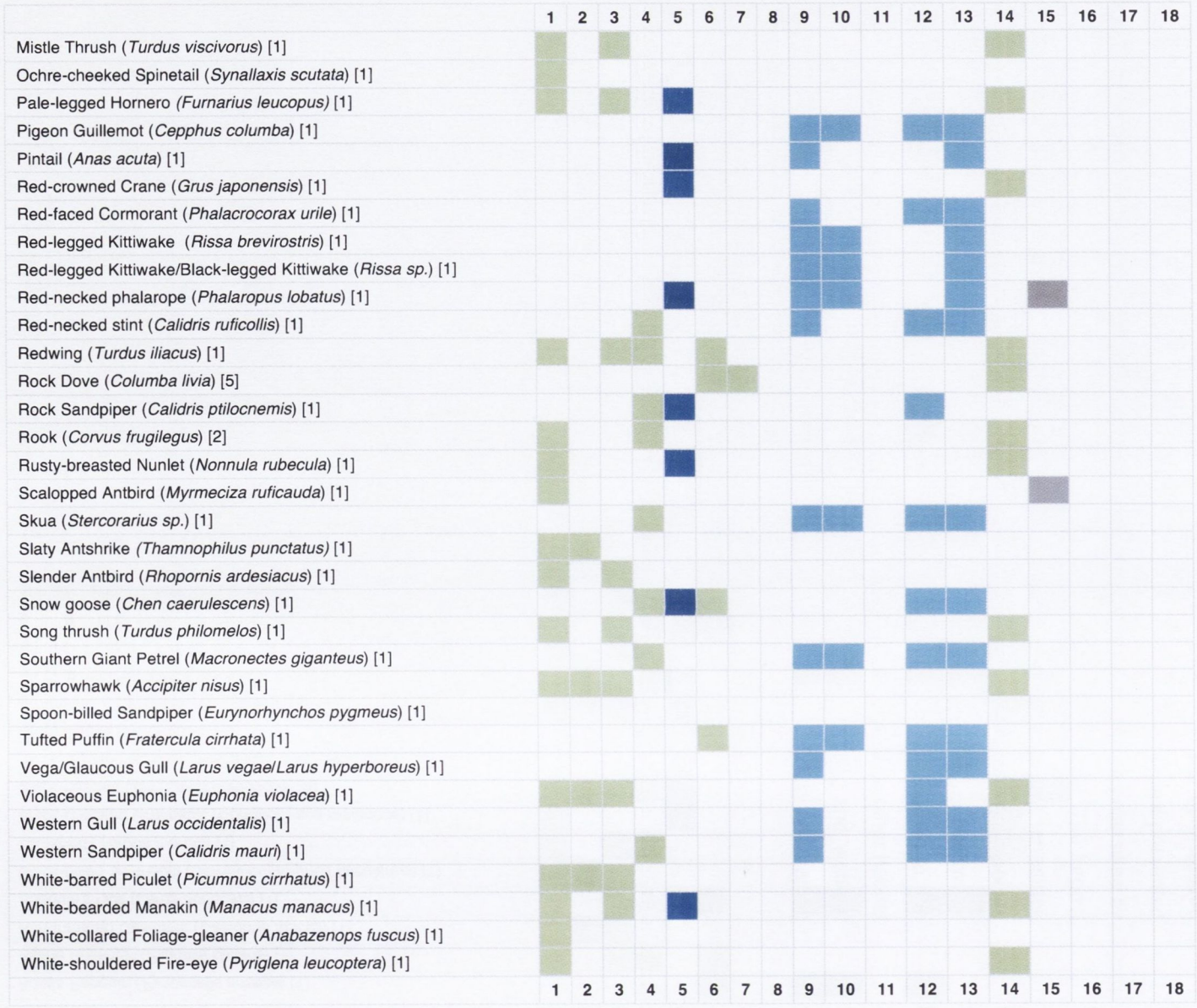
With regards to the species used, there was very little repetition between the studies, with the exception of the Black-headed Gull (*Larus ridibundus*) and the Rock Dove (*Columba livia*),

both of which were used in five of the included studies; the House Sparrow (*Passer domesticus*) which was used in three studies; and the Canada Goose (*Branta canadensis*), Caspian Gull (*Larus cachinnans*), Dunlin (*Calidris alpina*), and Common Buzzard (*Buteo buteo*), each of which were used in two studies.

System(s) and habitat type(s)

The species used were found to inhabit a variety of systems and habitats, which were classed with reference to the IUCN online database. The species used in the 37 studies are presented in Table 3.12, which also provides information on the system(s) that it inhabits (as classified by the IUCN), as well as the more specific habitat(s) in which the species is found. The largest proportion of species occupied either terrestrial (28%) or a combination of terrestrial, freshwater, and marine (28%) systems (Table 3.13). Slightly less common were species occupying a combination of terrestrial & marine systems (22.9%) or terrestrial & freshwater systems (17.6%). In terms of habitats that the species were designated as belonging to, the largest proportion (50%) belonged to the 'artificial – terrestrial' habitat class, while other habitat classes to which the species used belonged included 'marine (intertidal)' (43.2%) and 'wetland – inland' (40.2%) (Table 3.14). Most of the species were assigned to more than one habitat class, thus the summed proportions exceed 100%.


Species name [number of studies used in]
IUCN habitat classification code
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Adelie Penguin (Pygoscelis adeliae) [1]
Alpine Accentor (Prunella collaris) [1]
Ash-throated Casiornis (Casiornis fuscus) [1]
Audouin's Gull (Larus audouinii) [1]
Black-billed Scythebill (Campylorhamphus falcularius) [1]
Blackbird (Turdus merula) [1]
Black-cheeked Gnateater (Conopophaga melanops) [1]
Black-headed Gull (Larus ridibundus) [5]
Black-legged Kittiwake (Rissa tridactyla) [2]
Brent Goose (Branta bernicla) [1]
Broad-billed Sandpiper (Limicola falcinellus) [1]
Canada Goose (Branta canadensis) [3]
Caspian Gull (Larus cachinnans) [2]
Common Buzzard (Buteo buteo) [3]
Common Starling (Sturnus vulgaris) [1]
Cormorant/Shag (Phalacrocorax sp.) [1]
Creamy-bellied Thrush (Turdus amaurochalinus) [1]
Curlew Sandpiper (Calidris ferruginea) [1]
Double-crested Cormorant (Phalacrocorax auritus) [1]
Dunlin (Calidris alpina) [2]
Emperor Goose /Brent Goose (Chen canagica/Branta bernicla) [1]
European Starling (Sturnus vulgaris) [1]
Fieldfare (Turdus pilaris) [1]
Gentoo penguins (Pygoscelis papua) [1]
Glaucous-winged Gull (Larus glaucescens) [1]
Gull species (Larus sp) [1]
Herring Gull (Larus argentatus) [1]
Hooded Crane/White-naped Crane (Grus monacha/Grus vipio) [1]
House Martin (Delichon urbicum) [1]
House Sparrow (Passer domesticus) [3]
Iceland Gull/Glaucous Gull (Larus glaucoides/Larus hyperboreus) [1]
Kelp Gull (Larus dominicanus) [1]
Little Stint (Calidris minuta) [1]
Long-eared Owl (Asio otus) [1]
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Mistle Thrush (Turdus viscivorus) [1]
Ochre-cheeked Spinetail (Synallaxis scutata) [1]
Pale-legged Hornero (Furnarius leucopus) [1]
Pigeon Guillemot (Cepphus columba) [1]
Pintail (Anas acuta) [1]
Red-crowned Crane (Grus japonensis) [1]
Red-faced Cormorant (Phalacrocorax urile) [1]
Red-legged Kittiwake (Rissa brevirostris) [1]
Red-legged Kittiwake/Black-legged Kittiwake (Rissa sp.) [1]
Red-necked phalarope (Phalaropus lobatus) [1]
Red-necked stint (Calidris ruficollis) [1]
Redwing (Turdus iliacus) [1]
Rock Dove (Columba livia) [5]
Rock Sandpiper (Calidris ptilocnemis) [1]
Rook (Corvus frugilegus) [2]
Rusty-breasted Nunlet (Nonnula rubecula) [1]
Scalopped Antbird (Myrmeciza ruficauda) [1]
Skua (Stercorarius sp.) [1]
Slaty Antshrike (Thamnophilus punctatus) [1]
Slender Antbird (Rhopornis ardesiacus) [1]
Snow goose (Chen caerulescens) [1]
Song thrush (Turdus philomelos) [1]
Southern Giant Petrel (Macronectes giganteus) [1]
Sparrowhawk (Accipiter nisus) [1]
Spoon-billed Sandpiper (Eurynorhynchos pygmeus) [1]
Tufted Puffin (Fratercula cirrhata) [1]
Vega/Glaucous Gull (Larus vegae/Larus hyperboreus) [1]
Violaceous Euphonia (Euphonia violacea) [1]
Western Gull (Larus occidentalis) [1]
Western Sandpiper (Calidris mauri) [1]
White-barred Piculet (Picumnus cirrhatus) [1]
White-bearded Manakin (Manacus manacus) [1]
White-collared Foliage-gleaner (Anabazenops fuscus) [1]
White-shouldered Fire-eye (Pyriglena leucoptera) [1]
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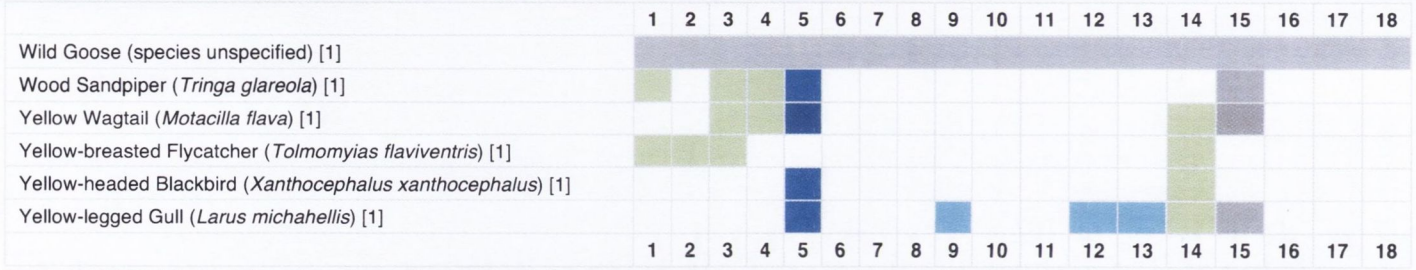


Table 3.12 Habitat and system types for each of the species used in the included studies. Both habitats and systems were classed with reference to the IUCN Red List online database.

Code	Habitat type
1	Forest and woodland
2	Savanna
3	Shrubland
4	Native grassland
5	Wetlands (inland)
6	Inland rocky areas
7	Caves & subterranean habitats
8	Desert
9	Marine - neritic
10	Marine - oceanic
11	Marine - deep ocean floor
12	Marine - intertidal
13	Marine - coastal/supratidal
14	Artificial - terrestrial
15	Artificial - aquatic
16	Introduced vegetation
17	Other
18	Unknown

System type
Terrestrial
Freshwater
Marine
Unknown/Cannot be classified

System – overall counts

Table 3.13 System(s) of birds used in the studies.

System	*n (%)
Terrestrial	21 (28.4%)
Terrestrial; Freshwater; Marine	21 (28.4%)
Terrestrial; Marine	17 (22.9%)
Terrestrial; Freshwater	13 (17.6%)
Unknown	2 (2.7%)

*n = 74 total species of birds used in the studies. Systems defined using the online IUCN Red List database (IUCN, 2012).

Habitat types – overall counts

Table 3.14 Habitat type(s) of the 74 bird species used in the studies.

Habitat type	n* (%)
Artificial - terrestrial	37 (50.0%)
Marine - intertidal	32 (43.2%)
Wetlands (inland)	30 (40.5%)
Forest and woodland	29 (39.2%)
Native grassland	29 (39.2%)
Marine - neritic	29 (39.2%)
Marine - coastal/supratidal	29 (39.2%)
Shrubland	22 (29.7%)
Artificial - aquatic	17 (23.0%)
Marine - oceanic	12 (16.2%)
Inland rocky areas	10 (13.5%)
Savanna	9 (12.2%)
Unknown	2 (2.7%)
Caves and subterranean habitats	1 (1.4%)
Desert	0 (0%)
Introduced vegetation	0 (0%)
Marine - deep ocean floor (benthic and demersal)	0 (0%)
Other	0 (0%)

*Total number of occurrences of habitat types is greater than 74 because many species were listed as occurring in multiple habitats. Habitat types were classed with reference to the online IUCN Red List Database.

Sample & isolate characteristics – sample type (cloacal, fecal, etc.)

The majority of studies (51.4%) used faecal samples, while most of the others (35.1%) used cloacal swabs for bacterial sample collection (Table 3.15). One study (2.7%) used a sample from the gastrointestinal tract. The remaining four studies used multiple sample types.

Table 3.15 Types of samples used in the 37 studies.

Sample type	n (%)
Faecal	19 (51.4)
Cloacal	13 (35.1)
Faecal or cloacal	3 (8.1)
Gastrointestinal tract	1 (2.7)
Faecal or gastrointestinal tract	1 (2.7)

Sample & isolate characteristics – bacterial species used in analysis

E. coli was the most frequently used species of bacteria, and was used in 21 of the studies (Table 3.16). *Enterococci* species were the second most common (used in 7 studies), while *Salmonella* were the third most commonly used (Table 3.16). One study (Nascimento *et al* 2003) identified bacteria only as Gram-negative.

Table 3.16 Bacteria species used in the 37 studies.

Bacteria species	n*
E. coli	21
Enterococci	7
Salmonella	6
Enterobacteriaceae	3
Campylobacter	2
Gram negative	1

*The sum is greater than 37 because several studies investigated multiple bacterial species

Antibiotics used – number of antibiotics tested per study

Between the 37 studies, a total of 73 different antibiotics were used in antibiotic resistance testing. The number of antibiotics used within each study ranged from five (Fogarty *et al.*, 2003) to 22 (Feng *et al.*, 2009), while the majority of the studies (70.3%) used twelve or fewer antibiotics (Table 3.17). Studies using either eight or twelve antibiotics were the most common (13.5% and 27.0% of included studies, respectively), likely corresponding to the number of antibiotic discs typically used in disc diffusion testing (the disc ejection machines typically eject either eight or twelve discs, depending on the size of petri dish being used).

Table 3.17 Number of antibiotics used in the 37 studies.

Number of antibiotics used	n (%)
5	2 (5.4)
6	1 (2.7)
7	1 (2.7)
8	5 (13.5)
9	2 (5.4)
10	2 (5.4)
11	3 (8.1)
12	10 (27.0)
13	2 (5.4)
14	1 (2.7)
15	1 (2.7)
16	2 (5.4)
17	2 (5.4)
18	1 (2.7)
19	0
20	0
21	1 (2.7)
22	1 (2.7)

Antibiotics used – names and classes

Used in 35 of the 37 included studies (94.6%), ampicillin was the most frequently used antibiotic in resistance testing (Table 3.18). Other antibiotics that were frequently used included chloramphenicol, tetracycline, gentamycin, and streptomycin (83.8%, 81.1%, 78.4%, and 75.7% of studies, respectively). There were 25 antibiotics that were only used in one study, while another 20 were used in only two or three studies.

Table 3.18 Antibiotics used in the 37 included studies.

Antibiotic	n studies (%)
Aminoglycoside antibacterials	
Amikacin	12 (32.4)
Apramycin	2 (5.4)
Gentamicin	29 (78.4)
Kanamycin	9 (24.3)
Neomycin	4 (10.8)
Nitrofurantoin	4 (10.8)
Streptomycin	28 (75.7)
Tobramycin	3 (8.1)
Amphenicols	
Chloramphenicol	31 (83.8)
Beta-Lactams, Penicillins	
Amoxicillin	3 (8.1)
Amoxicillin-clavulanic acid	15 (40.5)
Ampicillin	35 (94.6)
Ampicillin-sulbactam	2 (5.4)
Florfenicol	1 (2.7)
Mecillinam	3 (8.1)
Metronidazole	1 (2.7)
Piperacillin	1 (2.7)
Piperacillin-sulbactam	1 (2.7)
Piperacillin-tazobactam	1 (2.7)
Ticarcillin-clavulanic acid	1 (2.7)
Macrolides, Lincosamides and streptogramins	
Azithromycin	1 (2.7)
Clindamycin	1 (2.7)
Erythromycin	6 (16.2)
Quinupristin-dalafopristin	2 5.4)
Other antibacterials	
Colistin	2 (5.4)
Polymyxin	1 (2.7)
Teicoplanin	2 (5.4)
Vancomycin	6 (16.2)
Other Beta-Lactam antibacterials	
Aztreonam	4 (10.8)

Cefaclor	1 (2.7)
Cefadroxil	4 (10.8)
Cefazolin	2 (5.4)
Cefoperazone	1 (2.7)
Cefotaxime	7 (18.9)
Cefoxitin	6 (16.2)
Cefpiramide	1 (2.7)
Cefpirome	1 (2.7)
Cefpodoxime	2 (5.4)
Ceftazidime	13 (35.1)
Ceftiofur	4 (10.8)
Ceftizoxime	1 (2.7)
Ceftriaxone	3 (8.1)
Cefuroxime	3 (8.1)
Cephalexin	1 (2.7)
Cephalothin	12 (32.4)
Imipenem	6 (16.2)
Phosphonic acid derivatives	
Fosfomycin-Trometamol	1 (2.7)
Quinolone antibacterials	
Ciprofloxacin	24 (64.9)
Difloxacin	1 (2.7)
Doxyclycline	2 (5.4)
Enrofloxacin	5 (13.5)
Flumequine	3 (8.1)
Fosfomycin	4 (10.8)
Levofloxacin	2 (5.4)
Lincospectin	2 (5.4)
Nalidixic Acid	25 (67.6)
Norfloxacin	2 (5.4)
Ofloxacin	1 (2.7)
Orbifloxacin	1 (2.7)
Oxolinic acid	1 (2.7)
Oxytetracycline	4 (10.8)
Rifamycins	
Rifampin	1 (2.7)
Sulfonamides and trimethoprims	
Sulfamethoxazole	5 (13.5)
Sulfathiazole	1 (2.7)
Sulfisoxazole	3 (8.1)
Sulfonamides	7 (18.9)
Sultrim	1 (2.7)
Trimethoprim	6 (16.2)
Trimethoprim-sulfamethoxaxole	18 (48.6)
Tetracyclines	
Minocycline	1 (2.7)
Penicillin G	3 (8.1)
Tetracycline	30 (81.1)
Tigecycline* (new – glyclyclines)	3 (8.1)

Antibiotic were grouped into classes with reference to the Anatomical Therapeutic Chemical (ATC) classification system (WHO Collaborating Centre for Drug Statistics Methodology, 2011). The 73 different antibiotics that were used in the included studies belong to 11 classes. The most frequently used class of antibiotic were those belonged to 'Other Beta-Lactam Antibacterials' (19 of the 73 antibiotics used), followed by the 'Quinolone Antibacterials' (11 of the 73 antibiotics used) (Table 3.19).

Table 3.19 Classes of antibiotic represented amongst those used in the 37 included studies.

Antibiotic class	n (% of all antibiotics)
Aminoglycoside antibacterials	8 (10.8)
Amphenicols	1 (1.4)
Beta-Lactams, Penicillins	11 (14.9)
Macrolides, Lincosamides and streptogramins	4 (5.4)
Other antibacterials	4 (5.4)
Other Beta-Lactam antibacterials	19 (25.7)
Phosphonic acid derivitive	1 (1.4)
Quinolone antibacterials	14 (18.9)
Rifamycin	1 (1.4)
Sulfonamides and trimethoprim	7 (9.5)
Tetracyclines	4 (5.4)

3.4.3 Data analysis – subgroup analyses and patterns of resistance

Summary – prevalence of antibiotic resistance

Antibiotic resistant bacteria were identified in 34 of the 37 included studies, that is, at least one isolate in each of these 34 studies exhibited resistance to one or more of the antibiotics that were tested. In the other three studies, no antibiotic resistance was reported. Two of the three studies in which no antibiotic bacteria were identified were conducted in Antarctica, in penguins (Bonnedahl *et al.*, 2008) and in multiple bird species (Vigo *et al.*, 2011), while the third study that reported no resistance used a variety of wild bird species in England (Hughes *et al.*, 2008). In total, across the 34 studies in which resistant bacteria were identified, each of the bacteria genera or species demonstrated resistance, including *Campylobacter*, *E. coli*, *Enterobacteriaceae*, *Enterococci*, *Salmonella*, and unspecified Gram-negative isolates. Resistance was reported for 51 of the 73 antibiotics used across the studies; the 22 antibiotics for which no resistance was reported were as follows: Apramycin, Azithromycin, Cefazolin,

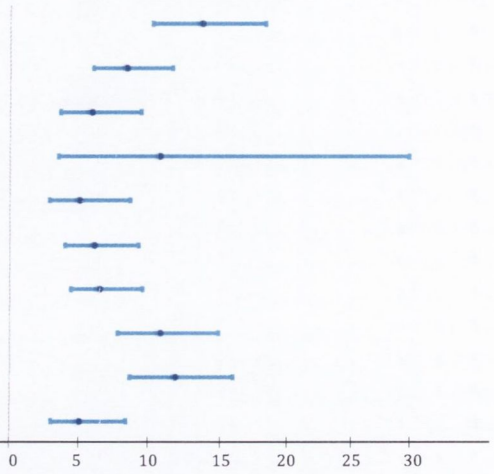
Cefaclor, Cefpiramide, Ceftriaxone, Cefpirome, Clindamycin, Colistin, Florfenicol, Norfloxacin, Ofloxacin, Oxolinic acid, Orbifloxacin, Piperacillin, Piperacillin-sulbactam, Piperacillin-tazobactam, Polymyxin, Sultrim Teicoplanin, Ticarcillin-clavulanic acid, and Tigecycline. Statistical analyses were conducted in R on ten of the 51 antibiotics to which resistance was demonstrated, to allow for the description of patterns of resistance with reference to subgroups. The results of these analyses are presented in the following section.

Subgroup analyses and patterns of resistance

The results of the meta-analysis using the random effects model indicated that resistance to the ten antibiotics ranged from 5.14% of samples (95% C.I. 3.02-8.63) for trimethoprim-sulfamethoxazole, to 14.41% (95% C.I. 10.72-19.09) for ampicillin (Table 3.20). The overall prevalence of resistance to ampicillin (14.41, 95% C.I. 10.72-19.09) was found to be significantly higher than that demonstrated to ciprofloxacin (6.18, 95% C.I. 3.83-9.84), gentamycin (5.21, 95% C.I. 2.96-8.99), kanamycin (6.34, 95% C.I. 4.13-9.61), nalidixic acid (6.73, 95% C.I. 4.54-9.87), and trimethoprim-sulfamethoxazole (5.14, 95% C.I. 3.02-8.63). In addition, the overall prevalence of resistance to tetracycline (12.31, 95% C.I. 8.9-16.6) was significantly greater than trimethoprim-sulfamethoxazole (5.14, 95% C.I. 3.02-8.63), and was borderline significantly greater than nearly ciprofloxacin (6.18, 95% C.I. 3.83-9.84) and gentamycin (5.21, 95% C.I. 2.96-8.99).

Table 3.20 Overall prevalence of resistance to each of the ten antibiotics, as determined using the Random Effects Model in R.

Antibiotic	n*	Resistance (%)	95% C.I.	Visualisation of resistance & 95% C.I.s
Ampicillin (AMP)	78	14.41	10.72-19.09	
Chloramphenicol (CHL)	85	8.78	6.27-12.16	
Ciprofloxacin (CIP)	54	6.18	3.83-9.84	
Erythromycin (ERY)	17	11.26	3.67-29.74	
Gentamycin (GEN)	59	5.21	2.96-8.99	
Kanamycin (KAN)	24	6.34	4.13-9.61	
Nalidixic Acid (NAL)	62	6.73	4.54-9.87	
Streptomycin (STR)	79	11.22	8.00-15.51	
Tetracycline (TET)	73	12.31	8.9-16.6	
Trimethoprim-sulfamethoxazole (SXT)	24	5.14	3.02-8.63	



*n = the total number of studies that assessed this antibiotic (e.g. not all studies examined all of the antibiotics). Studies that examined multiple species of bird were considered as individual studies.

The results of the random effects model (proportions and 95% confidence intervals) are provided for each of the ten antibiotics individually in forest plots (Figures 3.2-3.6), with reference to the three subgroups (geographical, taxonomic, and ecosystem). The overall means for each of the antibiotics are indicated in their respective forest plots. In these plots, where the 95% confidence intervals do not overlap, the differences between two were considered to be statistically significant. The vertical line on each of the plots represents the overall mean (proportion) for that antibiotic. Table 3.21 serves as a 'dictionary' that provides the common names for the various orders of bird that are referred to in Figures 3.2-3.6.

Table 3.21 Order name and common names of birds included

Order name	Includes
Anseriformes	Waterfowl
Charadriiformes	Gulls, button-quails, plovers, and allies
Columbiformes	Doves and pigeons
Falconiformes	Falcons, eagles, hawks, and allies
Gruiformes	Cranes and allies
Passeriformes	Passerines
Pelecaniformes	Pelicans and allies
Piciformes	Woodpeckers and allies
Procellariiformes	Albatrosses, petrels, and allies
Sphenisciformes	Penguins
Strigiformes	Owls

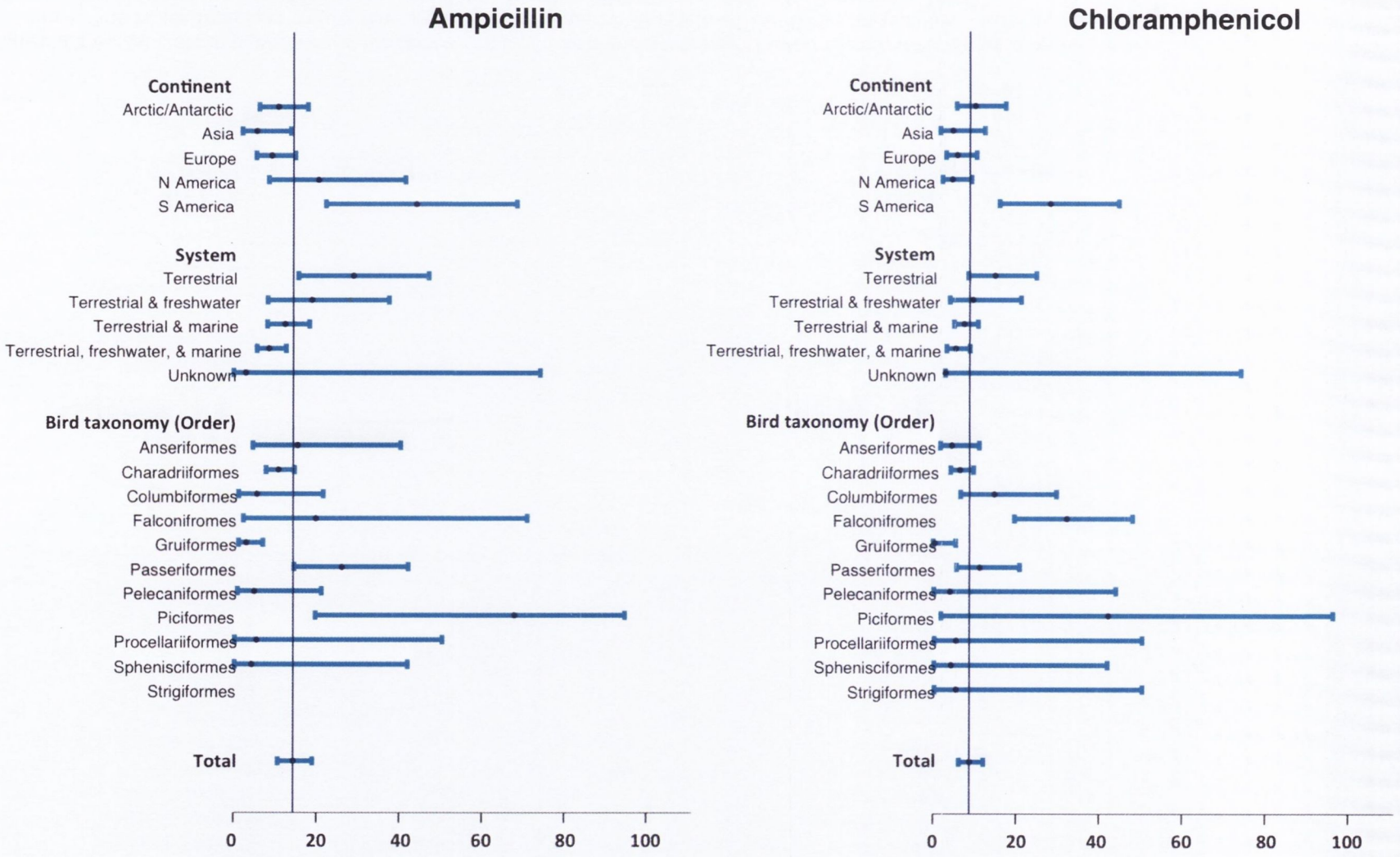


Figure 3.2 Forest plots of prevalence of resistance to ampicillin and chloramphenicol (proportions), with reference to the three subgroups: continent, system, and order. The points represent means (calculated with the Random Effects Model in R) for each of the variables, with 95% confidence intervals. Vertical reference lines indicate the overall mean for each of the antibiotics (95% C.I.s for these are indicated by 'Total').

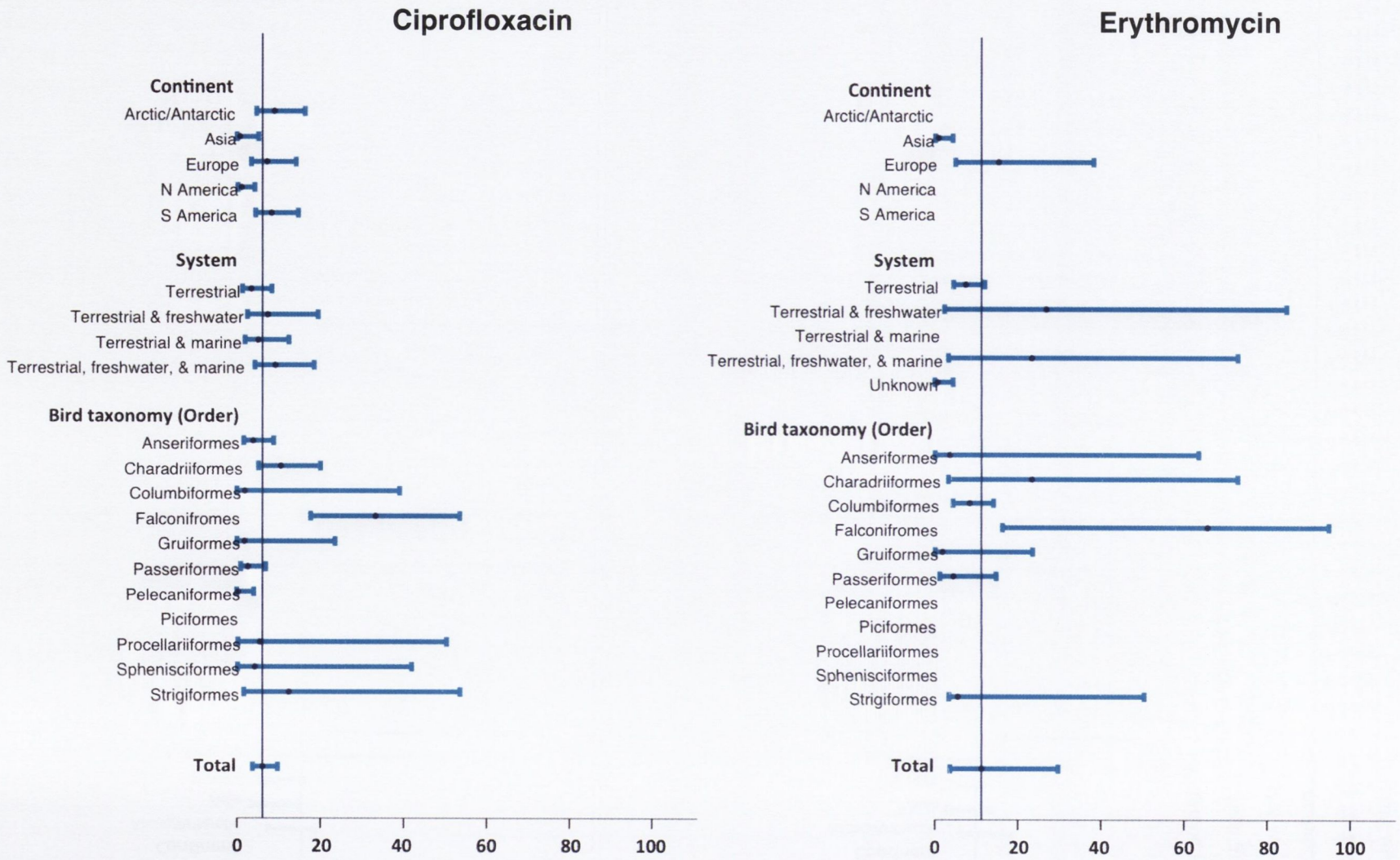


Figure 3.3 Forest plots of prevalence of resistance to ciprofloxacin and erythromycin (proportions), with reference to the three subgroups: continent, system, and order. The points represent means (calculated with the Random Effects Model in R) for each of the variables, with 95% confidence intervals. Vertical reference lines indicate the overall mean for each of the antibiotics (95% C.I.s for these are indicated by 'Total').

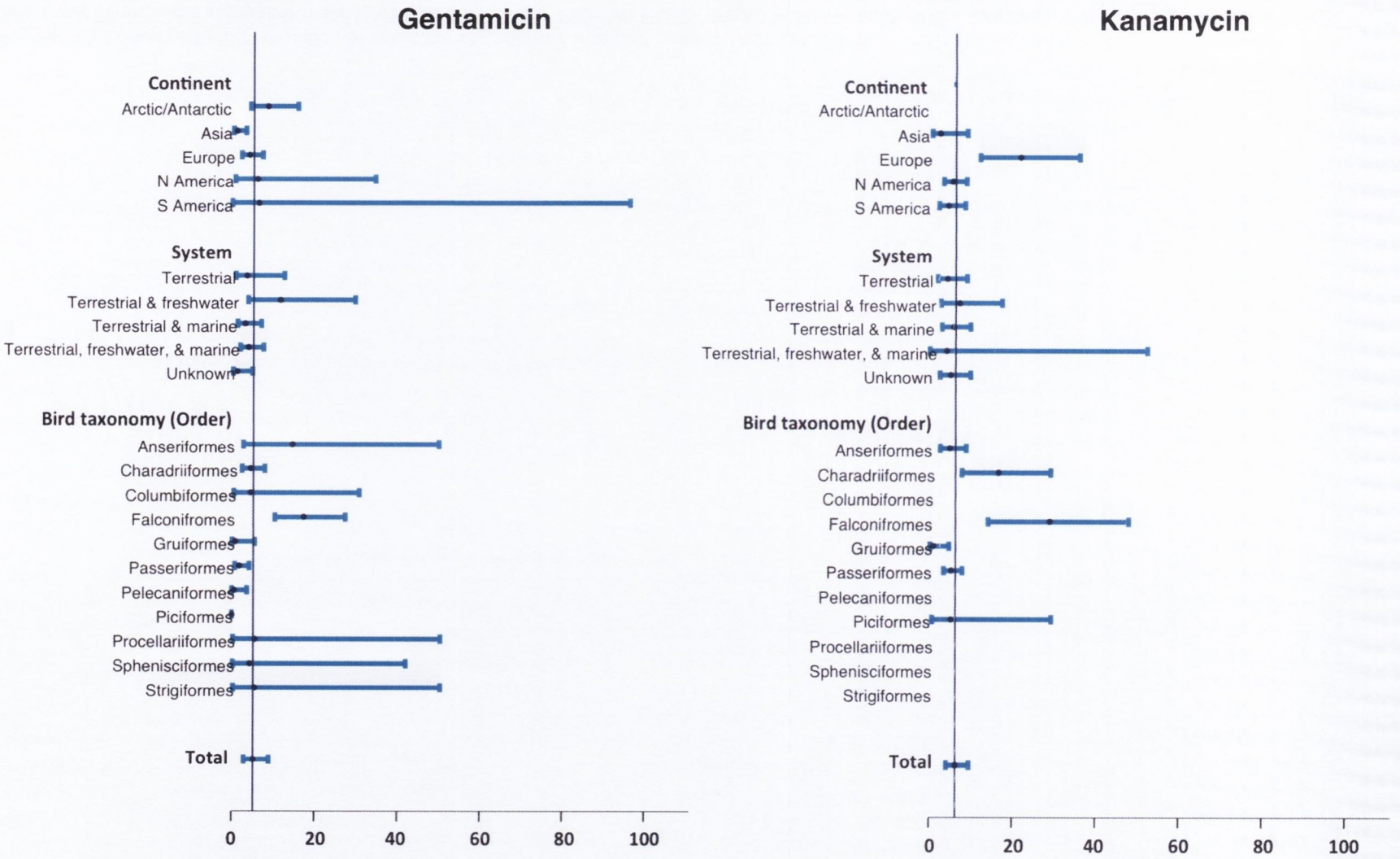


Figure 3.4 Forest plots of resistance to gentamicin and kanamycin (proportions), with reference to the three subgroups: continent, system, and order. The points represent means (calculated with the Random Effects Model in R) for each of the variables, with 95% confidence intervals. Vertical reference lines indicate the overall mean for each of the antibiotics (95% C.I.s for these are indicated by 'Total').

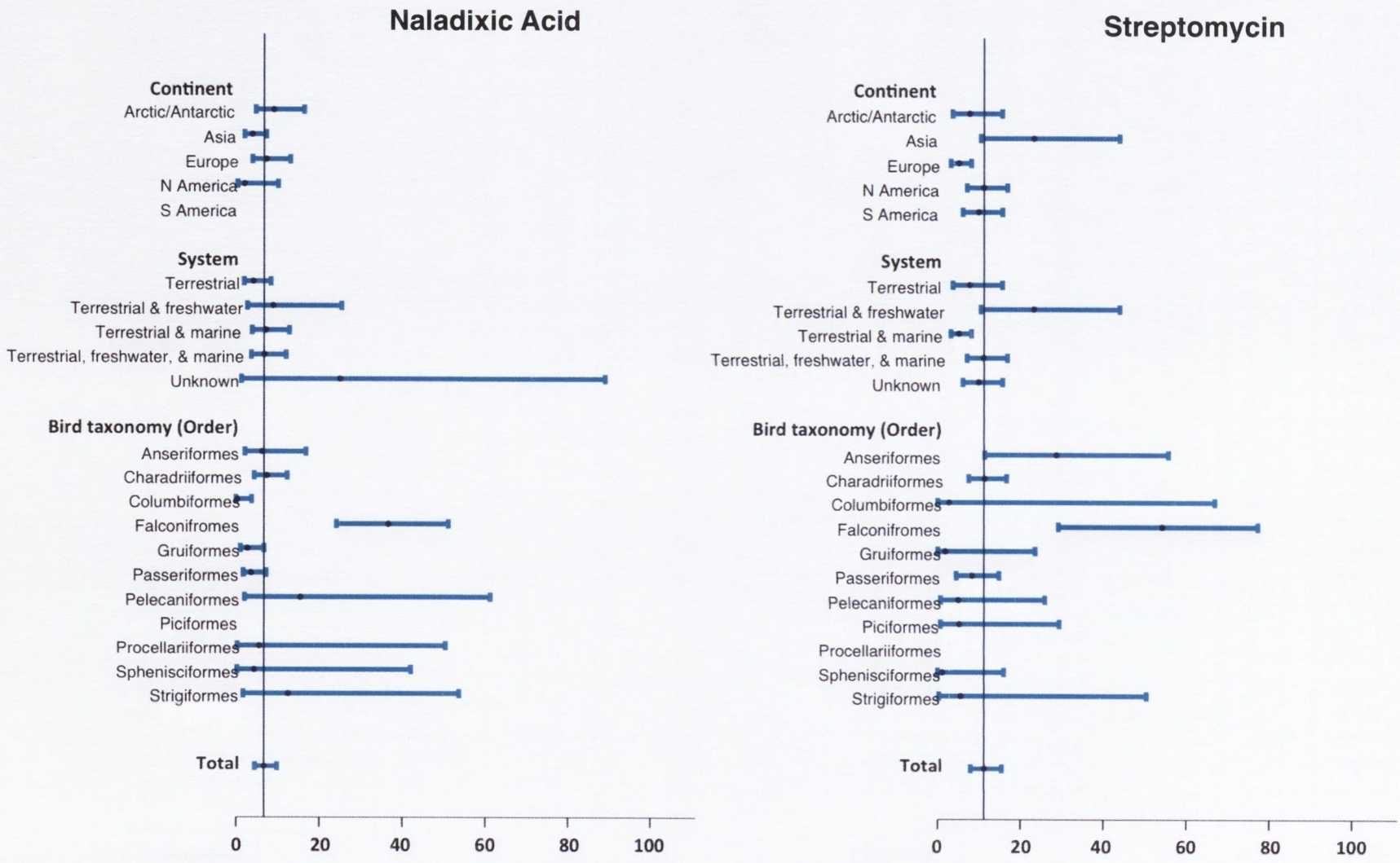
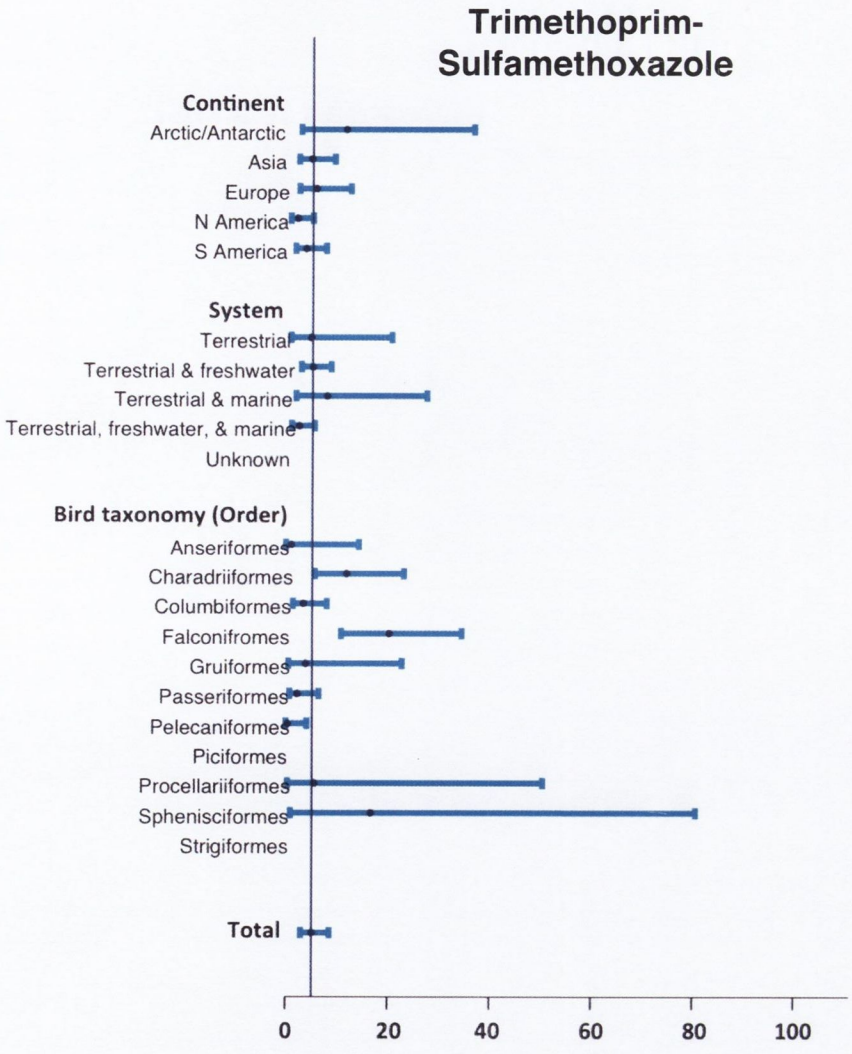


Figure 3.5 Forest plots of prevalence of resistance to nalidixic acid and streptomycin (proportions), with reference to the three subgroups: continent, system, and order. The points represent means (calculated with the Random Effects Model in R) for each of the variables, with 95% confidence intervals. Vertical reference lines indicate the overall mean for each of the antibiotics (95% C.I.s for these are indicated by 'Total').



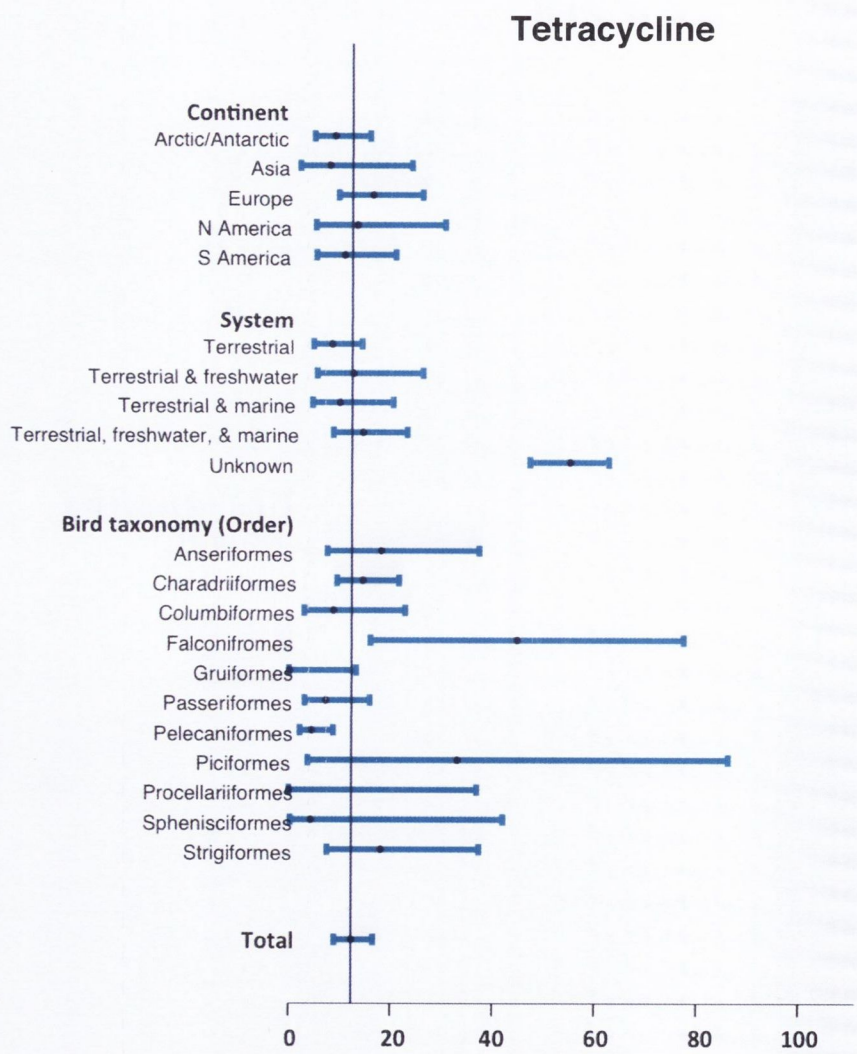


Figure 3.6 Forest plots of prevalence of resistance to trimethoprim-sulfamethoxazole and tetracycline (proportions), with reference to the three subgroups: continent, system, and order. The points represent means (calculated with the Random Effects Model in R) for each of the variables, with 95% confidence intervals. Vertical reference lines indicate the overall mean for each of the antibiotics (95% C.I.s for these are indicated by 'Total').

The forest plots were interpreted via examination of the respective 95% confidence intervals as follows. For each of the three subgroups (continent, system, and bird taxonomy), analysis was undertaken by comparing the 95% confidence intervals of the respective factors within each subgroup. In the forest plot that results from a meta-analysis, the resultant 95% confidence intervals allow for visual interpretation of the data by assessment of the extent to which they overlap (Condit *et al.*, 2000). Where the 95% confidence intervals do not overlap, the result can be considered significant, though there has been debate regarding whether or not this significance can be considered at the 5% level (Goldstein and Healy, 1995; Cochrane, 2002). Across the ten antibiotics, several statistically significant differences were found within the three subgroups. These differences are described, per antibiotic, in this section. All values reported are in percentages, which represent the prevalence of resistance amongst samples tested for that variable of the total number of samples tested amongst studies within the respective subgroup.

Ampicillin

With reference to geography, significantly more resistance to ampicillin was found in South America (44.16, 95% C.I. 22.3-68.55) than in the Arctic/Antarctic (10.69, 95% C.I. 6.17-17.87), Asia (5.56, 95% C.I. 2.12-13.79), and Europe (9.17, 95% C.I. 5.5-14.9). Birds described as living in terrestrial/freshwater/marine systems had less resistance (8.52, 95% C.I. 5.64-12.69) than those living in terrestrial systems alone (28.96, 95% C.I. 15.68-47.2). Several differences were found between the different orders of bird: Gruiformes (3.07, 95% C.I. 1.28-7.16) demonstrated less resistance than Passeriformes (26.16, 95% C.I. 14.63-42.26) and Piciformes (67.92, 95% C.I. 19.78-94.79), while Charadriiformes (10.83, 95% C.I. 7.86-14.74) demonstrated less resistance than Piciformes (67.92, 95% C.I. 19.78-94.79).

Chloramphenicol

As with Ampicillin, birds from South America demonstrated significantly more resistance to Chloramphenicol (28.21, 95% C.I. 16-44.76) than Asia (4.7, 95% C.I. 1.69-12.44), Europe (5.76, 95% C.I. 3.09-10.48) and North America (4.57, 95% C.I. 2.22-9.2). No differences were found between the different systems. Anseriformes (4.52, 95% C.I. 1.75-11.14), Charadriiformes (6.47, 95% C.I. 4.24-9.76), and Gruiformes (0.36, 95% C.I. 0-5.48) demonstrated significantly less resistance than Falconiformes (32.27, 95% C.I. 19.65-48.14), which was significantly more than the overall mean for chloramphenicol (8.78, 95% C.I. 6.27-12.16). The Gruiformes were the only order that was significantly lower than the overall mean, while the Falconiformes were the only order that was greater than the overall mean.

Ciprofloxacin

Samples taken from birds living in North America demonstrated a smaller proportion of resistance (1.14, 95% C.I. 0.29-4.35) than those from the Arctic/Antarctic (9.05, 95% C.I. 4.8-16.44) and South America (8.33, 95% C.I. 4.54-14.8). Differences between the systems were not statistically different. Between the different taxonomic orders, Anseriformes (3.93, 95% C.I. 1.69-8.9), Passeriformes (2.66, 95% C.I. 0.99-6.97), Pelecaniformes (0.27, 95% C.I. 0-4.1) demonstrated a lower prevalence of resistance to ciprofloxacin than Falconiformes (33.34, 95% C.I. 17.78-53.64).

Erythromycin

Erythromycin resistance was only investigated in studies on two continents. The isolates from Asia (0.92, 95% C.I. 0.18-4.41) were significantly less resistant than those from Europe (15.41, 95% C.I. 5.06-38.35). With regards to the systems, samples from birds that inhabit 'Unknown' systems had a lower prevalence of resistance (0.65, 95% C.I. 0-4.43) than those from terrestrial systems (7.47, 95% C.I. 4.54-12.05). Birds within the Columbiformes (8.39, 95% C.I. 4.41-14.2) and Passeriformes (4.46, 95% C.I. 1.24-14.85) orders demonstrated less resistance than those from the Falconiformes (65.37, 95% C.I. 16.35-94.95) order.

Gentamicin

Only one statistically significant difference between continents was found for gentamycin, and that was between Asia and the Arctic/Antarctic; Asia had an overall smaller prevalence of resistance (1.11, 95% C.I. 0.36-3.38) than the Arctic/Antarctic (8.59, 95% C.I. 4.46-15.9). No significant differences were found between systems, though several were found between the various orders of birds. Falconiformes (17.38, 95% C.I. 10.46-27.48) demonstrated a greater proportion of resistance than Charadriiformes (4.52, 95% C.I. 2.51-8), Gruiformes (0.81, 95% C.I. 0.11-5.56), Passeriformes (1.8, 95% C.I. 0.76-4.21), and Pelecaniformes (0.53, 95% C.I. 0-3.7).

Kanamycin

Samples from birds collected in Asia (2.25, 95% C.I. 0.68-9.15), North America (5.61, 95% C.I. 3.47-8.96), and South America (4.52, 95% C.I. 2.32-8.62) demonstrated significantly less resistance to kanamycin than those in Europe (21.96, 95% C.I. 12.26-36.19). There were no

differences between the systems. With regards to the orders, Falconifomres demonstrated significantly higher resistance (29.03, 95% C.I. 14.22-48.04) than Anseriformes (4.94, 95% C.I. 2.68-8.94), Gruiformes (0.99, 95% C.I. 0.2-4.76), and Passeriformes (5.31, 95% C.I. 3.51-7.98), while Charadriiformes (16.67, 95% C.I. 7.92-29.29) were significantly higher than Gruiformes and Passeriformes, but not Anseriformes.

Nalidixic Acid

There were no statistically significant differences found between continents or systems. Falconiformes (36.64, 95% C.I. 24.19-51.16) demonstrated a significantly higher prevalence of resistance than Anseriformes (6.28, 95% C.I. 2.17-16.85), Charadriiformes (7.45, 95% C.I. 4.4-12.34), Columbiformes (0.25, 95% C.I. 0-3.79), Gruiformes (2.76, 95% C.I. 1.1-6.75), and Passeriformes (3.62, 95% C.I. 1.76-7.29).

Streptomycin

Between continents, the proportion of resistant samples taken from birds in Europe (5.17, 95% C.I. 3.23-8.19) was significantly less than those from Asia (23.28, 95% C.I. 10.49-44.01). With reference to the systems that birds were defined as inhabiting, those in 'terrestrial and marine' systems (5.17, 95% C.I. 3.23-8.19) demonstrated less resistance than those in 'terrestrial and freshwater' systems (23.28, 95% C.I. 10.49-44.01). Between the various orders of birds, Falconiformes (54.29, 95% C.I. 29.23-77.35) had a higher prevalence of resistance than Charadriiformes (11.37, 95% C.I. 7.57-16.73), Gruiformes (1.85, 95% C.I. 0.11-23.64), Passeriformes (8.35, 95% C.I. 4.53-14.88), Pelecaniformes (5, 95% C.I. 0.78-25.95), and Spheniscoformes (1.16, 95% C.I. 0-16.05).

Trimethoprim-sulfamethoxazole

No statistically significant differences were found between continents or systems. The only differences that were evident were between bird orders, with Columbiformes (3.4, 95% C.I. 1.4-8.05), Passeriformes (2.21, 95% C.I. 0.73-6.47), and Pelecaniformes (0.27, 95% C.I. 0-4.1) demonstrating a lower proportion of resistant samples than Falconiformes (20.23, 95% C.I. 10.85-34.58).

Tetracycline

No statistically significant differences were found between continents. With regards to system, those classified as inhabiting 'unknown' systems had a higher prevalence of resistance (55.17, 95% C.I. 47.31-62.78) than each of the other four categories of system, which included Terrestrial (8.37, 95% C.I. 4.76-14.3), Terrestrial & Freshwater (12.57, 95% C.I. 5.47-26.3), Terrestrial & Marine (9.9, 95% C.I. 4.57-20.44), and Terrestrial, Freshwater, and Marine (14.52, 95% C.I. 8.71-23.21). In regards to bird order, the only statistically significant difference was between Falconiformes, Passeriformes, and Pelecaniformes; Falconiformes (44.83, 95% C.I. 16.04-77.56) were higher than Passeriformes (7.25, 95% C.I. 3.12-15.92) and Pelecaniformes (4.43, 95% C.I. 2.19-8.76).

3.5 Discussion

This study has, for the first time, used a systematic review and meta-analysis approach to investigate antibiotic resistant bacteria in wild birds. Whereas previous research efforts have provided case-study evidence of antibiotic resistance in bird species – usually within a single geographic region or (in most cases) with reference to one species of bird – the use of meta-analysis has meant that the results of such investigations could be pooled and further investigated. With this, the prevalence of resistance to a variety of antibiotics could be quantified more robustly than has typically been achieved within the context of individual investigations. The results of this meta-analysis have indicated that antibiotic resistant bacteria are prevalent in a variety of wild bird species that inhabit a range of ecosystems across the world, and in doing so, has also provided insights into factors that might be associated with resistance to a variety of antibiotics.

This meta-analysis has provided evidence of the widespread prevalence of antibiotic resistance in wild birds: of the 37 studies that were ultimately included, 34 reported resistance to at least one antibiotic. In pooling the data, the results of the meta-analysis have indicated that resistance to at least one antibiotic exists in all of the 11 orders of bird from which bacterial samples were taken, across each of the five continents where the research was conducted. This resistance was found to exist across terrestrial, marine, and freshwater systems. That resistance was found to be so prevalent supports the now widely established paradigm of antibiotic resistance as an emerging infectious disease that is a global threat (Bush *et al.*, 2011; Chan, 2012). The implications of this widespread prevalence of antibiotic resistance are discussed in the following section, first with reference to the overall prevalence of resistance to each of the ten antibiotics, and then with regards to variation in the prevalence of resistance with specific reference to the geographic, taxonomic, and ecosystem subgroups used in statistical analyses.

3.5.1 Overall prevalence of resistance

Several statistically significant differences were found between the overall prevalence of resistance to the ten antibiotics. The findings here suggest that, on a global scale, wild birds demonstrated a significantly higher prevalence of bacteria resistant to ampicillin than to ciprofloxacin, gentamycin, kanamycin, nalidixic acid, and trimethoprim-sulfamethoxazole, and also that resistance to tetracycline was greater than that demonstrated to trimethoprim-sulfamethoxazole. This confirms the results of similar research that has been conducted a sites across the world (which, for various reasons, were not amongst the studies included in this investigation). For example, in investigating antibiotic resistance in variety of bird species in Europe, Guenther *et al.* (2010) found that both ampicillin and tetracycline were amongst

the antibiotics to which the highest abundance of resistance was expressed. In investigating a variety of wildlife species in Portugal, Costa *et al.* (2005) also cited ampicillin and tetracycline as antibiotics to which resistance was most commonly determined. With reference to these two investigations and other relevant research, there are several possible explanations for the higher prevalence of resistance demonstrated to ampicillin and tetracycline. Based on research conducted in food animals, Bywater *et al.* (2004) suggested a link between the number of years that an antibiotic has been in use and the amount of resistance; older antibiotics (including both ampicillin and tetracycline) presented higher prevalence of resistance than newer antibiotics (such as ciprofloxacin). Although they do not offer an explanation, gentamycin was cited as an exception to this rule by Bywater *et al.* (2004); and interestingly, gentamycin was found to demonstrate a lower prevalence of resistance in the present study than the other antibiotics (and significantly lower than ampicillin). The parallels between the present study and the research by Bywater *et al.* (2004) (and similar research on food animals) are interesting in light of research into the potential pathways of resistance between wildlife and livestock in environmental systems (Sayah *et al.*, 2005; Blanco *et al.*, 2009; Kozak *et al.*, 2009). For example, Kozak *et al.* (2009) found high levels of resistance to tetracycline in small mammals living in the vicinity of swine farms (a high prevalence of resistance to tetracycline was also noted in the swine). Beyond vicinity to agricultural activity, a variety of factors may affect the prevalence of resistance within an animal to a given antibiotic; these include geographic, taxonomic, and ecosystem-characteristics. Use of these factors in this meta-analysis has provided several interesting insights that are discussed with reference to relevant literature – both that conducted specifically on wild birds, and that which has investigated antibiotic resistance in environmental systems more generally – in the following section.

3.5.2 Geographic, taxonomic, and ecosystem factors

With regards to bird taxonomy, this research has provided interesting insights into variation between different taxonomic orders of bird. Guenther *et al.* (2010) cited birds of prey, waterfowl, and passerines as notable reservoirs of antibiotic-resistant *E. coli* in Europe, and the evidence from a wider geographic area provided by the present study supports this claim. For example, Falconiformes (which includes birds of prey) were found to demonstrate a significantly higher prevalence of resistance to nine of the ten antibiotics used in this investigation, relative to their respective means. Marrow *et al.*, 2009 suggest that given their position on the food chain, birds of prey can acquire antibiotic resistant bacteria from their prey. The likelihood of this is evidenced in light of research that indicates that small mammals (including rodents upon which birds of prey feed) are reservoirs of antibiotic resistance (Gilliver *et al.*, 1999; Sherley *et al.*, 2000; Kozak *et al.*, 2009). Similarly, Lemus *et*

al., (2008) cite livestock (which have been given antibiotics) as a potential source of antibiotic resistance in birds that are scavengers (some of which are also included in the Falconiformes order), via either the transfer of resistant bacteria or the acquisition of antibiotic residues. Although Guenther *et al.*, (2010) suggested that waterfowl and passerines are also notable reservoirs of antibiotic resistant bacteria, the results here do not entirely support this; for each of these, the overall resistance demonstrated was higher only for one of the ten antibiotics. With regards to waterfowl, the Anseriformes demonstrated a significantly higher prevalence of resistance for streptomycin only. That resistance to streptomycin was relatively high is particularly interesting given the assertion by Kemper (2008) that the use of streptomycin has largely become obsolete in many countries, though it should be noted that its use in livestock in the United States for disease prevention has continued (Sarmah *et al.*, 2006). Thus, the prevalence of antibiotic resistance amongst the included studies might provide evidence of the extent to which antibiotics can persist in a given environment (Halling-Sørensen *et al.*, 1998). Indeed, antibiotics (including those used in a veterinary context) have been recognized as important micropollutants in both terrestrial and aquatic environments, and are therefore of particular concern with regards to antibiotic resistance pathways (Halling-Sørensen *et al.*, 1998; Kumar *et al.*, 2005; Kemper, 2008). However, it is also important to recognize that soil microorganisms can produce streptomycin, and thus, resistance may naturally develop in environmental systems from which wildlife (including birds) could acquire resistance (Kümmerer, 2009). Similar to streptomycin, despite also being suggested by Guenther *et al.*, (2010) as an order for which resistance to multiple antibiotics was higher, the Passerines order demonstrated a significantly higher resistance than the mean for only one antibiotic, ampicillin. However, the Passerines represent the largest order of bird (>6000 species), and to elucidate the factors underlying this finding would require specific investigation of the patterns of resistance – and life history factors that might be driving it – in the various genera and species that comprise this taxa (Ricklefs, 2012). Variaration between taxa, and the extent to which their respective life histories might be driving this, provide a good example of what a One Health approach might offer given the potential links between bird behavior (i.e. knowledge provided by ecologists) and the bacteria they acquire (i.e. described by microbiologists).

It was anticipated that variation with reference to geography would be found, but very few significant differences between continents were found. One notable finding was a significantly higher prevalence of resistance to ampicillin and chloramphenicol of samples taken from birds in South America, relative to the respective overall means for these antibiotics. Interesting to consider in regards to what might be driving this is the suggestion by Levy (2001) that a common practice in South America was the spraying of fruit trees with antibiotics (though he notes that the practice was not exclusive to there). Given that the majority of the birds used amongst the included studies conducted in South America were

passerines (which live in terrestrial environments) (e.g. Silva *et al.*, 2009), it is interesting to consider that this might result in the exposure of wild birds to antibiotics from this practice. Interestingly, a systematic review of research on antibiotic resistance in *E. coli* that focused on investigations of resistance in a clinical context (i.e. in humans) also found a higher prevalence of resistance in South America relative to the United States and (Central) Europe (Erb *et al.*, 2007). In general, the lack of many significant differences between continents could be due to the fact that the migratory status of bird species was not considered in this meta-analysis (nor were non-seasonal movements that might take a bird across international borders). Further to this, the age of the birds from which samples taken were rarely mentioned in the included studies. Given research by van Dongen *et al.* (2013) that found significant variation in the microbial flora of wild birds with reference to age, it is interesting to consider that the prevalence of resistant bacteria in an individual bird may change over time – and that not only is it possible for a bird to acquire resistant bacteria, but also that the potential may exist for a bird to lose resistant bacteria as it ages.

An unexpected finding was that there were very few significant differences between the systems (terrestrial, freshwater, and marine). This may be evidence of the fact that resistance can spread throughout environments, so birds living in aquatic systems are ultimately exposed to the same resistant bacteria and/or antibiotic residues as those that occupy terrestrial systems. Indeed, although they were for some time considered distinct 'self-contained' entities, the links between terrestrial and aquatic systems are increasingly seen as interconnected (Schneider *et al.*, 2002; McCallum *et al.*, 2004). In discussing the management implications of this, Schneider *et al.* (2002) cite two primary mechanisms by which terrestrial and aquatic systems interface: the movement of organisms between the two, and the flow of water which links the two systems. Although they did not consider the implications of this in an infectious disease context, both of the pathways that they suggest are highly relevant in considering antibiotic resistance; these are used as a basis for discussing the results of this investigation in the following section.

The movement of wild birds from site to site inherently lends itself to the acquisition and spread of resistant bacteria. Proximity to agricultural areas was cited by many of the authors of the included studies as a factor driving the various resistances identified in wild bird species across the globe, including Portugal (Radhouani *et al.*, 2010), France (Bonnedahl *et al.* 2009), the Czech Republic (Dolejska *et al.*, 2007), and the United States (Gibbs *et al.*, 2007). Several studies have provided evidence that direct contact between wildlife and livestock was likely *not* the cause of resistance demonstrated in wild birds (Dolejska *et al.*, 2008; Rybarikova *et al.*, 2010). These authors and others have suggested alternative pathways by which resistance could spread from agricultural areas to wild birds, including via livestock feed (Gaukler *et al.*, 2009; Radimersky *et al.*, 2010) and faecal material of livestock

(Cole *et al.*, 2005; Radhouani *et al.*, 2010). The waterways that link terrestrial and aquatic environments (as suggested by Schneider *et al.* 2002), may serve as a pathway by which resistance can spread. Indeed, in addition to exposure at farms themselves, water bodies have been noted as providing a pathway of resistance via agricultural runoff including in the United States (Gaukler *et al.* 2009; Middelton and Ambrose, 2005), the Czech Republic (Rybarikova *et al.*, 2010), and South Korea (Han *et al.*, 2011). Beyond farms, human sewage and/or discharge from wastewater treatment plants were also cited as a potential source of resistance demonstrated by the wild bird species by many of the authors of included studies, including in the United States (Gaukler *et al.* 2009; Middelton and Ambrose, 2005), and the Czech Republic (Dolejska *et al.*, 2007). It is important to note that pathways by which resistant bacteria are spread can go in both directions (i.e. not only from livestock and agricultural environments to humans). In their investigation of resistant *Salmonella* isolates in Iran, Mirzaie *et al.* (2010) cite the potential for resistance-harbouring house sparrows to transfer resistant bacteria to poultry. Similarly, Waldenstrom *et al.* (2005) reference a 'feed-back loop' whereby resistance can pass from farm to farm via wildlife reservoirs, which serves to widen the potential threat to food animals and, consequently, to humans. Wild birds as vectors of antibiotic resistant bacteria are critical to consider in considering the implications for human health, and indeed, one of the emerging paradigms of antibiotic resistance is the recognition of the importance of considering environmental reservoirs of antibiotic resistant bacteria (Finley *et al.*, 2013). Although this study focused on antibiotic resistant bacteria, evidence of exchange of resistance genes between environmental bacteria and clinical pathogens in particular has also become recognized as an issue that warrants further investigation (Forsberg *et al.*, 2012; Rolain *et al.*, 2012)

3.5.3 Challenges and limitations of this investigation

There were several limitations of this systematic review and meta-analysis, many of which are limitations that have previously been noted using such an approach. The criticisms of meta-analysis are wide-ranging; including criticisms such as that which has referred to it as 'mega-silliness' and the aurally appealing yet slightly ridiculous "meta-analysis shmeta-analysis" (1994). Recent work in the field of meta-analysis has challenged some of the more popular criticisms, and these are addressed with reference to the present investigation in the section that follows.

One of the most commonly cited problems with meta-analyses is publication bias (often referred to as the 'file drawer problem'), in reference to the fact that negative results (i.e. 'non-findings') are often not published (Stroup *et al.*, 2000; Sterne *et al.*, 2001). In the context of antibiotic resistance, this would imply that investigations that find resistant bacteria are

more likely to be published than studies in which antibiotics are found to be effective. However, in reality, this is likely not as problematic as in other fields. Firstly, many of the published studies on antibiotic resistance used multiple antibiotics in screening for resistance, and in most cases, the results indicated a range of sensitivities/resistances to the numerous antibiotics on the panels. In addition, it has been suggested that in regards to antibiotic resistance, a 'non-result' (i.e. where the antibiotic is effective) is an interesting finding in itself – given that within the literature resistance has become the norm (Halpern *et al.*, 2005). As a result, this was not considered to affect this meta-analysis (particularly considering that only studies published between 2002 and 2012 were included – a time during which a study presenting absent (or even low) levels of resistance would be interesting.

The hallmark of a systematic review and meta-analysis is that it provides a highly structured methodology by which the literature is searched and studies returned are subjected to a selection process using a pre-determined list of inclusion/exclusion criteria. While this objectivity is one of its greatest strengths, it can also prove to be a weakness in cases where studies with a subject of particular interest may not be included amongst the studies that are reviewed and analysed. In the case of the meta-analysis conducted here – and considering the focal species of the following chapters of this thesis (i.e. the Greater Flamingo) – the fact that none of the included studies used flamingos as a study organism is worthy of comment. Its absence is due to several reasons, firstly is that very little research has been conducted on antibiotic resistance in any of the six species of flamingo, and none of the research that did use flamingos that was returned during the search process could be included. For example, one study conducted by Sato *et al.* (2009) investigated antibiotic resistant bacteria in the Lesser Flamingo (*Phoeniconaias minor*), however, the samples were taken from captive flamingos and as such, the study was excluded during the selection process as it was ineligible (as detailed in Table 3.3, the inclusion criteria was that "Samples must be from 'wild' birds – that is, birds from which samples were taken must be described or classified within the study as 'wild', 'free-living' or 'feral'"). Another study conducted by Dib *et al.* (2009) that was also returned during the literature search process investigated antibiotic resistance in faeces samples from flamingos in Andean lakes. However, this study was excluded because the results provided were insufficient (as per Table 3.3, eligibility criteria was that 'Full results must be provided').

With reference to the results of the statistical analysis, another limitation of this investigation concerns the fact that little variation was found between many of the characteristics used in the subgroup analyses. In reality, it is likely a combination of factors that ultimately affect the prevalence of resistance in a given bird, and influences are likely more complex than the three generalized factors (geography, taxonomy, and ecosystem) that were considered here.

Thus, there remain several areas for which further research would likely add to what was found here.

There are several ways in which the results of a systematic review and meta-analysis can be used to inform future the study design of future research. In proposing a framework by which the results of systematic reviews can be used in designing primary research studies, Thompson *et al.* (2012) suggest that such an approach is necessary to avoid 'research wastage' (which they define as 'suboptimal use of research funding'). The benefits that they, and other authors writing on the topic, cite include highlighting gaps in research as well as informing certain aspects of study design, such as sample size; the use of systematic reviews and meta-analyses in all stages of empirical research – i.e. planning, designing, and conducting – is well-regarded (Sutton *et al.*, 2007; Thompson *et al.*, 2012; Jones *et al.*, 2013). Although the framework proposed by Thompson *et al.* (2012) is specific to the design of research in a human health context (e.g. clinical trials), the same principles can be applied to other research fields. The suggestion that systematic reviews can (and should) be used in the design of empirical research is valuable to consider in regards to the present systematic review and meta-analysis. The results of this meta-analysis, and in particular, the characteristics of the included studies (described in Section 3.4.2 'Data analysis – study characteristics'), could be used to inform the study design of primary research. For example, an evaluation of the antibiotics that were most frequently used in these studies can provide a basis for the selection of antibiotics in a future study during the planning stages of the investigation, both in the specific antibiotics used (e.g. ampicillin's frequent use across most of the included studies), as well as in the quantity of antibiotics that might be appropriate to use in a similar study (e.g. most studies used either eight or twelve antibiotics). Similarly, consideration of the bacterial species that were commonly analysed amongst studies in the meta-analysis (*E.coli* was most frequently used) can inform the selection of bacteria in future research, as can the species of bird under investigation – the Passeriformes (passerines) and Charadriiformes (gulls, button-quails, plovers, and allies). Methodological choices (type of bacterial sample taken, method of analysis of resistance in the laboratory, etc.) can also be informed by previous research efforts – including those that were commonly used, as well as (where specific comments are made) those which were found to work well, and those that did not. Thus, this meta-analysis not only provides an interesting insight into patterns of antibiotic resistance in wild birds retrospectively, but also can offer suggestions for future, field-based research efforts.

3.6 Conclusions and suggestions for future research

The potential for wild birds to serve as reservoirs of zoonotic disease has long been recognised (Allen *et al.*, 2010). Through use of a meta-analysis approach, this investigation has provided insights into patterns of antibiotic resistant bacteria in wild birds, with reference to factors that have previously been suggested to play a role – including geography, taxonomy, and ecosystem characteristics. This research has indicated that:

- Falconiformes demonstrated a high overall prevalence of resistance to multiple antibiotics, which may be the result of certain aspects of their life history (e.g. scavenging and predatory feeding habits).
- Very few significant differences between systems were found, which is suggestive of the fact that resistance spreads between terrestrial and aquatic systems
- Relatively few significant differences were found with reference to geography (i.e. continent), which might reflect the fact that many species of bird have ranges across multiple continents, thereby increasing the likelihood of being exposed to a variety of resistant bacteria, antibiotics/residues, and resistance genes.

To gain further insights into patterns of antibiotic resistance in wild birds – and the factors that might affect it – it would be important for future research to investigate both wildlife and food animals within the same meta-analysis. With this, links between the two could be investigated. If such research were to include studies conducted over a long time frame (e.g. 20-30 years), it might be possible to elucidate temporal variation in the emergence and spread of antibiotic resistant bacteria. It may also be interesting to conduct field-based research of antibiotic resistance in wild bird species to address some of the challenges encountered in evaluating factors that may affect the prevalence of resistance. For example, in the included studies, the age of bird was typically not noted, and because research conducted on adult birds means the bird has likely visited multiple sites, geography as a driving factor is 'lost'. Use of bird chicks (that are unable to fly) may remove this uncertainty. Field-based research may also validate some of the findings of this meta-analysis; for example, the antibiotics to which resistance was frequently determined in the included studies could be empirically tested.

3.7 References

Anzures-Cabrera, J. And Higgins, J.P.T. 2010. Graphical displays for meta-analysis: an overview with suggestions for practice. Research Synthesis Methods **1,** 66-80.

Al-Bahry, S., Mahmoud, I., Elshafie, A., Al-Harthy, A., Al-Ghafri, S., Al-Amri, I. & Alkindi, A. 2009. Marine Pollution Bulletin **58,** 720-725.

Alanis, A.J. 2005. *Resistance to antibiotics: are we in the post-antibiotic era?* Archives of Medical Research **36,** 697-705.

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J. and Handelsman, J. 2010. *Call of the wild: antibiotic resistance genes in natural environments.* Nature Reviews Microbiology **8,** 251-259.

Aminov, R.I. 2009. The role of antibiotics and antibiotic resistance in nature. Environmental Microbiology **11,** 2970-2988.

Andersson, D.I. & Hughes, D. *Antibiotic resistance and its cost: is it possible to reverse resistance?* Nature Reviews Microbiology **8,** 260-271.

Anzures-Cabrera, J., & Higgins, J. 2010. Graphical displays for meta-analysis: An overview with suggestions for practice. Research Synthesis Methods **1**, 66-80.

Arnqvist, G. and Wooster, D. 1995. Meta-analysis: synthesizing research findings in ecology and evolution. Ecology and Evolution **10,** 236-240.

Baquero, F., Martínez, J.- L., Cantón, R. 2008. Antibiotics and antibiotic resistance in water environments. Current Opinion in Biotechnology **19,** 260-265.

Berman, N. G., & Parker, R. A. 2002. Meta-analysis: neither quick nor easy. BMC Medical Research Methodology **2**, 10.

Bibby, C.J. 1999. Making the most of birds as environmental indicators. In: Adams, N.J. & Slotow, R.H. (eds) Proc. 22 Int. Ornithol. Congr., Durban. Ostrich 70 (1): 81–88.

BirdLife International, 2012.

Blackburn, J.K., Mitchell, M.A., Blackburn, M-C.H., Curtis, A., Thompson, B.A. 2010. *Evidence of antibiotic resistance in free-swimming, top-level marine predatory fishes.* Journal of Zoo and Wildlife Medicine **41,** 7-16.

Blanco, G., Lemus, J.A. & Grande, J. 2009. *Microbial pollution in wildlife: linking agricultural manuring and bacterial antibiotic resistance in red-billed choughs.* Environmental Research **109,** 405-412.

Bonnendahl, J., Olsen, B., Waldenström, J., Broman, T., Jalava, J., Huovinen, P. & Österblad, M. 2008. *Antibiotic susceptibility of faecal bacteria in Antarctic penguins.* Polar Biology **31,** 759-763.

Borenstein, M., Hedges, L. V., Higgins, J. P., & Rothstein, H. R. 2011. *Introduction to meta-analysis.* John Wiley & Sons.

Bhullar, K., Waglechner, N., Pawlowski, A., Koteva, K., Banks, E.D., Johnston, M.D., Barton, H.A. and Wright, G.D. 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS ONE. Available online at: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0034953. Accessed 30 August 2013.

Brand, R. A. 2009. Editorial: Standards of Reporting: The CONSORT, QUORUM, and STROBE Guidelines. *Clinical Orthopaedics and Related Research®* **467**, 1393-1394.

Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., Jacoby, G.A., Kishony, R. *et al.* 2011. Tacking antibiotic resistance. Nature Reviews Microbiology **9,** 894-896.

Bywater, R., Deluyker, H., Deroover, E., De Jong, A., Marion, H., McConville, M., ... & Walters, J. 2004. A European survey of antimicrobial susceptibility among zoonotic and commensal bacteria isolated from food-producing animals. Journal of Antimicrobial Chemotherapy **54**, 744-754.

Carmel, Y., Kent, R., Bar-Massada, A., Blank, L., Liberzon, J., et al. 2013. Trends in ecological research during the last three decades – a systematic review. PLoS ONE **8**, doi:10.1371/journal.pone.0059813.

Cars, O., Hedin, A. and Heddini, A. 2011. *The global need for effective antibiotics—moving towards concerted action.* Drug Resistance Updates **14,** 68-69.

Chan, M. 2012. Antimicrobial Resistance in the European Union and the World. Keynote address at conference on Combating Antimicrobial Resistance: Time For Action, Copenhagen, Denmark, 14 March 2012.

Cohen, M.L. 1992. *Epidemiology of drug resistance: implications for a post-antimicrobial era.* Science **257,** 1050-1055.

Cole, D., Drum, D.J.V., Stallknecht, D.E., White, D.G., Lee, M.D., Ayers, S., Sobsey, M. and Maurer, J.J. 2005. Free-living Canada Geese and antimicrobial resistance. Emerging Infectious Diseases **11,** 935-938.

Cook, D.J., Guyatt, G.H., Clifton, J., Buckingham, L., Willan, A., McIlroy, W. and Oxman, A.D. 1993. Should unpublished data be included in meta-analyses? Current convictions and controversies. JAMA **269,** 2749-2753.

Cooper, H.M. 1998. Synthesizing Research: A Guide for Literature Review. Sage Publications, Thousand Oaks, California, 205pp.

Côté, I.M. and Reynolds, J.D. 2012. *Meta-analysis at the intersection of evolutionary ecology and conservation.* Evolutionary Ecolgy

Davies, J.E. Origins, acquisition and dissemination of antibiotic resistance determinants. In: Antibiotic Resistance: Origins, Evolution, Selection, and Spread. Wiley, Chichester (Ciba Foundation Symposium 207), pp. 15-35.

D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N. and Wright, G.D. 2011. Antibiotic resistance is ancient. Nature **477,** 457-461.

Dolejska, M., Cizek, A. & Literak, I. 2007. *High prevalence of antimicrobial-resistant genes and integrons in* Escherichia coli *isolates from black-headed gulls in the Czech Republic.* Journal of Applied Microbiology **103,** 11-19.

Erb, A., Stürmer, T., Marre, R. and Brenner, H. 2007. Prevalence of antibiotic resistance in *Escherichia coli*: overview of geographical, temporal, and methodological variations. European Journal of Clinical Microbiology and Infectious Diseases **26,** 83-90.

Esiobua, N., Armentaa, L. and Ikea, J. 2002. Antibiotic resistance in soil and water environments. International Journal of Environmental Health Research **12,** 133-144. DOI:10.1080/09603120220129292 pages 133-144

European Technology Assessment Group (ETAG). 2006. *European Parliament Policy Department Economic and Scientific Policy: Antibiotic Resistance.* Available online at: http://www.europarl.europa.eu/stoa/publications/ studies/stoa173_en.pdf. Accessed 13 October 2010.

European Union. 2002. *Press release: questions and answers on antibiotics in feed.* MEMO/02/66. Available online at: http://europa.eu/rapid/pressReleasesAction.do?reference=MEMO/02/66&format=HTML&aged =0&l anguage= EN&guiLanguage=en. Accessed 15 December 2010.

Eysenck, H. J. 1952. *The effects of psychotherapy: an evaluation.* Journal of Consulting Psychology **16**, 319-324.

Eysenck, H.J. 1978. *An exercise in mega-silliness.* American Psychologist **33**, 517.

Falagas, M.E., Pitsouni, E.I., Malietzis, G.A. and Pappas, G. 2008. Comparison of PubMed, Scopus, Web of Science, and Google Scholar: strengths and weaknesses. The FASEB Journal **22,** 338-342.

Fèvre, E.M., Bronsvoort, B.M.C., Hamilton, K.A. and Cleaveland, S. 2006. *Animal movements and the spread of infectious disease.* Trends in Microbiology **14,** 125-131.

Finley, R.L., Collignon, P., Larsson, D.G., McEwen, S.A., Li, X.Z., Gaze, W.H., Reid-Smith, R., Timinouni, M., Graham, D.W. and Topp, E. The scourge of antibiotic resistance: the important role of the environment. Clinical Infectious Diseases **57,** 704-710. doi: 10.1093/cid/cit355.

Flather, M. D., Farkouh, M. E., Pogue, J. M., & Yusuf, S. 1997. Strengths and limitations of meta-analysis: larger studies may be more reliable. Controlled clinical trials **18**, 568-579.

Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A. and Dantas, G. 2012. The shared antibiotic resistome of soil bacteria and human pathogens. Science **31,** 1107-1111.

Furness, R.W. 1993. Birds as monitors of pollutants. In: Furness, R.W. and Greenwood, J.J.D. (eds). Birds as Monitors of Environmental Change. Springer. pp. 86-143.

Garg, A.X., Hackam, D. and Tonelli, M. 2008. Systematic Review and Meta-analysis: When One Study Is Just not Enough. Clinical Journal of the American Society of Nephrology **3,** 253-260.

Gates, S. 2002. Review of methodology of quantitative reviews using meta-analysis in ecology. Journal of Animal Ecology **71,** 547-557.

Gaukler, S. M., Linz, G. M., Sherwood, J. S., Dyer, N. W., Bleier, W. J., Wannemuehler, Y. M., ... & Logue, C. M. 2009. Escherichia coli, salmonella, and mycobacterium avium subsp. paratuberculosis in Wild European starlings at a Kansas cattle feedlot. Avian Diseases **53**, 544-551.

Gillen, S., Schuster, T., zum Büschenfelda, C.M., Friess, H. and Kleef, J. 2010. Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of response and resection percentages. PLOS Medicine, DOI: 10.1371/journal.pmed.1000267.

Gilliver, M., Bennett, m., Begon, M., Hazel, S., and Hart, C. 1999. *Antibiotic resistance found in wild rodents.* Nature **401,** 233-234.

Glass, G.V. and Smith, M.L. 1978. *Reply to Eysenck.* American Psychologist **33**, 517-519.

Gould, I.M. 2009. *Controversies in infection: infection control or antibiotic stewardship to control healthcare-acquired infection?* Journal of Hospital Infection **73,** 386-391.

Gould, I.M. 2012. *The antibiotic paradox.* In: Antibiotic Policies. Eds Gould, I.M. and Meer, J.W.M. Springer New York. 15-25.

Guenther, S., Grobbel, M., Lübke-Becker, A., Goedecke, A., Friedrich, N. D., Wieler, L. H., & Ewers, C. 2010. Antimicrobial resistance profiles of< i> Escherichia coli</i> from common European wild bird species. Veterinary microbiology **144**, 219-225.

Gurevitch, J., Curtis, P.S. and Jones, M.H. 2001. Meta-analysis in ecology. Advances in Ecological Research **32**, 199–247.

Gurevitch, J., & Hedges, L. V. 1999. Statistical issues in ecological meta-analyses. Ecology **80**, 1142-1149.

Haggbloom, S. J., Warnick, R., Warnick, J.E., Jones, V.K., Yarbrough, G.L., Russell, T.M., Borecky, C.M., GcGahhey, R., Powell J.L. III, Beavers, J. and Monte, E. (2002). *The 100 most eminent psychologists of the 20th century.* Review of General Psychology, **6**, 139–152.

Halling-Sørensen, B., Nielsen, S.N., Lanzky, P.F., Lützhøft, H.C.H. and Jørgensen, S.E. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment – a review. Chemosphere **36,** 357-393.

Halpern, M.T., Schmier, J.K., Snyder, L.M., Asche, C., Sarocco, P.W., Lavin, B., Nieman, R. and Mandell, L.A. 2005. *Meta-analysis of bacterial resistance to macrolides.* Journal of Antimicrobial Chemotherapy **55,** 748-757.

Han, D., Unno, T., Jang, J., Lim, K., Lee, S. N., Ko, G., ... & Hur, H. G. 2011. The occurrence of virulence traits among high-level aminoglycosides resistant *Enterococcus* isolates obtained from feces of humans, animals, and birds in South Korea. International Journal of Food Microbiology **144**, 387-392.

Hancock, R.E.W. 2007. *The end of an era?* Nature Reviews Drug Discovery **6,** 28.
Hart, B., Lundh, A. and Bero, L. 2012. Effect of reporting bias on meta-analyses of drug trials: reanalysis of meta-analyses. BMJ **344,** doi: http://dx.doi.org/10.1136/bmj.d7202.

Hasan, B., Sandegren, L., Melhus, Å., Drobni, M., Hernandez, J., Waldenström, J., Alam, M. and Olsen, B. 2012. Antimicrobial Drug–Resistant Escherichia coli in Wild Birds and Free-range Poultry, Bangladesh. Emerging Infectious Diseases **18,** 2055-2058.

Hedges, L.V., Gurevitch, J. and Curtis, P.S. 1999. The meta-analysis of response ratios in experimental ecology. Ecology **80,** 1150-1156. http://dx.doi.org/10.1890/0012-9658(1999)080[1150:TMAORR]2.0.CO;2

Hess, G. 1996. *Disease in metapopulation models: implications for conservation.* Ecology **77,** 1617-1632.

Hillebrand, H. 2008. Meta-analysis in ecology. eLS. DOI: 10.1002/9780470015902.a0003272

Ioannidis, J.P.A. 2005. *Why Most Published Research Findings Are False.* PLoS Med 2(8): e124. doi:10.1371/journal.pmed.0020124

Ioannidis, J. P., & Lau, J. 1999. Pooling research results: benefits and limitations of meta-analysis. The Joint Commission journal on quality improvement **25**, 462-469.

IUCN. 2007. Habitat Classification Scheme. Available online at: http://www.iucnredlist.org/documents/June_2012_Guidance_Habitats_Classification_Scheme.pdf. Accessed 22 July 2012.

IUCN, 2012. Summary Statistics, The IUCN Red List of Threatened Species. Available online at: http://www.iucnredlist.org/about/summary-statistics. Accessed 5 August 2012.

Jayawardena, R., Ranasinghe, P., Galappatthy, P., Malkanthi, R., Constantine, G. and Katulanda, P. Effects of zinc supplementation on diabetes mellitus: a systematic review and meta-analysis. Diabetology and Metabolic Syndrome **4** a systematic review and meta-analysis. doi:10.1186/1758-5996-4-1

Jones, A.P., Conroy, E., Williamson, P.R., Clarke, M. and Gamble, C. 2013. The use of systematic reviews in the planning, design and conduct of randomised trials: a retrospective cohort of NIHR HTA funded trials. BMC Medical Research Methodology **13,** doi:10.1186/1471-2288-13-50.

Keeling, M.J., Danon, L., Vernon, M.C. and House, T.A. *Individual identity and movement networks for disease metapopulations.* Proceedings of the National Academy of Sciences of the United States of America **107,** 8866-6670.

Kemper, N. 2008. Veterinary antibiotics in the aquatic and terrestrial environment. Ecological Indicators **8,** 1-13.

Kitadai, N., Obi, T., Yamashita, S., Murase, T., & Takase, K. 2012. Antimicrobial susceptibility of Escherichia coli isolated from feces of wild cranes migrating to Kagoshima, Japan. The Journal of veterinary medical science/the Japanese Society of Veterinary Science **74**, 395-397.

Knapp, G. and Hartung. J. 2003. Improved tests for a random effects meta-regression with a single covariate. Statistics in Medicine **22,** 2693-2710.

Koskimies, P. 1989. Birds as a tool in environmental monitoring. In *Annales Zoologici Fennici* (Vol. 26, No. 3, pp. 153-166).

Koskimies, P. 1989. Birds as a tool in environmental monitoring. Annales Zoologici Fennici **26,** 153-166.

Kozak, G.K., Boerlin, P., Janecko, N., Reid-Smith, R.J. and Jardine, C. 2009. Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. Applied and Environmental Microbiology **75,** 559-566.

Kumar, K., Gupta, S.C., Baidoo, S.K., Chander, Y. and Rosen, C.J. 2005. Antibiotic uptake by plants from soil fertilized with animal manure. Journal of Environmental Quality **34,** 2082-2085.

Kümmerer, K. 2009. Antibiotics in the aquatic environment–a review–part I. Chemosphere **75**, 417-434.

Larsen, P.O. and von Ins, M. 2010. *The rate of growth in scientific publication and the decline in coverage provided by Science Citation Index.* Scientometrics **84,** 575-603.

Lee, E., Dobbins, M., DeCorby, K., McRae, L., Tirilis, D. and Husson, H. 2012. An optimal search filter for retrieving systematic reviews and meta-analyses.BMC Medical Research Methodology **12,** doi:10.1186/1471-2288-12-51

Lemeshow, A.R., Blum, R.E., Berlin, J.A., Stoto, M.A. and Colditz, G.A. 2005. Searching one or two databases was insufficient for meta-analysis of observational studies. Journal of Clinical Epidemiology **58,** 867-873.

Levy, S.B. 2001. Antibiotic resistance: consequences of inaction. Clinical Infectious Diseases **33,** S124-S129.

Levy, S.B. and Marshall, B. 2004. *Antibiotic resistance worldwide: causes, challenges, and responses* Miranda, C.D. and Zemelman, R. 2001. *Antibiotic resistant bacteria in fish from the Concepción Bay, Chile.* Marine Pollution Bulletin **42,** 1096-1102.

Liu, D., Xiao, W., Lu, J. and Zhang, Z. *Review of research progress of infectious diseases in wild birds.* Shengtai Xuebao/Acta Ecologica Sinica **31,** 6959-6966.

Livermore, D.M., Warner, M., Hall, L.M.C., Enne, V.I., Projan, S.J., Dunman, P.M., Wooster, S.L. & Harrison, G. *Antibiotic resistance in bacteria from magpies (Pica pica) and rabbits (Oryctolagus cuniculus) from west Wales.* Environmental Microbiology **3,** 658-661.

Luborsky, L. 1954. *A note on Eysenck's article "The effects of psychotherapy: an evaluation."* British Journal of Psychology **45**, 129-131.

Mallory, M.L., Robinson, S.A., Hebert, C.E. and Forbes, M.R. 2010. Seabirds as indicators of aquatic ecosystem conditions: a case for gathering multiple proxies of seabird health. Marine Pollution Bulletin **60**, 7-12. http://dx.doi.org/10.1016/j.marpolbul.2009.08.024.

Marrow, J., Whittington, J. K., Mitchell, M., Hoyer, L. L., & Maddox, C. 2009. Prevalence and antibiotic-resistance characteristics of Enterococcus spp. isolated from free-living and captive raptors in central Illinois. Journal of wildlife diseases **45**, 302-313.

Martínez, J.L. 2008. Antibiotics and Antibiotic Resistance Genes in Natural Environments. Science **321,** 365-367. DOI: 10.1126/science.1159483

Martinez, J.L. 2009a. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environmental Pollution **157,** 2893-2902.

Martinez, J.L. 2009b. The role of natural environments in the evolution of resistance traits in pathogenic bactiera. Proceedings of the Royal Society B **276,** 2521-2530.

McCallum, H. I., Kuris, A., Harvell, C. D., Lafferty, K. D., Smith, G. W., & Porter, J. 2004. Does terrestrial epidemiology apply to marine systems? Trends in Ecology & Evolution **19**, 585-591.

Middleton, J. H., & Ambrose, A. 2005. Enumeration and antibiotic resistance patterns of fecal indicator organisms isolated from migratory Canada geese (Branta canadensis). Journal of Wildlife Diseases **41**, 334-341.

Mirzaie, S., Hassanzadeh, M., & Ashrafi, I. 2010. Identification and characterization of Salmonella isolates from captured house sparrows. Turkish Journal of Veterinary and Animal Sciences **34**, 181-186.

Moher, D., Cook, D.J., Eastwood, S., Olkin, I., Rennie, D., Stroup, D.F. *et al.* 1999. *Improving the quality of reports of meta-analyses of randomized controlled trials: the QUOROM statement.* The Lancet **354,** 1896-1900.

Morrison, M.L. 1986. Bird populations as indicators of environmental change. Current Ornithology **3,** 429-451.

Nascimento, A. M., Cursino, L., Gonçalves-Dornelas, H., Reis, A., Chartone-Souza, E., & Marini, M. Â. 2003. Antibiotic-resistant Gram-negative bacteria in birds from the Brazilian Atlantic forest. The Condor **105**, 358-361.

Neu, H.C. 1992. *The crisis in antibiotic resistance.* Science **257,** 1064-1073.

Olsen, B., Munster, V.J., Wallensten, A., Waldenström, J., Osterhaus, A.D.M.E. and Fouchier, R.A.M. 2006. Global Patterns of Influenza A Virus in Wild Birds. Science **312**, 384-388. DOI: 10.1126/science.1122438

Osenberg, C.W., Sarnelle, O., Coopers, S.D. and Holt, R.D. 1999. Resolving ecological questions through meta-analysis.: goals, metrics, and models. Ecology **80,** 1105-1117.

Osterblad, M., Norrdahl, L., Korpimaki, E. & Huovinen, P. 2001. *How wild are wild mammals?* Nature **409,** 233-234.

Pallecchi, L., Bartoloni, A., Paradisi, F. & Rossolini, G.M. 2008. *Antibiotic resistance in the absence of antimicrobial use: mechanisms and implications.* Expert Review of Anti-Infective Therapy **6,** 725-732.

Palumbi, S.R. 2001. Humans as the world's greatest evolutionary force. Science **293,** 1786-1790.

Pappas, G., Kiriaze, I.J. and Falagas, M.E. 2008. *Insights into infectious disease in the era of Hippocrates.* International Journal of Infectious Diseases **12,** 347-350.

Parker, LA., Saez, NG, Porta, M., Hernandez-Aguado, I., and Lumbreras, B. 2012. The impact of including different study designs in meta-analyes of diagnostic accuracy studies. Eur J Empidemol.

Piddock, L.J.V. 2011. *The crisis of no new antibiotics—what is the way forward?* The Lancet Infectious Diseases **12,** 249-253.
Poeta, P., Costa, D., Sáenz, Y., Klibi, N., Ruiz-Larrea, F., Rodrigues, J., & Torres, C. 2005. Characterization of antibiotic resistance genes and virulence factors in faecal enterococci of wild animals in Portugal. Journal of Veterinary Medicine, Series B **52**, 396-402.

Pullin, A.S. and Knight, T.M. 2001. *Effectiveness in conservation practice: pointers from medicine and public health.* Conservation Biology **15,** 50-54.

Radhouani, H., Pinto, L., Coelho, C., Sargo, R., Araujo, C., Lopez, M., ... & Poeta, P. 2010. MLST and a genetic study of antibiotic resistance and virulence factors in vanA-containing Enterococcus from buzzards (Buteo buteo). Letters in Applied Microbiology **50**, 537-541.

Radimersky, T., Frolkova, P., Janoszowska, D., Dolejska, M., Svec, P., Roubalova, E., Cikova, P. & Literak, I. 2010. *Antibiotic resistance in faecal bacteria (Escherichia coli, Enterococcus spp.) in feral pigeons.* Journal of Applied Microbiology **109,** 1687-1695.

Ricklefs, R.E. 2012. Species richness and morphological diversity of passerine birds. PNAS **109,** 14482-14487.

Rifkin, J. L., Nunn, C. L., & Garamszegi, L. Z. 2012. Do animals living in larger groups experience greater parasitism? A meta-analysis. The American Naturalist **180**, 70-82.

Rolain, J.M., Canton, R. and Cornaglia, G. 2012. Emergence of antibiotic resistance: need for a new paradigm. Clinical Microbiology and Infection **18,** 615-616.

Rolland, R.M., Hausfater, G., Marshall, B. & Levy, S.B. 1985. *Antibiotic-resistant bacteria in wild primates: increased prevalence in baboons feeding on human refuse.* Applied and Environmental Microbiology **49,** 791-794.

Rose, S. 2010. *Hans Eysenck' controversial career.* The Lancet **376,** 407-408.

Rosengren, L.B., Gow, S.P. and Weese, J.S. 2010. Antimicrobial use and resistance in pigs and chickens: A review of the science, policy and control practices from farm to slaughter – executive summary. Canadian Journal of Infectious Diseases and Medical Microbiology **21,** 123-124.

Rosenzweig, S. A transvaluation of psychotherapy: a reply to Hans Eysenck. The Journal of Abnormal and Social Psychology **49**, 298-304.

Rybaříková, J., Dolejská, M., Materna, D., Literák, I., & Čížek, A. 2010. Phenotypic and genotypic characteristics of antimicrobial resistant *Escherichia coli* isolated from symbovine flies, cattle and sympatric insectivorous house martins from a farm in the Czech Republic (2006–2007). Research in Veterinary Science **89**, 179-183.

Russell, R.L. and Orlinksy, D.E. 1996. Psychotherapy research in historical perspective. Archives of General Psychiatry **53,** 708-715.

Saldaña, J. 2010. *Modelling the spread of infectious diseases in complex metapopulations.* Mathematical Modelling of Natural Phenomena **5,** 22-37.

Sampson, M., Barrowman, N.J., Moher, D., Klassen, T.P., Pham, B., Platt, R., St John, P.D., Viola, R. and Raina, P. 2003. Should meta-analysts search Embase in addition to Medline? Journal of Clinical Epidemiololgy **6**, 943-55.

Sarmah, A.K., Meyer, M.T. and Boxall, A.B.A. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (Vas) in the environment. Chemosphere **65,** 725-759.

Sato, M., Ahmed, A.M., Noda, A., Watanabe, H., Fukumoto, Y. and Shimamoto, T. 2009. Isolation and characterisation of multidrug-resistant Gram-negative bacteria from imported flamingos in Japan. Acta Veterinaria Scandinavica **51:**46, doi:10.1186/1751-0147-51-46.

Sayah, R. S., Kaneene, J. B., Johnson, Y., & Miller, R. 2005. Patterns of antimicrobial resistance observed in Escherichia coli isolates obtained from domestic-and wild-animal fecal samples, human septage, and surface water. Applied and Environmental Microbiology **71**, 1394-1404.

Schaefer, A.M., Goldstein, J.D., Reif, J.S., Fair, P.A. & Bossard, G.D. 2009. Antibiotic-resistant organisms cultured from Atlantic bottlenose dolphins (Tursiops truncatus) inhabiting estuarine waters of Charleston, SC and Indian River Lagoon, FL. EcoHealth **6,** 33-41.

Schultz, K.F., Altman, D.G., Moher, D. 2010. *CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials.* BMJ **340,** 698-702.

Schneider, R.L., Mills, E.L. and Josephson, D.C. 2002. Aquatic-terrestrial linksages and implications for landscape management. In: Liu, J. and Taylor, W.W. (eds) Integrating Landscape ecology into Natural Resource Management. Cambridge University Press, 480pp.

Schwarzer, G. 2014. Meta-analysis with R. Available online at: http://cran.r-project.org/web/packages/meta/meta.pdf. Accessed 14 April 2014.

Séveno, N. A., Kallifidas, D., Smalla, K.; van Elsas, J. D., Collard, J.-M.; Karagouni, A. D. and Wellington, E. M. H. 2002. Occurrence and reservoirs of antibiotic resistance genes in the environment. Reviews in Medical Microbiology **13,** 15-27.

Shaprio, S. 1994. Meta-analysis/Shmeta-analysis. American Journal of Epidemiology **140,** 771-778.

Sherley, M., Gordon, D.M. and Collignon, P.J. 2000. Variations in antibiotic resistance profile in Enterobacteriaceae isolated from wild Australian mammals. Environmental Microbiology **2,** 620-631.

Silbergeld, E.K., Graham, J. And Price, L.B. 2008. Industrial food animal production, antimicrobial resistance, and human health. The Annual Review of Public Health **29,** 151-169.

Silva, V. L., Nicoli, J. R., Nascimento, T. C., & Diniz, C. G. 2009. Diarrheagenic Escherichia coli strains recovered from urban pigeons (Columba livia) in Brazil and their antimicrobial susceptibility patterns. Current Microbiology **59**, 302-308.

Singer, R.S., Ward, M.P and Maldonado, G. 2006. *Can landscape ecology untange the complexity* Smith, P., Hiney, M.P. & Samuelson, O.B. 1994. *Bacterial resistance to antimicrobial agents used in fish farming: a critical evaluation of method and meaning.* Annual Review of Fish Diseases **4,** 273-313.

Sjölund, M., Bonnedahl, J., Hernandez, J., Bengtsson, S., Cederbrant, G., Pinhassi, J., Kahlmeter, G. and Olsen, B. 2008. *Dissemination of multidrug-resistant bacteria into the Arctic.* International Polar Year **14,** 70-72.

Smith, M.L. and Glass, G.V. 1977. *Meta-analysis of psychotherapy outcome studies.* American Psychologist **32,** 752-760.

Smits, J.E.G. and Fernieb, K.J. 2013. Avian wildlife as sentinels of ecosystem health. Comparative Immunology, Microbiology and Infectious Diseases **36,** 333-342.

Spector, T.D. and Thompson, S.G. 1990. The potential and limitations of meta-analysis. Journal of Epidemiology and Community Health **45,** 89-92.

Stachowiak, M., Clark, S.E., Templin, R.E. and Baker, K.H. 2009. *Tetracycline-resistant* Escherichia coli *in a small stream receiving fish hatchery effluent.* Water, Air, & Soil Pollution.

Sterne, J. A., Egger, M., & Smith, G. D. 2001. Investigating and dealing with publication and other biases in meta-analysis. BMJ **323**, 101-105.

Stewart, G. 2009. *Meta-analysis in applied ecology.* Biology Letters **6,** 78-81.

Stock, I. & Wiedemann, B. 2001. *Natural antibiotic susceptibilities of* Edwardsiella tarda, E. icaluri *and* E. hoshinae. Antimicrobial Agents and Chemotherapy **45,** 2245-2255.

Stroup, D.F., Berlin, J.A., Morton, S.C., Olkin, I., Williamson, G.D., Rennie, D., Moher, D., Becker, B.J., Sipe, T.A., and Thacker, S.B. 2000. *Meta-analysis of observational studies in epidemiology – A proposal for reporting.* Journal of the American Medical Association **283,** 2008-2012.

Strupp, H.H. 1993. Exploring applied psychology: origins and critical analyses. Master lectures in psychology. American Psychological Association, Washington D.C., USA. 193pp.

Sutherland, W.J., Pullin, A.S., Dolman, P.M. and Knight, T.M. 2004. The need for evidence-based conservation. Trends in Ecology and Evolution **19,** 305-308.

Sutton, A.J., Cooper, N.J., Jones, D.R., Lambert, P.C., Thompson, J.R. and Abrams, K.R. 2007. Evidence-based sample size calculations based upon updated meta-analysis. Statistical Methods **26,** 2479-2500.

Thaller, M.C., Migliore, L., Marquez, C., Tapia, W., Cedeño, V., Rossolini, M. & Gentile, G. 2010. *Tracking acquired antibiotic resistance in commensal bacteria of Galápagos land iguanas: no man, no resistance.* PLoS ONE **5:** e8989. doi:10.1371/journal.pone.0008989

Thompson, M., Tiwari, A., Fu, R., Moe, E., and Buckley, D.I. 2012. A framework to facilitate the use of systematic reviews and meta-analyses in the design of primary research studies. Prepared by the Oregon Evidence-based Practice Center under contract HHSA 290-2007-10057-I. AHRQ Publication No. 12-EHC009-EF. Rockville, MD: Agency for Healthcare Research and Quality. January 2012. www.effectivehealthcare.ahrq.gov/reports/final.cfm.

Valkama, E., Uusitalo, R., Ylivainio, K., Virkajärvi, P., & Turtola, E. 2009. Phosphorus fertilization: a meta-analysis of 80 years of research in Finland. Agriculture, ecosystems & environment **130**, 75-85.

van Dongen, W.F.D., White, J., Brandl, H.B., Moodley, Y., Merkling, T., Leclaire, S., Blanchard, P., Danchin, E., Hatch, S.A. and Wagner, R.H. 2013. Age-related differences in the cloacal microbiota of a wild bird species. BMC Ecology **13**:11, doi:10.1186/1472-6785-13-11.

Via,E., Radua, J., Cardoner, N., Happé, F. and Mataix-Cols, D. 2011. Meta-analysis of gray matter abnormalities in autism spectrum disorder: should Asperger disorder be subsumed under a broader umbrella of autistic spectrum disorder? Archives of General Psychiatr **68**, 409-418. doi: 10.1001/archgenpsychiatry.2011.27.

Waldenström, J., Mevius, D., Veldman, K., Broman, T., Hasselquist, D., & Olsen, B. 2005. Antimicrobial resistance profiles of Campylobacter jejuni isolates from wild birds in Sweden. Applied and environmental microbiology **71**, 2438-2441.

Waldvogel, F.A. 2004. *Infectious diseases in the 21st century: old challenges and new opportunities.* International Journal of Infectious Disease **8,** 5-12.

Walker, E., Hernandez, A. V., & Kattan, M. W. 2008. Meta-analysis: its strengths and limitations. Cleveland Clinic Journal of Medicine **75**, 431-439.

Wampold, B.E. 2001. The Great Psychotherapy Debate: Models, Methods, and Findings. Lawrence Erlbaum Associates, Inc. Mahwah, New Jersey.

Wenzel, R.P. 2004. *The antibiotic pipeline – challenges, costs, and virtues.* New England Journal of Medicine **351,** 523-526.

Wilson, D.B. and Lipsey, M.W. Practical Meta-Analysis. Applied Social Research Methods Series **49,** 251pp.

World Health Organization. 2000. *Overcoming antimicrobial resistance.* World Health Report on Infectious Diseases. Available online at: http://www.who.int/infectious-disease-report/2000/other_versions/index-rpt2000_text.html. Accessed 8 June 2012.

World Health Organization. 2002. *Fact sheet number 194: antibiotic resistance.* Available online at: http://www.who.int/mediacentre/factsheets/fs194/en/. Accessed on 10 October 2010.

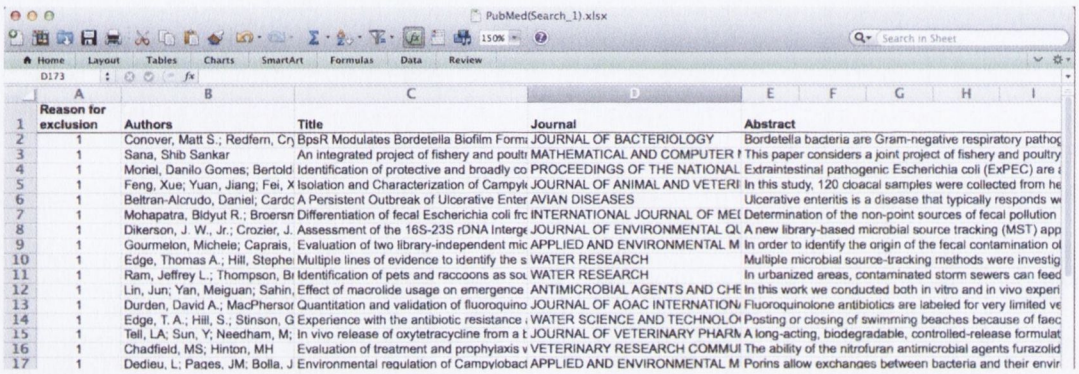
World Health Organization. 2005. Containing antimicrobial resistance. *Policy Perspectives on Medicines.* World Health Organization, Geneva. Available online at http://hinfo.humaninfo.ro/gsdldata/collect/ medicinedocs/pdf/s7920e/s7920e.pdf. Accessed 9 October 2010.

World Health Organization Collaborating Centre for Drug Statistics Methodology. 2012. Guidelines for ATC classification and DDD assignment. Oslo, 2011.

Wright, G.D. and Sutherland, A.D. 2007. *New strategies for combating multidrug-resistant bacteria.* Trends in Molecular Medicine **13,** 260-267.

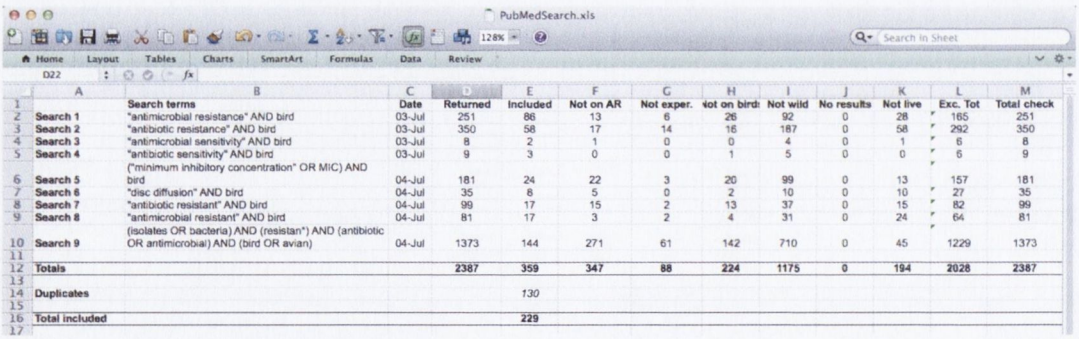
3.8 Appendix I. Example of article review process.

The first spreadsheet shows an example of the articles returned from one search (e.g. Search_1, which used search terms, "antimicrobial resistance" AND bird). The reason for each of the article's exclusion is indicated in the first column. Included articles were exported to a separate database. The second spreadsheet shows the compiled information for all of the searches (within one of the databases), including the search terms used, the number of articles returned, the number of articles ultimately included, and the number of articles that were excluded (and the reasons they were).



PubMed(Search_1).xlsx

	Reason for exclusion	Authors	Title	Journal	Abstract
2	1	Conover, Matt S.; Redfern, Cr	BpsR Modulates Bordetella Biofilm Forma	JOURNAL OF BACTERIOLOGY	Bordetella bacteria are Gram-negative respiratory pathog
3	1	Sana, Shib Sankar	An integrated project of fishery and poultr	MATHEMATICAL AND COMPUTER I	This paper considers a joint project of fishery and poultry
4	1	Moriel, Danilo Gomes; Bertold	Identification of protective and broadly co	PROCEEDINGS OF THE NATIONAL	Extraintestinal pathogenic Escherichia coli (ExPEC) are a
5	1	Feng, Xue; Yuan, Jiang; Fei, X	Isolation and Characterization of Campylo	JOURNAL OF ANIMAL AND VETERI	In this study, 120 cloacal samples were collected from he
6	1	Beltran-Alcrudo, Daniel; Cardc	A Persistent Outbreak of Ulcerative Enter	AVIAN DISEASES	Ulcerative enteritis is a disease that typically responds w
7	1	Mohapatra, Bidyut R.; Broersn	Differentiation of fecal Escherichia coli frc	INTERNATIONAL JOURNAL OF MEI	Determination of the non-point sources of fecal pollution
8	1	Dikerson, J. W., Jr.; Crozier, J.	Assessment of the 16S-23S rDNA Interge	JOURNAL OF ENVIRONMENTAL QU	A new library-based microbial source tracking (MST) app
9	1	Gourmelon, Michele; Caprais,	Evaluation of two library-independent mic	APPLIED AND ENVIRONMENTAL M	In order to identify the origin of the fecal contamination ol
10	1	Edge, Thomas A.; Hill, Stephe	Multiple lines of evidence to identify the s	WATER RESEARCH	Multiple microbial source-tracking methods were investig
11	1	Ram, Jeffrey L.; Thompson, Br	Identification of pets and raccoons as sou	WATER RESEARCH	In urbanized areas, contaminated storm sewers can feed
12	1	Lin, Jun; Yan, Meiguan; Sahin,	Effect of macrolide usage on emergence	ANTIMICROBIAL AGENTS AND CHE	In this work we conducted both in vitro and in vivo experi
13	1	Durden, David A.; MacPherson	Quantitation and validation of fluoroquino	JOURNAL OF AOAC INTERNATION	Fluoroquinolone antibiotics are labeled for very limited ve
14	1	Edge, T. A.; Hill, S.; Stinson, G	Experience with the antibiotic resistance	WATER SCIENCE AND TECHNOLO	Posting or closing of swimming beaches because of faec
15	1	Tell, LA; Sun, Y; Needham, M;	In vivo release of oxytetracycline from a t	JOURNAL OF VETERINARY PHARM	A long-acting, biodegradable, controlled-release formulat
16	1	Chadfield, MS; Hinton, MH	Evaluation of treatment and prophylaxis v	VETERINARY RESEARCH COMMUI	The ability of the nitrofuran antimicrobial agents furazolid
17	1	Dedieu, L; Pages, JM; Bolla, J	Environmental regulation of Campylobact	APPLIED AND ENVIRONMENTAL M	Porins allow exchanges between bacteria and their envir



PubMedSearch.xls

	Search terms	Date	Returned	Included	Not on AR	Not exper.	Not on bird	Not wild	No results	Not live	Exc. Tot	Total check
Search 1	"antimicrobial resistance" AND bird	03-Jul	251	86	13	6	26	92	0	28	165	251
Search 2	"antibiotic resistance" AND bird	03-Jul	350	58	17	14	16	187	0	58	292	350
Search 3	"antimicrobial sensitivity" AND bird	03-Jul	8	2	1	0	0	4	0	1	6	8
Search 4	"antibiotic sensitivity" AND bird	03-Jul	9	3	0	0	1	5	0	0	6	9
Search 5	("minimum inhibitory concentration" OR MIC) AND bird	04-Jul	181	24	22	3	20	99	0	13	157	181
Search 6	"disc diffusion" AND bird	04-Jul	35	8	5	0	2	10	0	10	27	35
Search 7	"antibiotic resistant" AND bird	04-Jul	99	17	15	2	13	37	0	15	82	99
Search 8	"antimicrobial resistant" AND bird	04-Jul	81	17	3	2	4	31	0	24	64	81
Search 9	(isolates OR bacteria) AND (resistan*) AND (antibiotic OR antimicrobial) AND (bird OR avian)	04-Jul	1373	144	271	61	142	710	0	45	1229	1373
Totals			2387	359	347	88	224	1175	0	194	2028	2387
Duplicates				130								
Total included				229								

4 An investigation of antibiotic resistant bacteria in the Greater Flamingo (*Phoenicopterus roseus*) in the Mediterranean region

4.1 Introduction

4.1.1 Use of sentinels

Throughout history, a variety of animal species have been used to monitor pollutants in a given environment. Perhaps one of the best-known examples is the canary, which was commonly used to detect carbon monoxide poisoning in mines starting in the 19th Century; it was deemed to be advantageous because, "they are much more sensitive to the poisonous action of the gas than are men" (Burrell and Siebert, 1916). Such animals are often referred to as 'sentinels', a term likely derived from the Latin *sentire,* meaning 'perceive, feel, experience' (Cawley, 2013). The use of sentinels in scientific research began to increase in the mid-20th century due to a growing awareness about the extent and potential effects of anthropogenic contaminants affecting supposedly 'natural' environments (van der Schalie *et al.*, 1999). This awareness, and the environmental movement that came with it, was in part a result of the publication of Rachel Carson's book Silent Spring in 1962, which exposed the increasingly negative effects of anthropogenic activity on environmental health (Palmer *et al.*, 1998; Heckel, 2012; Mascarelli, 2012). The book's title implies a future without birds, which she noted had been dying due to the overuse of pesticides (Carson, 1962). In asserting, "Our fate is connected with the animals", she noted that wildlife health is closely connected to human health, and thus, warning signs should not be ignored. It is therefore unsurprising that in the decades that followed the publication of Silent Spring, a vast body of research has used a variety of wildlife species to assess the extent of contamination in both aquatic and terrestrial ecosystems (National Research Council, 1991; for reviews see Phillips, 1970; Rabinowitz *et al.*, 2005).

In addition to their use in monitoring chemical contaminants such as heavy metals and pesticides, wildlife species are also useful in assessing the prevalence of infectious disease – an important component of an environments' overall condition (Rabinowitz *et al.*, 2009). In a human health context, wildlife can be particularly useful where the infectious disease is zoonotic (Patz *et al.*, 2004; Rabinowitz *et al.* 2009). Zoonotic diseases are diseases that can be transferred between animals (either domestic or wild) and humans, and comprise 75% of emerging infectious diseases in humans (Taylor *et al.*, 2001; Patz *et al.*, 2004). Research that uses animal species to evaluate the prevalence of zoonotic disease can be used to forecast the threat of disease outbreaks in human populations (van der Schalie *et al.*, 1999; Halliday *et al.* 2007). It is therefore unsurprising that a large body of research has developed that uses wildlife in such a context (Rabinowitz *et al.*, 2005).

Within this literature, the terminology used to describe the use of wildlife to evaluate environmental health is varied and inconsistent, and includes the following: 'indicators', 'sentinels', 'monitors', 'biomonitors', 'bioindicators', and 'bioaccumulators', amongst others (O'Brien *et al.*, 1993; Berthet, 2013). Most authors make an attempt to state which term they are using and justify their selection by providing a definition (e.g. Rainbow, 1995), though the definitions given vary considerably between authors (O'Brien *et al.*, 1993; Zacharias and Roff, 2001). As a result, although many authors have offered their own definitions of the respective terms, no consensus has been reached (O'Brien *et al.*, 1993; Beeby, 2001; Zacharias and Roff, 2001; Halliday *et al.*, 2007). In this study, the term *sentinel* is used following definitions proposed by Beeby (2001) and Halliday *et al.* (2007), who define a sentinel as a species used to monitor the prevalence of infectious disease in a given environment. That is, a particular aspect of environmental condition (here, antibiotic resistance) is not assessed by a given animal's presence or absence (which is how an 'indicator' is commonly described) – but rather, through the detection of a response within the animal itself. A similar definition is used by Zacharias and Roff (2001), who assert that the term sentinel is synonymous with the term 'condition indicator'. Further to offering specific definitions of sentinels, these authors and others have provided a list of criteria for the selection of an 'ideal' sentinel, usually with reference to one or more contaminants and/or diseases that can be monitored. Such lists are useful in ensuring that the species used is appropriate, which has implications for both practical considerations for fieldwork and interpreting the results of laboratory analysis of any samples taken. While selection criteria exist for a broad range of diseases and contaminants, none has been established that is specific to antibiotic resistant bacteria, despite the fact that a variety of wildlife species have been determined to be reservoirs of these potentially dangerous bacteria. The prevalence of these in environmental systems is described in the following section, which is followed by an overview of a candidate sentinel, the Greater Flamingo.

4.1.2 Antibiotic resistance

Since their introduction, the use of antibiotics in hospitals, homes, and sewage treatment plants has been widespread. The prevalence of antibiotic resistant bacteria (ARB) in urban locations is therefore not unexpected; for example, there are not-infrequent news stories describing outbreaks of drug resistant pathogens in hospitals. It is believed that antibiotic resistance was largely limited to hospitals until the mid-1960s (Alanis, 2005). After this, evidence suggesting the spread of ARB into supposedly 'natural' environments became available. Given their frequent use in agriculture, this is unsurprising; transport through water systems can also provide ARB with an opportunity to spread from urban areas (Cole, 2005). Indeed, proximity to human activity has been cited as a factor that serves to increase the

incidence of ARB, though this has been the subject of debate within the literature, given the potential for naturally occurring resistance to arise (Allen *et al.*, 2010). Research describing the presence of ARB in isolated areas, such as in birds in the Arctic, may be indicative of a more widespread problem (Gilliver *et al.*, 1999; Sjölund *et al.*, 2008; Miller *et al.*, 2009; Guenther *et al.*, 2010). In many of the studies, multi-drug resistance has been noted – suggestive of an even more severe and complex problem (Rosenblatt-Farrell, 2009).

Beyond home and hospital use, antibiotics are used in a variety of arenas, including in agriculture, fish farming, wastewater treatment, and industry – all of which have been suggested as putative sources of ARB in natural environments (McEwen *et al.*, 2006; Schaefer *et al.*, 2009). However, given their appearance where no such activities are present, or where human activity of any type is minimal, it is clear that much remains to be understood regarding the sources and mechanisms by which ARB result in natural environments (Blackburn *et al.*, 2010). The need for establishing the extent to which resistance patterns occur in nature, both spatially and temporally, is also widely recognized, as this will provide a basis from which possible sources and mechanisms of transfer of antibiotic resistance can be explored (Aminov, 2009). One of the challenges in investigating patterns of antibiotic resistance in environmental systems is determining the driving factors. To date, geographical variation in the distribution and abundance of ARB has been indicated, with resistances to particular antibiotics often (causally) linked to their use in the vicinity of the sampling site (Blanco *et al.*, 2007). Although some research has indicated that human activity is an important factor driving the prevalence of ARB in natural environments, other researchers have cautioned against assuming direct linkages between the co-occurrence of ARB and antibiotic use in a given environment (Singer *et al.,* 2006). Indeed, although a significant body of research now provides us with case-study evidence of the presence of ARB in natural environments, many of these studies were single-site investigations, limiting the potential for comparative analyses across a large geographic range. A landscape ecology approach has been recommended for the elucidation of processes underlying antibiotic resistance prevalence in natural environments (Singer *et al.*, 2006). It is clear that the need for establishing the extent to which resistance patterns occur in nature, both spatially and temporally remains (Aminov, 2009), and that use of a multi-site approach is a key in addressing these remaining questions; several recent efforts have adopted such an approach in an effort to address this gap in the research (Blanco *et* al., 2007; Guenther *et al.,* 2010). The selection of an appropriate study organism is of critical importance, and must take into account a variety of factors – including its geographical distribution (i.e. it must be found at multiple sites), as well as a number of more practical factors (i.e. where resources are limited, cost-effective sampling strategies must be possible).

4.1.3 The Greater Flamingo – a candidate sentinel of antibiotic resistance

Research on antibiotic resistance in animals has been conducted within agricultural, urban, and rural landscapes using a variety of sentinels including livestock, domestic pets, and wildlife species (Lloyd, 2007; Allen *et al.*, 2010; Wright, 2010; Bush *et al.*, 2011). The use of sentinels in monitoring antibiotic resistance is important, as they can provide an indication of the extent to which environmental systems serve as reservoirs of antibiotic resistance. This information can be used in examining factors (e.g. human activity, landscape, etc.) that contribute to the prevalence of antibiotic resistance in a given environment – a debate that has centered on the relative contribution of human activity. The selection of an appropriate species, in which a response can be detected (i.e. sensitivity or resistance to antibiotics), is thus of critical importance. The Greater Flamingo was proposed as a candidate sentinel of antibiotic resistance for this study. It was initially proposed as a candidate sentinel because it is a well-studied species, about which much has been published in the scientific literature. As a result, much is known about its life history, ecology and distribution; as a widely studied bird that is also one of the more charismatic species of birds (and can be considered a flagship species), many focused research projects exist for which large numbers of flamingos are captured on an annual basis, providing an ideal opportunity for sample collection.

The genus *Phoenicopterus* is comprised of six species of flamingo. Between them, the six species have ranges across Europe, Asia, Africa, North America, and South America (Bird Life International, 2012). They can be distinguished from one another by a variety of visual factors including size, colouration (of plumage, legs, bills, and eyes), bill shape, and by the presence/absence of a hind toe (Flamingo Resource Centre, 2008). Flamingos are a popular attraction both in- and ex-situ, serving as draws at zoos (nearly 70% of zoos in Europe have flamingos, for example) and within their natural habitats, where ecotourists flock to see them (Galicia and Baldassarre, 1997; King and van Weeren, 2005; Azcárate, 2010). Given their charismatic appeal, flamingos are amongst the most well-known and widely recognized birds and are therefore often used as a flagship species for wetland conservation and environmental campaigns in general; they recently featured in a biodiversity awareness campaign in Ireland (Plate 4) (Schmitz *et al.*, 1990; Baldassarre and Arengo, 2000; BiodiversityInOurLives, 2013).

Plate 4. A 'biodiversity beermat' that featured a flamingo chick, in providing a link between biodiversity and people's every day lives in an awareness campaign developed to share research findings with the public. The beermats appeared in dozens of pubs in Ireland between 2012 and 2013 (www.biodiversityinourlives.com).

Their popularity is further evidenced by the frequency with which flamingos appear in news media; they recently made the news when an account in the Daily Mail online covered a report from a zoo, which noted a connection between the music played during mating to breeding success...Barry White, naturally, was found to improve breeding success (Shead, 2012). Although seemingly trivial at times, their celebrity likely contributes to their draw as a popular research species and resultant efforts to address some of the frequently asked questions about flamingos – the answers to some of which have remained elusive. As recently as 2010, for example, Anderson and Williams investigated, *Why do flamingos stand on one leg*? while other recent research by Amat *et al.* (2011) has provided further information to another oft-asked question, *Why are flamingos pink?* (their research determined that one of the species, the Greater Flamingo, uses secretions from the uropygial gland – commonly referred to as the 'preen' or 'oil' gland – as a sort of 'make-up' to supplement the pink colour they get from their diet).

Geographic distribution and preferred habitats

Found on three continents (Europe, Asia, and Africa), the Greater Flamingo is the most geographically widespread of the genus *Phoenicopterus* (Geraci *et al.* 2012). Variation in effort across its large range makes estimation of its total global population difficult; surveys are not conducted on an annual basis at each of the sites where it breeds and the results from the individual surveys that do exist are rarely compiled; thus, the process of data aggregation is complicated. Estimates have placed the population at between 500,000 and 600,000 birds worldwide, making it the third most abundant species of flamingo (after the Lesser and Caribbean) (Johnson and Arengo, 2003; Birdlife International, 2012). Despite its large range and population, it breeds at a relatively small number of sites (~35) (Johnson and Cézilly, 2007). At each of these sites it exists as what is termed a 'breeding colony', each of which consists of anywhere between several hundred and tens of thousands of breeding pairs (Lee *et al.*, 2011; Geraci *et al.* 2012). Flamingos typically live in wetlands, including lagoons, marshes, and lakes, and as with the other species of flamingo, the Greater Flamingo prefers a saline environment (Cézilly and Johnson, 1995; Tourenq *et al.*, 2001; Nager *et al.*, 2010). As a result, their populations are frequently associated with saltpans – they inhabit both active and abandoned saltpans in addition to natural wetlands (Paracuellos *et al.*, 2002; Bechet *et al.*, 2012). The location and number of breeding sites for the Greater Flamingo varies from year to year, depending on weather and in particular, the accumulation of rainfall – the lack of which can leave traditional breeding sites dry in a given year (Cezilly and Johnson, 1995; Bechet and Johnson, 2008; Nager *et al.*, 2010). Management of potential breeding and nesting sites is therefore often practiced, including the maintenance of breeding islands and the artificial management of water levels (Cezilly *et al.* 1996; Martos and Johnson, 1996; Bechet and Johnson, 2008).

A large amount of research has been conducted on the Greater Flamingo in the Mediterranean Region, where over 300,000 individuals are found (Balkız *et al.*, 2007). The coastline of the Mediterranean has been used for salt production for centuries, and although many of these operations have ceased, the sites continue to provide an optimal habitat for many species of waterfowl (Sánchez *et al.*, 2006). Within this region, the Greater Flamingo was until recently considered to exist as several metapopulations, referred to as the Western Mediterranean, Eastern Mediterranean (believed to include colonies in Asia), and North African metapopulations (Balkız *et al.*, 2007; Boucheker, 2011). It was believed that each of these three metapopulations consisted of several breeding colonies, between which occasional exchanges (i.e. migration and other movements) occurred, but that the three metapopulations themselves were distinct from one another. However, Balkız *et al.* (2007) provided evidence, based on band-resighting data, of dispersal between the Western and Eastern Mediterranean metapopulations (particularly involving flamingos from Turkey, which had been long considered to be part of the Eastern Mediterranean metapopulation); thus the

two metapopulations came to be considered the European metapopulation. Further research by Boucheker *et al.* (2011) has indicated 'extensive interchange' between this European metapopulation and the North African one and thus, it seemed likely that the entire Mediterranean metapopulation of Greater Flamingos could be classed as one. Indeed, in 2012 genetic testing was, for the first time, used to differentiate breeding colonies; results confirmed that all colonies within the Mediterranean region should be considered as a single, interbreeding population with frequent movement and dispersal between sites (Geraci *et al.*, 2012). This finding has implications with regards to establishing conservation plans, and also in considering the potential for transfer of infectious disease between sites.

Life history

Flamingo chicks are typically born in the summer, following an incubation period that lasts up to 30 days, during which both the male and female parents mind the nest (Cézilly *et al.*, 1994). Once hatched, the flamingo chicks gather in a crèche (also commonly referred to as a nursery), which can contain thousands of flamingos (Cézilly *et al.*, 1994; Tourenq *et al.*, 1995). The adults care for the flamingo chicks in the crèche, and although they are left during the day, reunion readily occurs due to the unique call of each of the chicks, which its parents recognize (Childress *et al.*, 2006). Born with grey feathers, flamingo chicks lack the pink colouring for which flamingos are renowned. It is primarily from their food that flamingos get their pink colour, accumulating carotenoid pigments (which they cannot digest) as they mature (Fox, 1962; McGraw, 2006). In addition to obtaining their pink colouring from food, recent research has also provided evidence of flamingos gaining colouration from the application of oils derived from uropygial secretions (preening oils) as a sort of 'make-up', to enhance their colouration for mating purposes (Amat *et al.*, 2011).

The Greater Flamingo is a filter feeder, and ingests its primary source of food, aquatic invertebrates, using lamellae (Rodríguez-Pérez *et al.*, 2007). These are a specialized comb-like feature that allows them to take in food, while filtering out substances like mud that they do not need to ingest (Zweers *et al.*, 1995). The Greater Flamingo has been observed feeding on a number of additional sources of food, including seeds of plants and, at sites in France and Spain, rice (Zweers *et al.* 1995; Johnson, 2000; Deville *et al.*, 2013). Flamingo chicks are fed exclusively by their parents, which typically forage within a 10km range but have (very infrequently) been recorded foraging as far as 400km from the breeding site; foraging occurs both during the day and at night (Rendos-Martin and Johnson, 1996; Amat *et al.*, 2005). Flamingo chicks are fed beak-to-beak via crop secretions, which are produced by both parents and provide the chicks with essential vitamins (Eraud *et al.*, 2008). In the first few weeks, the crop secretions are referred to as 'crop milk' and are a distinctive red colour due to the presence of carotenoid pigments (Brown *et al.*, 2009). Although they display preliminary feeding behaviour as early as two weeks, flamingo chicks are reliant on their parents for food

for 10-12 weeks, until their beak is curved sufficiently such that they are able to feed themselves (Martin *et al.*, 2005; Brown *et al.*, 2009).

As described above, our knowledge of the nature of the Greater Flamingo metapopulation(s) in the Mediterranean region has evolved over the past several years. An improved understanding of the connectedness of the various populations has come as a result of the large dataset of reported sightings of ringed birds, made possible by annual ringing operations and, on a more limited scale, VHF radio telemetry and satellite tracking (Amat *et al.*, 2005; Javed *et al.*, 2006). This dataset provides a continuous source of evidence with which the extent and timings of migrations and movements of the Greater Flamingo between sites can be resolved. Most non-migratory movements occur for the purpose of foraging and in general, foraging trips are less frequent during chick-rearing (Amat *et al.*, 2005). Less is known about dispersal of the Greater Flamingo post-breeding season, when large-distance migration is believed to occur, due to the fact that observation effort is low in sites outside Southern Europe (Amat *et al.*, 2005; Sanz-Aguilar *et al.*, 2012). In 2005, a satellite tracking study indicated that the Greater Flamingo may travel over 2000km, from Southern Spain to sites in Mauritania in Northwest Africa (Amat *et al.*, 2005). Further evidence for dispersal to Mauritania was provided by Diawara *et al.* through use of a band-resighting study (2009). Band-resighting has also provided evidence of 'extensive exchange' between sites in Southern Europe and North Africa, where it is believed that some proportion of the Greater Flamingo overwinters (Samraoui *et al.*, 2009). Details about the nature of the Greater Flamingo's migratory movements have yet to be fully resolved, however, and are unlikely to be until further satellite tracking investigations are undertaken and/or observation efforts across the entirety of its potential range are improved.

Conservation status

In the Mediterranean region (where predators are few), the Greater Flamingo has a life span that can exceed 30 years (Johnson, 1997). In contrast to several of the other species of flamingo that are highly protected (e.g. the Andean Flamingo, classed as 'vulnerable' by the International Union for Conservation of Nature (IUCN), has a range that is limited to several sites in South America), the Greater Flamingo has retained a large range and its population levels have remained relatively stable (Bird Life International, 2012). Consequently, it is described as a species of 'least concern' by the IUCN. Despite this, the Greater Flamingo remains protected under several conventions; it is listed in Appendix II of the Convention on Trade in Endangered Species (CITES) and in Appendix A of Commission Regulation (EU) No. 709/2010 (on the 'protection of species of wild fauna and flora by regulating trade therein').

4.2 Aims

The aim of this chapter is to evaluate the Greater Flamingo as a sentinel of antibiotic resistance. This will be achieved by achieving the following objectives:

1. Produce a comprehensive list of criteria with which a sentinel species of antibiotic resistance can be selected.
2. Conduct preliminary screening of antibiotic resistant bacteria (ARB) in the Greater Flamingo (a candidate sentinel), involving:
    - Selection of appropriate study sites
    - Collection of bacterial samples from live Greater Flamingos at each of the study sites
    - Susceptibility testing of the bacterial samples against a panel of antibiotics
    - Evaluation of the extent of differences in resistance(s) between the study sites

4.3 Methods

4.3.1 Criteria for sentinel selection

Using Beeby (1999), Landres *et al.* (1988), Bowman and Schulte-Hostedde (2009) and Sayeh *et al.* (2005) as key references, a literature review was conducted to determine criteria that have traditionally been used to select wildlife sentinels for the surveillance and monitoring of environmental health (e.g. chemical pollution and infectious diseases). With this review, a preliminary list of criteria for the selection of wildlife sentinels was developed. The criteria compiled in this initial literature review were modified with reference to studies that evaluated antibiotic resistance in environmental systems. Those with potential relevance to antibiotic resistance were included; others were adapted to make them suitable for the purposes of selecting a sentinel of antibiotic resistance.

4.3.2 Determination of antibiotic resistant bacteria in the Greater Flamingo

4.3.2.1 Site selection

With reference to research publications and websites of relevant research groups (e.g. the Flamingo Specialist Group), a literature review was conducted to determine the primary breeding sites for the Greater Flamingo. From this review, a list of prospective study sites was generated with reference to the status of active research (i.e. ringing operations) at each of the breeding sites. For the purposes of this investigation, only sites within the Western Mediterranean metapopulation were considered for fieldwork, as it is more accessible than the sites located in the Eastern Mediterranean and Africa.

A list of twelve prospective sites with active ringing operations was generated (Table 4.1). Individuals conducting research on the Greater Flamingo at each of the sites and/or working at associated institutions were contacted to determine their interest in collaborating. The campaign to contact relevant researchers was aided by Dr. Arnaud Bechet, the Director of the Greater Flamingo Specialist Group. Dr. Bechet kindly offered to send out a letter on my behalf to researchers working with the ringing operations across the entire network, thus lending credibility to my research and helping in my efforts to arrange collaborations. Not all of the sites contacted had ringing operations planned for 2010; other sites did not respond to the request. In total, fieldwork was arranged at six sites (Table 4.1). However, in mid-April it became clear that fieldwork would not be possible at one of these sites – that located in Sardinia, Italy. Despite coming together at the typical breeding site at two different times, the flamingos there did not breed; it is believed that the failed attempts were likely due to a disturbance (dogs) at the site, and thus the ringing operation was cancelled for 2010 (Zucca, pers. comm., 2010). Consequently, samples were collected from five study sites: Ebro Delta, Spain; Rio Odiel, Spain; and Fuente de Piedra, Spain; Camargue, France; and Comacchio, Italy. Details of these sites are given in the following section.

Table 4.1. List of sites at which flamingo ringing has previously occurred, the response to the request for collaboration, and the sites at which fieldwork was ultimately arranged.

Site name	Notes	Fieldwork arranged?
Morocco		
Iriki Depression, Ourzazate	No ringing operations planned for 2010	
Spain		
Donana (Huelva-Sevilla)		Yes
Laguna de Fuenta de Piedra (Malaga)		Yes
Ebro Delta		Yes
Laguna Petrola (Albacete)		
France		
Salin de Giraud saltpans, Camargue, (Bouches-du-Rhone)		Yes
Italy		
Cagliari (Stagno di Molentargius di Quartu and Santa Gilla), Sardinia	Flamingos did not breed in 2010 due to disturbance	Yes*
Margherita di Savoia		
Orbetello		
Valle di Comacchio saltpans (Ferrara)		Yes
Greece		
Lake Messi		
Algeria		
Garaet Ezzemoul	Ringing not planned for 2010 as flamingos only recently began breeding here.	
El Goléa	No response	
Turkey		
Gediz Delta	No ringing operations planned	
Camalti	No response	

4.3.2.2 Sample collection

Permissions

Ethical approval for sample collection was necessary due to the involvement of live wildlife. This approval was requested and subsequently granted from the Trinity College Dublin Bioresources Unit. In some instances, additional permission (e.g. for sample collection within a protected areas) was required for fieldwork and sample collection at individual sites.

Given the potentially hazardous nature of the samples, permission to import the samples into Ireland was also required. This two-stage process was coordinated through the Product Import

Section, Food Safety Liaison Division, in the Department of Agriculture, Fisheries and Food (DAFF) and required the following:

(i) completion of an initial application ('*Registration of Importers of Live Animals*') to be placed on the DAFF register of importers of live animals, and

(ii) submission of a consignment-specific application ('*VET 15: Application for License to Import Samples of Animal products for Diagnostic/Education/research/Exhibition Purposes*') to obtain authorization to import specific samples (the authorization process includes the granting of a license, which must be attached to each of the shipments).

As TCD was already registered as an importer of products of animal origin, stage (i) of the registration process was not necessary. However, submission of form VET 15, specific to these samples, was required. This application was successful, and an importation license was granted ('*Importation of Carcases and Animal Products (Prohibition) Orders, 1967 and 1992, Poultry, Poultry Carcases, Poultry Eggs and Poultry Products Restriction on Importation Order, 1971*').

Capture and sampling of flamingos

Once ethical approval, fieldwork permissions, and import licenses were granted, sample collection could commence. The fieldwork took place in July and August, 2010 (Table 2).

Table 4.2. Dates on which fieldwork was conducted.

Site name	Date of sampling
Rio Odiel, Spain	17 July 2010
Comacchio, Italy	20 July 2010
Ebre Delta, Spain	1 August 2010
Camargue, France	4 August 2010
Fuente de Piedra, Spain	7 August 2010

Flamingos were captured with the assistance of participants in annual flamingo ringing operations at each of the five study sites, the specific dates for which are arranged on an annual basis with reference to the specific dates of egg-laying and the resultant age of chicks (Rendón-Martos *et al.*, 2009). The aim is to capture flamingos prior to their ability to fly – which occurs between 2 and 2.5 months – when they are still primarily under the care of adult

flamingos in the crèche (Johnson *et al.*, 1993). At each of the five sites, corral trapping was used. This is a technique suited to the capture of wild, flightless birds – whether they be moulted adults or in this case, pre-flight chicks. The capture process involves the coordination of several large teams of people, who herd the birds from the water (where the crèche is located) into a pen on land (FAO, 2007). This method is advantageous relative to others (e.g. a grid network of noose/traps immersed in water, commonly used for the capture of adult wading birds) in that it allows large numbers of flamingos (as many as 1,000) to be captured (Childress and Jarrett, 2005).

The flamingo ringing operations are typically arranged by local research institutes and involve between one hundred and two hundred people, including local volunteers – many of whom are amateur ornithologists – and scientists from regional academic institutions and conservation groups (Rendón-Martos *et al.*, 2009). The process is organized in advance of the day that ringing operation actually occurs; much of the preparatory work for the capture, including the organization of equipment and the preparation of pens is coordinated by the local research institution in the days preceding the capture (Plate 5). The day prior to the ringing operation, there is an informational meeting at which details of the process are given, and individuals are assigned to their relevant teams – which are divided according to roles, including 'biological sampling', 'ringing', 'measurements' and 'porters'.

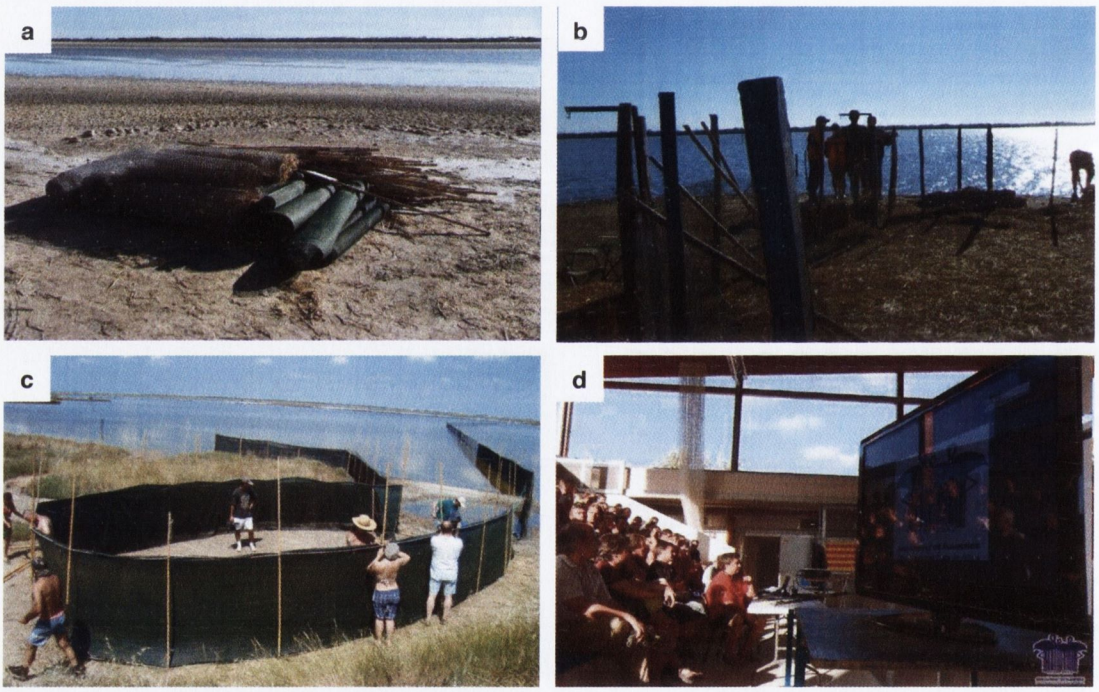


Plate 5 Preparation for flamingo capture begins the day before, when **a)** Materials for the pen are brought to the site, and **b) and c)** The pen is assembled adjacent to the body of water where the crèche is located. **d)** An informational meeting is held, at which teams and tasks are assigned and details of the capture operation are provided.

The 'capture' portion of the day itself typically takes between one and two hours. The operation begins with participants assembling around the perimeter of the breeding site (i.e. lagoon or lake) just prior to sunrise – with the exception of the Comacchio, Italy site, where capture took place in the afternoon (Plate 6).

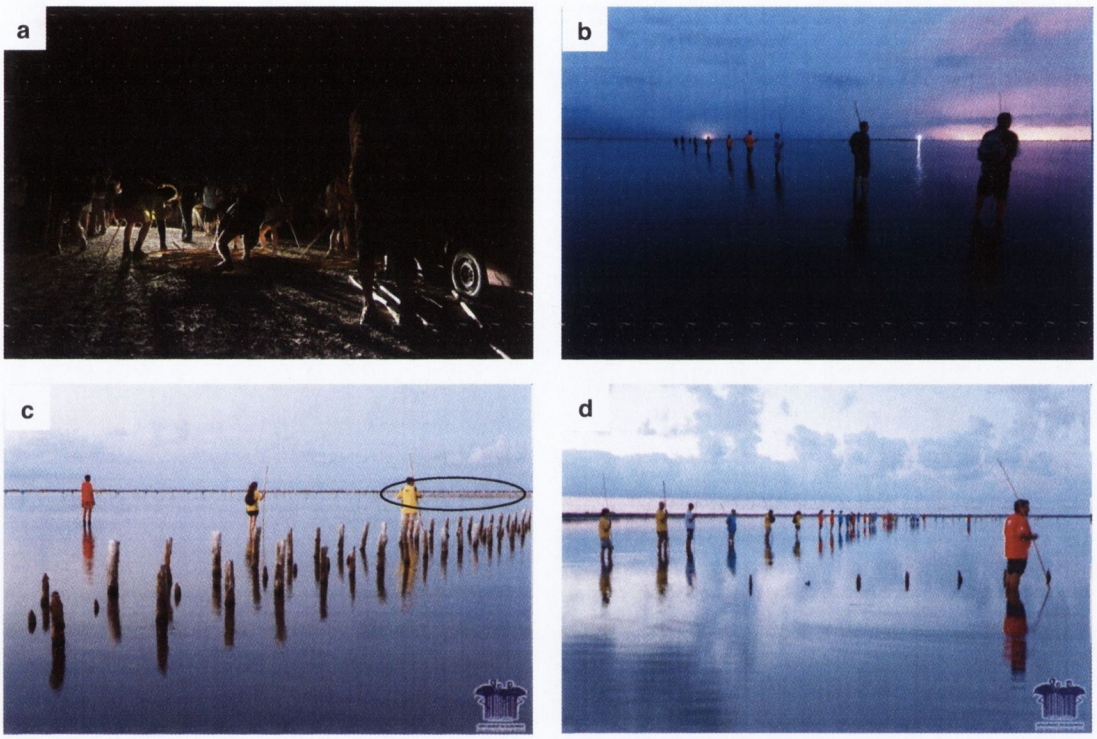


Plate 6 a) Preparations for capture begin when it is still dark, as participants collect 'bats'. **b)** Participants in capture assemble in the water as the sun begins to rise. **c)** Participants get into place in lines surrounding the flamingo colony (colony is within the black circle). **d)** There are usually several leaders appointed who guide the teams during the capture process.

As the sun begins to rise, the teams move towards each other through the water, which is mid-calf to waist deep (or slightly higher on those of us who are a bit shorter!), herding the flamingos from their crèche towards the pen that has been constructed beforehand at one end of the water body (Plate 7). At several of the sites, the effort was assisted by a team working from small boats to direct the birds appropriately towards the pen. Adults near the crèche fly away during this procedure (note that some of the adult birds are absent at this time of day as they are away from the site foraging for food), leaving the fledgling flamingos to be herded into the pen.

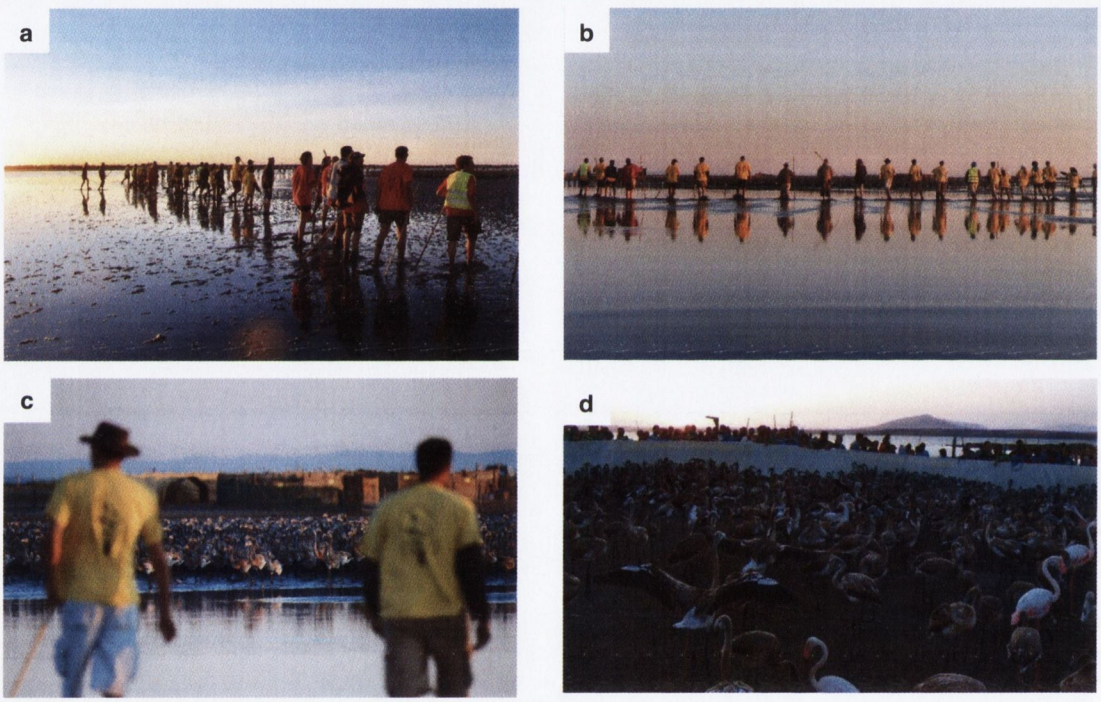


Plate 7 a) and b) Capture of the flamingos begins as the separate teams move towards each other. **c)** Flamingos are herded towards the shore, where the pen has been set up. **d)** Although the aim is to capture juvenile flamingos only, several adult flamingos are usually captured as well.

Once captured, 'porters' carry the flamingos through what can best be described as an assembly line, which consists of several stations at which various team members measure and weigh, ring, and take biological samples from the flamingos (Plate 8). The specific biological sample(s) taken varies between the sites – typically blood and feather samples are taken; oral and cloacal swabs are also sometimes taken (Johnson, 2000). Brooks and Childress (2005) provide detailed guidelines regarding the care and handling of flamingos during the capture and sampling process.

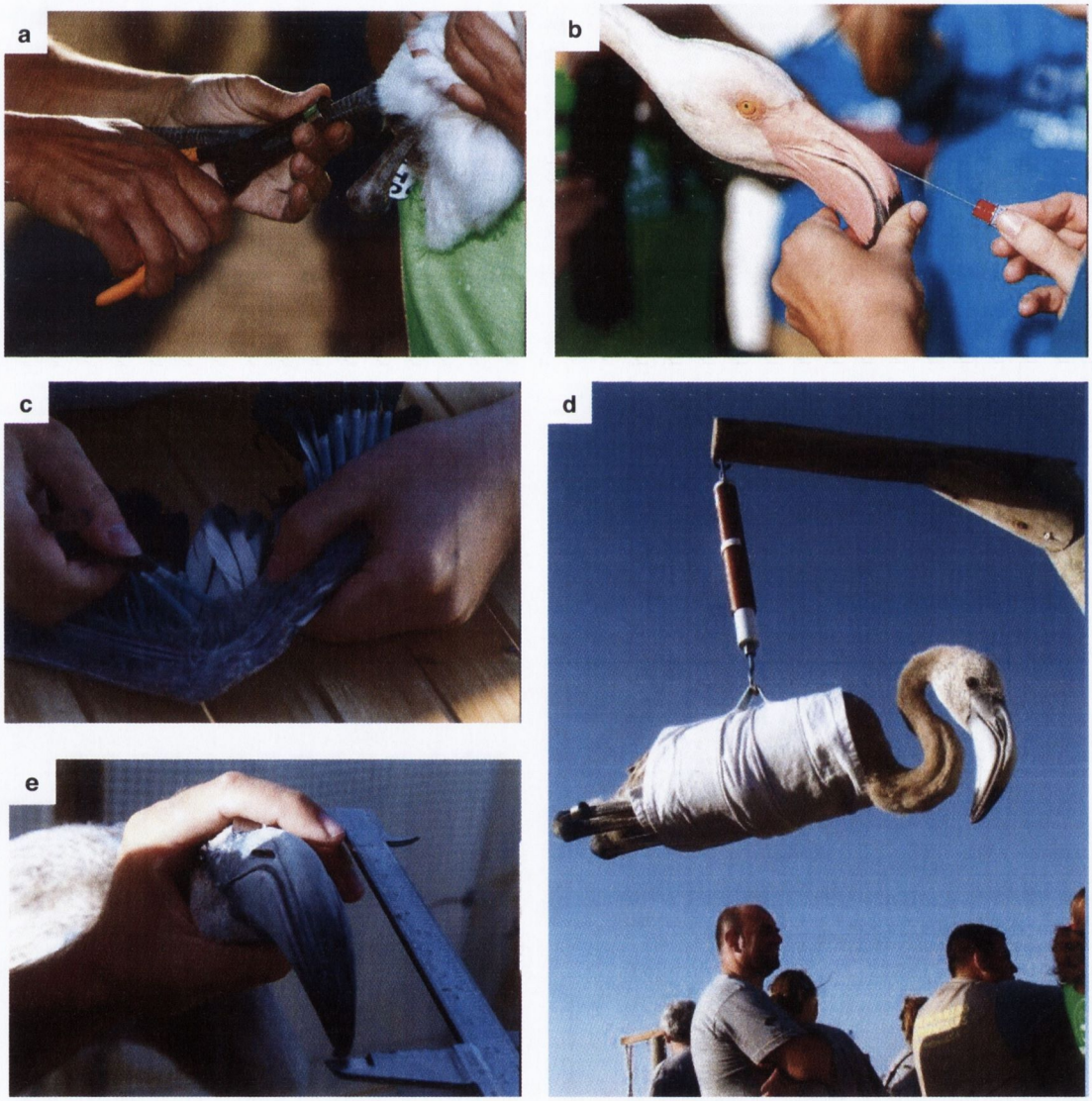


Plate 8 Many scientists use the capture of flamingos for the purposes of their own research. **a)** Ringing, **b) and c)** Sampling, **d)** Weighing, and **e)** Measuring of captured flamingos all typically occur.

Once ringing, measuring, and sampling of an individual bird has been completed, it is released (Plate 9). In most cases, not all birds that are captured go through the ringing/sampling process, due to a limited number of rings. Once all of the available rings have been used, the 'extra' birds are released from the pen (Plate 9).

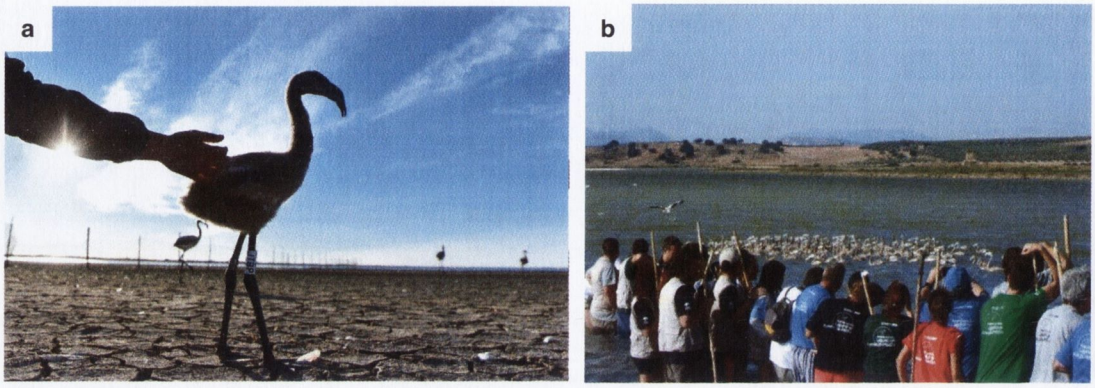


Plate 9 a) Once a flamingo chick has been through each of the stations (ringing, measuring, and biological sampling), it is released. **b)** Once all of the rings have been used up, the flamingos that did not go through the process are released from the pen.

For the purposes of this research, cloacal samples were collected using Sterilin Amies clear gel transport swabs. FAO (2007) guidelines on disease sampling procedures were followed in taking cloacal swabs from the flamingos. The procedure involves inserting the tip of the swab into the cloaca, rotating it several times while applying gentle pressure, before removing the swab and placing it into the tube containing the transport medium. Sterile methods were employed at all times during the swabbing to reduce the chance of contamination e.g. holding the collection tube upside down to minimize contaminants falling in, and wearing gloves (Plate 10).

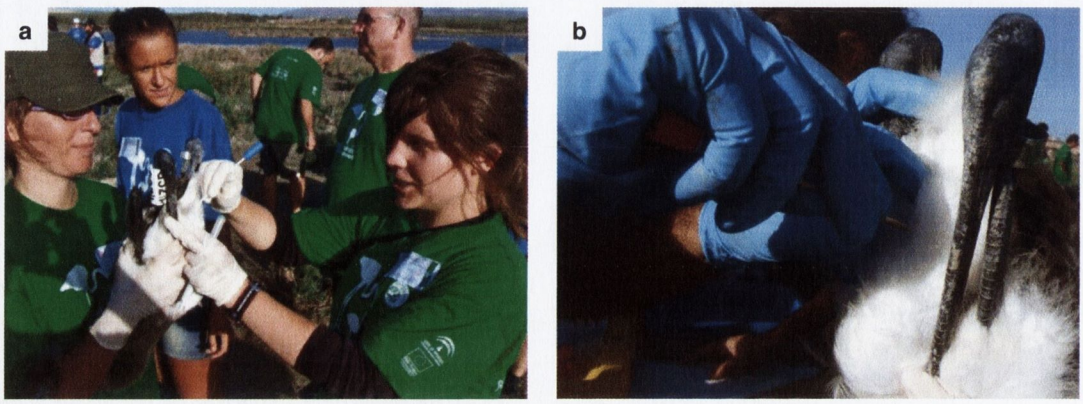


Plate 10 a) and b) Cloacal swabbing of a Greater Flamingo chick using sterile methods.

A total of 350 swabs were obtained: 75 samples were taken at each of the sites, with the exception of Ebre Delta, at which 50 samples were taken. Each sample was clearly labeled with a five digit alphanumeric code to indicate the study site and sample number (e.g. EB001 and CO056 indicate sample number one at Ebre Delta and sample number fifty-six at Comacchio, respectively). Samples were refrigerated at 4°C within several hours (maximum) of collection. As biological samples that are a potential biohazard, the samples were classed as UN 3733 and were packaged for shipping following UN guidelines – which requires use of a triple packaging system (Figure 4.1). The samples were shipped within 24 hours to the Microbiology Research Unit at the Dublin Dental University Hospital, where they were stored at 4°C until laboratory analysis took place.

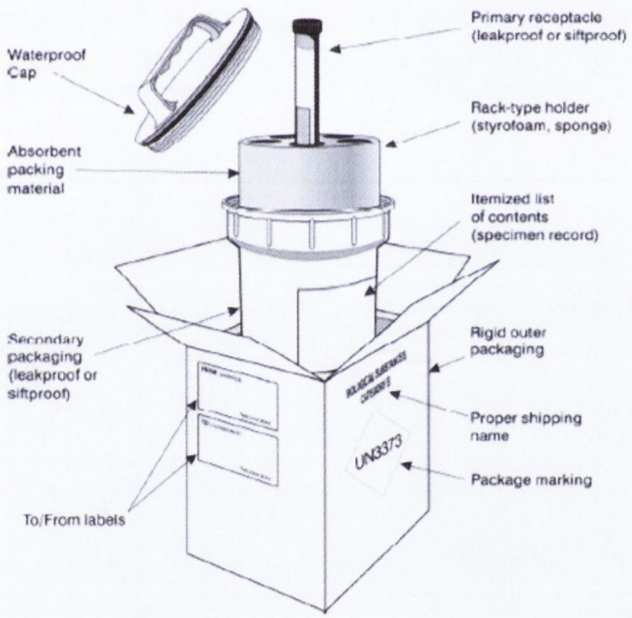


Figure 4.1 Triple packaging system required for the shipment of the potentially hazardous samples, substances assigned to class UN3373 (World Health Organization, 2007-2008.)

4.3.2.3 Laboratory analysis

Selection of antibiotics

Antibiotics were selected using a multi-stage process. First, a literature search was conducted to find a diverse range of studies that examined antibiotic resistance in wild animals. The aim was to determine which antibiotics have commonly been used in this field of research, to aid in the selection of antibiotics for this investigation. The 46 studies returned in the (unstructured)

search were published between 1986 and 2010, and used a variety of animals, including mammals, reptiles, and birds, in investigating antibiotic resistance across the globe. Combined, these studies used a total of 87 antibiotics, and from these, a list was compiled of the antibiotics that were more commonly used. From this list, eight antibiotics were selected with the aim of ensuring that antibiotics from different classes were represented (antibiotics are traditionally grouped, or 'classed', with reference to their mode of action). In consultation with a professional working in a clinical environment, consideration was also given to using antibiotics that are of relevance to human health (i.e. those which are clinically important), so documents like the World Health Organisations 'List of Critically Important Antimicrobials for Human Medicine' (2011) were consulted.

The eight antibiotics that were ultimately selected comprised seven different classes of antibiotics (Table 4). The antibiotics (and their respective dosages) selected for use in this investigation were: ampicillin (10μg), chloramphenicol (15μg), ciprofloxacin (5μg), erythromycin (15μg), gentamycin (30μg), kanamycin (10μg), tetracycline (30μg), and sulphamethoxazole/trimethoprim (25μg) (Table 4.3).

Table 4.3 Consolidated list of the names, concentrations, and respective abbreviations of the antibiotic discs used in this study.

Antibiotic	Dosage	Abbreviation
Ampicillin	10μg	AMP
Chloramphenicol	15μg	C
Ciprofloxacin	5μg	CIP
Erythromycin	15μg	E
Gentamycin	30μg	CN
Kanamycin	10μg	KAN
Tetracycline	30μg	TET
Trimethoprim-sulfamethoxazole	25μg	SXT

Susceptibility testing

Antibiotic susceptibility testing was undertaken using Kirby-Bauer disc diffusion, with reference to guidelines provided by the CLSI (2006). A total of one hundred samples were analysed, which represented twenty samples randomly selected from each of the five study sites. (One sample represents a single swab taken from one flamingo). Each of these samples was plated onto a Mueller-Hinton agar plate using a direct plating technique (Bartoloni *et al.* 2006). Immediately upon plating, eight antibiotic discs (Oxoid Antimicrobial Susceptibility Testing Discs, sourced from Fisher Scientific) were applied to each plate (one disc of each of the eight antibiotics). The plates were incubated for 24 hours at 30°C. Each plate (i.e. one sample) was

evaluated for microbial growth; resistance profiles were compiled for those which yielded growth as follows.

Following incubation, each of the plates that yielded bacterial growth was assessed to determine which antibiotics the bacterial sample was resistant to. Visual descriptions were recorded, which noted the presence/absence of a zone of inhibition, whether or not the lawn (i.e. bacterial growth) was touching the antibiotic disc, and the presence of any colonies within the zone of inhibition. From these descriptions, a general classification of sensitive or resistant (to each of the eight antibiotics) was assigned to the one hundred samples for the purposes of evaluating the Greater Flamingo as a sentinel of antibiotic resistance.

4.3.2.4 Statistical analysis

Resistance profiles

Resistance profiles for each of the samples were compiled using a binomial classification scheme with reference to the response of the sample (i.e. total bacteria) to each of the 8 antibiotics, in which 0 = sensitive and 1 = resistant. So, if a given sample was resistant to all eight of the antibiotics, its profile would read 11111111, whereas if it was resistant to only one antibiotic (e.g. ampicillin), its profile would read 10000000. The frequency of the different profiles that resulted was assessed. Only samples for which a full profile was generated (i.e. resistant or susceptible was indicated to each of the eight antibiotics) were used for further statistical analyses, as described below.

Multidrug resistance

Multidrug resistance was evaluated by determining the number of antibiotics (out of a possible eight) to which a given sample was resistant. Samples resistant to two or more antibiotics were considered to be multidrug resistant.

Overall effectiveness of antibiotics and variation between sites

The overall effectiveness of the antibiotics across the five sites was evaluated by calculating the percent of samples resistant to each of the eight antibiotics. The effectiveness of the eight antibiotics was also analysed with reference to the site at which they were taken, in order to investigate geographic variation in the effectiveness of each of the antibiotics. Pearson chi-square tests were used to evaluate differences between sites, with reference to the proportion of samples resistant to each of the antibiotics. Chi square tests were selected because they

are appropriate for us when comparing proportions, and have been used in similar research comparing the prevalence of antibiotic resistance between sites (Guardabassi *et al.*, 1998; Shaefer *et al.*, 2009).

Landscape analysis – land cover characterization and quantification and relationship to antibiotic resistance

The influence of landscape on the prevalence of antibiotic resistance was assessed through use of land cover data. Those data were obtained from the EU's 'coordination of information on the environment' (CORINE) programme that provides a database that includes 44 land cover classes across Europe (CORINE, 2013). Land cover around each of the five study sites was determined using CORINE Land Cover 2006 raster data with a spatial resolution of 100m (Version 16) in ArcGIS (Version 10.1). Buffers were created around each of the five study sites at three intervals: 1k, 5k, and 10km, and maps were generated in ArcGIS for each of the study sites to visualize land use. The areas (in m^2) of all of the polygons within each of the respective buffers were extracted from the attributes table, and imported into Microsoft Excel. Because most of the buffers were comprised of multiple polygons of the same land cover type, the totals for each land cover had to be calculated. The proportions of the total area of the buffers that the various land cover classes comprised were calculated, and these data were imported into SPSS (Version 20) for statistical analysis.

CORINE land cover nomenclature provides descriptions of land cover at three levels; the most specific of which has 44 classes ('Level 3'), which at the next level are grouped into 15 classes ('Level 2'), which are then grouped into five categories ('Level 1'). For the purpose of this investigation, the Level 1 categories were used for statistical analyses, as this level provides an indication of the system generally; the five categories include 'artificial surfaces', 'agricultural areas', 'forest and semi-natural areas', 'wetlands', and 'water bodies'. Using SPSS (Version 20), stepwise regression models were used to determine the extent to which landscape (i.e. land cover) factors served as predictors for the prevalence of antibiotic resistance. Stepwise regression models are useful in determining which variables, of a suite of potential ones, are most useful in predicting a given outcome; the method is frequently used in ecology and has been described as a 'dynamic approach' (MacNally, 2000). The models included each of the land cover classifications/spatial scale combinations as independent variables for each of the study sites to identify which metrics were the best at predicting resistance (Landau and Leeuwen, 2012). This process was undertaken separately for each of the eight antibiotics, because it was expected that the relationships between land cover, antibiotic resistance and spatial scale, would be different for the various antibiotics, given that the contexts within which antibiotics are used varies considerably.

3 Site descriptions

Fieldwork was conducted at five Great Flamingo breeding sites in the Mediterranean region (Figure 4.2 and Table 4.4). The sites represent a selection of the known locations commonly used as breeding sites by the Greater Flamingo, though some inter-annual variation occurs (including that due to climatic variation and occasional disturbances at breeding sites that disrupt breeding in a given year). Descriptions of each site are provided on the following pages, along with satellite images of the sites (Plates 10-14).

Figure 4.2 Sites at which cloacal swabs were collected from Greater Flamingos are indicated, including three sites in Spain (Rio Odiel, Fuente de Piedra, and Ebro Delta), one in France (Camargue), and one in Italy (Comacchio).

Table 4.4. Details of each of the five study sites, including its official name, common name, geographic location, and designations.

Official site name (common name/that used in this dissertation is given in brackets)	Site location	Coordinates	Special designations
Marismas del Odiel (Rio Odiel)	Huelva, Spain	37°14'N 6°59'W	Biosphere Reserve (1983) Special Protected Bird Area Internationally Important Wetland (1989) (RAMSAR convention)
Laguna de Fuente de Piedre (Fuente de Piedre)	Fuente de Piedre, Spain	37°06'N 4°48'W	Internationally Important Wetland (1983) (RAMSAR convention) Specially Protected Area for Birds (SPA)
Punte de la Banya (Ebre Delta)	Ebre Delta, Spain	40°35'N 0°40'E	Internationally Important Wetland (1993) (RAMSAR convention) National Park (1983)
Etang du Fangassier, Salin de Giraud (Camargue)	Camargue, France	43°25'N 4°37'E	Biosphere Reserve (1977) Internationally Important Wetland (1986) (RAMSAR convention)
Valli di Comacchio (Comacchio)	Comacchio, Italy	44°38'N 12°12'E	Internationally Important Wetland (1981) (RAMSAR convention)

Marismas del Odiel, Spain

Marismas del Odiel (Rio Odiel) is located on the Mediterranean coast in Southwestern Spain at the confluence of the Odiel and Tinto Rivers on the Gulf of Cádiz, across the Odiel River from Huelva, a city of approximately 150,000 people. The coastal wetland site is comprised of over 7,000 ha of intertidal mudflats and saltpans (Rendon-Martos *et al.*, 2008). The region has been described as 'very polluted'; in addition to receiving contamination from the two rivers that feed into it, the site is also influenced by an industrial area in the vicinity of Huelva (Grande *et al.*, 2003; Oías *et al.*, 2004). Numerous research efforts have investigated the extent and impacts of the pollution, much of it focusing on heavy metal contamination (reviewed in Oías *et al.*, 2004). The site is largely influenced by mining activity, which has taken place in the region since prehistoric times (Oías *et al.*, 2004). Acid mine drainage continues to serve as an input of heavy metal contamination into the area, which has been described as one of the most heavily contaminated heavy metal estuaries worldwide (Sainz *et al.*, 2004; Barba-Brioso *et al.* 2010).

Despite this, the site is renowned for its avian diversity (Sanchez *et al.*, 2006). As a result, it has been designated as a Biosphere Reserve, a Special Protected Bird Area, and an Internationally Important Wetland (Rendon-Martos *et al.*, 2008; Oías *et al.*, 2004). Although historically several attempts at breeding have occurred (with limited success), it wasn't until 2008 that large-scale breeding by Greater Flamingos was observed at Rio Odiel (Rendon-Martos *et al.*, 2008). This was likely the result of targeted modification of the site in the late 1980s and early 1990s, including the construction of artificial islands intended to support flamingo breeding (Rendon-Martos *et al.*, 2008). Today, Greater Flamingo breeding occurs in a saltpan complex measuring over 1,000ha (Sanchez *et al.*, 2013).

Plate 11. Satellite images of the fieldwork site at Rio Odiel, in Spain. 100km and 2000m scale bars are provided in the bottom left of each image, respectively (Google Earth, 2012).

Fuente de Piedra

Fuente de Piedra is a 1400 ha inland saline lake that is located in Southern Spain, 50km from the coast (Martos and Johnson, 1996). The shallow lake (it typically has a maximum depth of less than 0.6m) is dependent upon rainfall and as such, is characterised by seasonal flooding and drying (García and Neill, 1993; García et al., 1995; Rendón-Martos et al., 2009). The variation in water level can be hard to predict, and there are years in which the lake has been completely dry and alternatively, years during which there was sufficient rainfall for it to behave as a more permanent lake (García et al., 1997). The (generally) cyclical fluctuation in water levels impacts not only the lake's physical and chemical properties, but also affects its ability to support biological communities – ranging from phytoplankton and zooplankton to larger wildlife species (García and Neill, 1993; García et al., 1997).

Notable amongst the species it supports is the Greater Flamingo, which uses the lake as a breeding site in years during which water levels are suitably high (Rendón-Martos *et al.,* 2009). The Greater Flamingo has intermittently used Fuente de Piedra as a breeding site for over 100 years (Rendón-Martos *et al.,* 2009). In more recent years, the lake has become one of the most important breeding sites for the bird in the Western Mediterranean, with as many as 40,000 flamingos recorded at the lake (Rendón-Martos *et al.,* 2009). Since the early 1980s, a certain amount of management of the site has occurred, including maintenance of breeding islands and control of water levels, to encourage breeding colonies use of the site (Rendon-Martos and Johnson, 1996). Today, the site is protected as a RAMSAR site, a natural reserve, and a specially protected area for birds (SPA) (Rendon-Martos *et al.*, 2009).

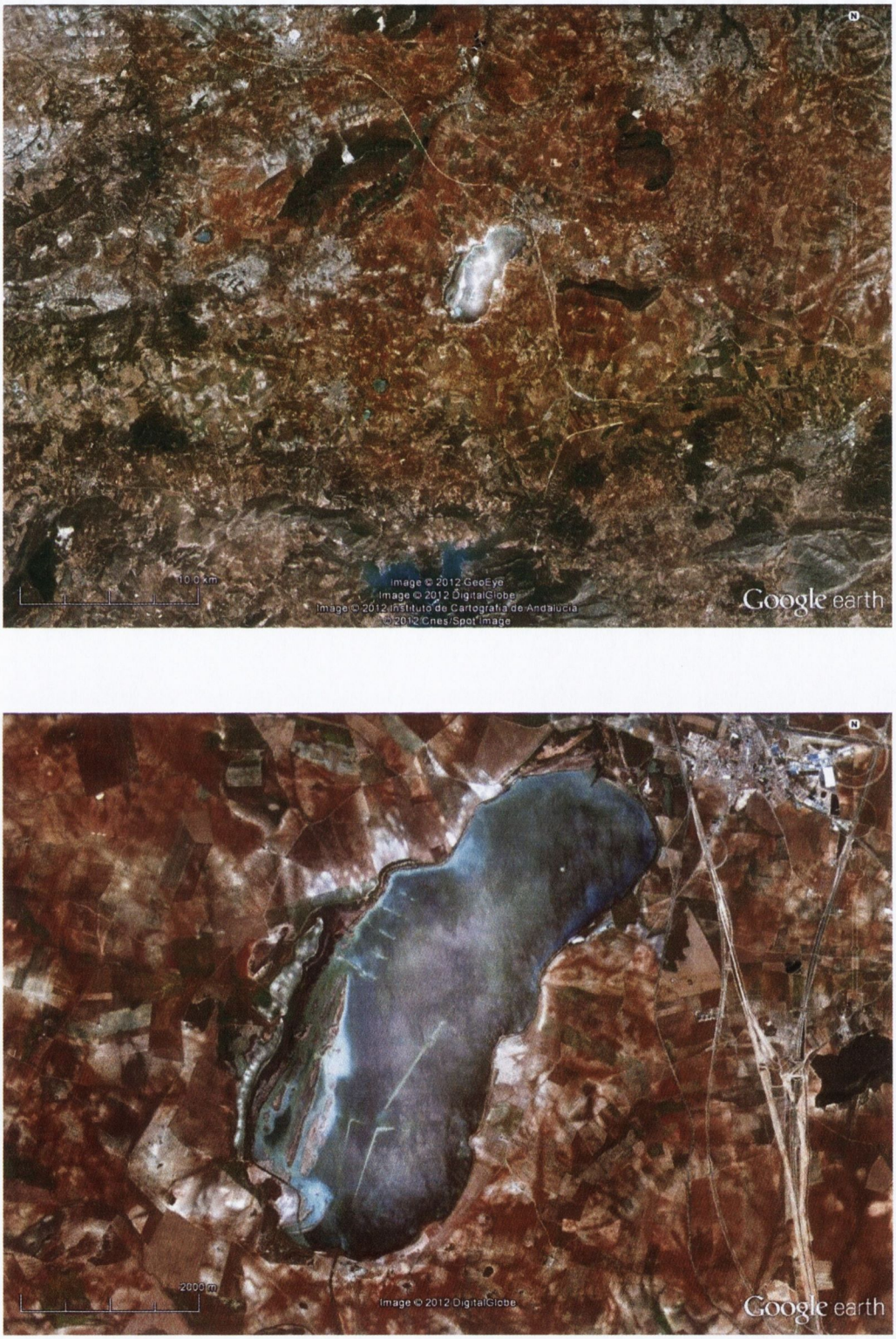


Plate 12. Satellite images of the fieldwork site at Fuente de Piedre, in Spain. 100km and 2000m scale bars are provided in the bottom left of each image, respectively (Google Earth, 2012).

Comacchio

The Comacchio saltpans at which the Greater Flamingo breeds comprise an area of 400ha (Albanese *et al.*, 2009). The saltpans are part of a larger wetland complex, Valli di Comacchio (12,500ha) that consists of a series of inter-connected lagoons (Albanese *et al.*, 2009). The wetlands lie within the Po Delta National Park (Frascari *et al.*, 2002; Munari, 2011). Previously used for commercial salt operations, salt extraction at the site ceased in the mid-1980s (Albanese et al., 2009). The lagoons have both freshwater (Reno River) and drainage water inputs; drainage into the system is largely from agricultural land (Borghesi *et al.*, 2011). Intensive fish farming activities served to contribute organic matter (via sewage discharge) to the site until the early 1990s (Munari *et al.*, 2003). Together with other anthropogenic activities, this served to transform the ecosystem – eventually causing its collapse, described as an 'ecological catastrophe' by Sorokin *et al.* (1996), evidenced by the proliferation of pycocianobacteria and the resultant depletion of the benthic fauna, including zooplankton and associated benthic communities (Sorokin *et al.*, 1996). The biotic communities today remain affected, and are comprised of different species than similar lakes in the Mediterranean; it is believed that agricultural run-off still serves to influence the site (Munari and Mistri, 2012).

The site has been designated as a Special Area of Conservation as well as a Specially Protected Area for birds. The breeding flamingo colony at Comacchio is relatively recent; breeding was first noted there in 2000 (Bacetti et al., 2008; Albanese et al., 2009). Despite an increase in the number of chicks that have fledged since breeding was first observed, no specific management for the flamingo populations existed as of 2009 (Albanese et al., 2009). Nonetheless, annual flamingo ringing operations have been conducted at Comacchio since 2000, providing researchers with an opportunity to collect data at what is an interesting and dynamic site.

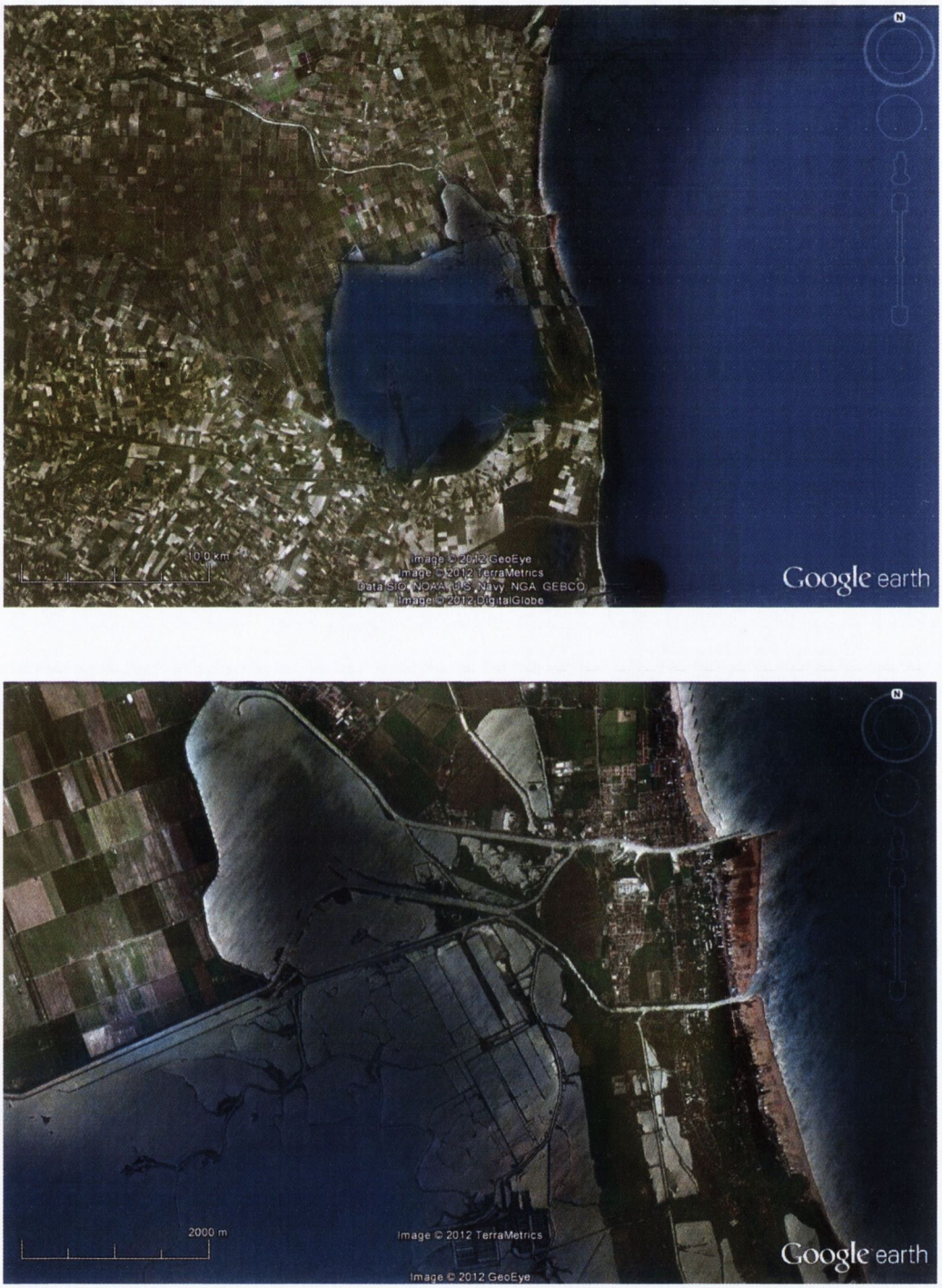


Plate 13. Satellite images of the fieldwork site at Comacchio, in Italy. 100km and 2000m scale bars are provided in the bottom left of each image, respectively (Google Earth, 2012).

Camargue, France

The Camargue is a well-known region in Southern France. Located south of Arles, it lies within the Rhône River Delta, its boundaries delineated by the Petite Rhône and Grande Rhône rivers (Chauvelon, 1998). The Camargue covers over 145,000ha in total, within which lies the Salin de Giraud, which at 11,000ha was at one time Europe's largest commercial saltpan (Britton and Johnson, 1987). Today, the region continues to be actively managed for salt production (including artificial control of water levels, in particular), and although significantly reduced today, salt is still exported (Plate 14) (Johnson, 2000; Deville *et al.*, 2013). Rice farming, livestock farming (predominantly bovine and equine grazing) and tourism have occurred in the region for some time and continue today, with much of the present-day tourism focusing on the region's wild birds (Tamisier, 1991; Tourenq *et al.*, 1999; Ernoul *et al.*, 2013).

Plate 14. Salt from the Camargue, France packaged and available for sale at a shop in Ireland provide evidence of the continued productivity of the salt pans there

The Camargue is divided into 'etangs' (lagoons), which are very shallow, highly saline environments, some of which have been used in salt production and many of which provide a unique habitat for a variety of flora and fauna (Britton and Podlejski, 1981; Britton and Johnson, 1997). It is one of these, the Etang du Fangassier that, with the help of management strategies, has served as a breeding site for the Greater Flamingo for many decades (Martos and Johnson, 1996). The Greater Flamingo breeding colony at the Camargue has been said to be its most important in Europe and is notable for the amount of research conducted within it to date (Johnson, 1997). The Greater Flamingo has been ringed at the Camargue since 1947, and organised research efforts have been ongoing since the 1970s (Johnson, 2000; Tour du Valat, 2013). Management of the site for the Greater Flamingo has occurred for a long time; for example, the building of artificial nest sites to support breeding (Nager *et al.*, 1996). Erosion of the nest sites has been counteracted through the restoration efforts of landowners (and in some cases, financed by the salt companies), who rebuild the sites to maintain them as viable breeding sites (Rendon-Martos and Johnson, 1996; Johnson, 2000).

Plate 15. Satellite images of the fieldwork site at the Camargue, in France. 100km and 2000m scale bars are provided in the bottom left of each image, respectively (Google Earth, 2012).

Delta de l'Ebre

The Ebre Delta is located on the coast in northwestern Spain, where the Ebre River meets the Mediterranean Sea. The area measures 330km^2 and is comprised of numerous salt marshes, including several commercial saltpans (Oro *et al.*, 2011). The delta is affected by a variety of sources including urban, industrial, and agricultural inputs – rice production in this region predominates (Gonzáles-Solís *et al.*, 1996). Heavy metal inputs are received from industrial activities; they are carried via the Ebre River to the delta (Mañosa *et al.*, 2001; Cotin *et* al., 2011; Ochoa *et al.*, 2012). The river also carries domestic and agricultural wastes (including pesticides used in rice production) and the impacts of these inputs on the delta have been widely researched – typically with reference to heavy metals (Mateo *et* al., 1997, 1998; Ochoa *et al.*, 2013).

Much of the area was designated as a National Park in 1983, and the wetlands support numerous bird species and fisheries (Ibàñez *et al.*, 1997). Although reports of their presence in the region exist from centuries ago, evidence supporting the breeding of the Greater Flamingo in the Punta de la Banya saltpans only exists since 1993 (Curcó *et al.*, 2009). Over this time, a comprehensive management programme has been instituted, consisting of on-the-ground habitat maintenance combined with research efforts (Curcó *et al.*, 2009). Banding operations have taken place here since 2004, and data from these efforts indicates that breeding success has risen over time (Curcó *et al.*, 2009).

Plate 16. Satellite images of the fieldwork site at Ebre Delta, in Spain. 100km and 2000m scale bars are provided in the bottom left of each image, respectively (Google Earth, 2012).

4.5 Results

4.5.1 Criteria for sentinel selection

Twelve criteria for selecting a sentinel were found from the literature review; these fell into two categories: those relating to the candidate species itself, and those relating to practical considerations for fieldwork. The criteria – and their rationale for use in selecting a sentinel of antibiotic resistance in particular – are provided in Table 4.5. Beeby (1999) recommended, as a starting point, the selection of a species about which much is known, as well-researched species allow for more meaningful conclusions to be drawn. Many of the further criteria suggested by Beeby (1999) related to the species' life history and physiology, as these were considered to impact its interaction with the environment as well as its susceptibility to respond to a contaminant. Several of the criteria related to a candidate species geographic distribution, which was considered to be important for making inter-site comparisons. To further facilitate such comparisons, it was suggested that a sentinel should be found as distinct populations at sites and between which there is little movement (Bowman & Schulte-Hostedde, 2009; Beeby 1999; Landres *et al.* 1988, Sayeh *et al* 2005). This would ensure that samples could be associated with a specific site in investigating potential factors that drive variation. Beeby (1995) also noted the importance of selecting a species that was abundant at a particular location to ensure the availability of an adequate sample size, from which statistically significant conclusions could be drawn. The other commonly suggested criteria related to practical considerations for sample collection and fieldwork, particularly where resources were limited. For example, the selection of a species found in readily accessible sites could help where time and financial resources were limited. Similarly, one should consider the ease with which a candidate species could be captured – as a difficult capture could add a significant amount of time on to fieldwork. Along these lines, selecting a species for which a sampling regime already existed could ease some of the organizational burden (such species were often, but not always, the 'charismatic' species). Arranging for fieldwork with a pre-existing sampling regime may also help where the candidate species was highly protected, as these species often necessitate additional permits for sampling and shipment of samples.

Table 4.5. Criteria for selection of a sentinel of antibiotic resistance

Criteria	Rationale	Reference(s)
Species life history & physiology		
Large body of knowledge about the species	Helps in selection of an appropriate species, and providing a context for discussion of results	Beeby 1999
Limited home range, does not move from the environment in which it is found	Abundance of antibiotic resistance within the species can be linked to its environment	Bowman & Schulte-Hostedde, 2009/Beeby 1999, Landres *et al.* 1988, Trainer, 1970
Found as distinct populations	Comparison of driving factors of resistance	
Found at multiple sites	To enable comparison of driving factors	Sayeh et al 2005
Potential sources of antibiotic resistance can be identified	For drawing conclusions about links between the species environment and the prevalence of resistance	Beeby 1999
Easily identified	To ensure the correct species is sampled	Trainer, 1970; Beeby 1999
Abundant	Ensures statistically significant sample size	Beeby 1999; Trainer, 1970
Detectable response to contaminant	To determine if the contaminant in question exists in the environment	
Practical factors		
Sampling/capture operations already established	Cost effective, less to organize, resource efficiency	Noted by author
Not highly protected	Easier to get permit for taking samples	Noted by author
Easy to capture	Fewer researchers/fieldwork assistants required, less stressful for animal	EROCIPS
Found at accessible sites	Cost effective, time efficient	Noted by author

This list was compiled from a literature review of criteria suggested for the selection of sentinels for environmental health monitoring. The criteria are grouped into two categories: 1) factors relating to the species itself (i.e. life history & physiological considerations), and factors about the species in relation to practical considerations for fieldwork

4.5.2 Determination of antibiotic resistant bacteria in the Greater Flamingo

4.5.2.1 The Greater Flamingo: a candidate sentinel

Having been proposed as a candidate sentinel of antibiotic resistance, it was necessary to evaluate the Greater Flamingo as a sentinel prior to proceeding with fieldwork, with reference to the list of criteria as developed through the literature review. As noted in the introduction, there is a large body of research on the Greater Flamingo. An extensive ringing programme across its range in Southern Europe has provided a wealth of information regarding the location, size, and interconnectedness of the individual populations, thus it was confirmed that the Greater Flamingo existed as a metapopulation, consisting of several breeding populations between which there was occasional exchange. Although this is not ideal with reference to one of the criterion (ideally, there would be no exchange between the populations to meet the criteria of 'a limited home range'), it was decided that by using fledgling flamingos, which would not yet have left the site at which they hatched, this criterion could be met. Related to this is the criterion of selecting a species that is easy to capture. Given their inability to fly, the selection of fledgling flamingos was ideal, because they can simply be 'herded' into a pen (on land) from the body of water in which their crèche is located. This methodology allows large numbers of individuals (as many as one thousand) to be captured, and could be achieved with the Greater Flamingo because of its large population size (another criteria that must be met). Regarding the size of its population, the breeding sites routinely see hundreds, if not thousands, of flamingos hatch each year, thus there was no concern regarding the ability to obtain an adequate sample size.

With reference to practical considerations, the Greater Flamingo was considered to be an ideal sentinel. Due to the interest in the Greater Flamingo, and the pre-existing collaborative research network, a well-organized scheme of ringing operations was already in existence. The head of this network was able to put me in contact with the lead organizers of each of the ringing operations, thus this research was given a degree of credibility. This was immensely important, as the ringing operations are costly to organize and require the coordination of hundreds of volunteers that alone, I would not have been able to achieve. Further to this, the cost of these operations is quite large and that would also have proven to be restrictive. The Greater Flamingo is listed as a species of 'least concern' by the IUCN, thus, there were no restrictions regarding sample collection or shipment of samples across borders (with reference to its protection status, other unrelated restrictions were in place with reference to the importation of potentially hazardous specimens). Early on in research planning, consideration was given to using the Lesser Flamingo as a sentinel. However, it is listed as threatened under the IUCN; and given its natural distribution (primarily East Africa), sample collection would have been costly, thus it was decided not to use the Lesser Flamingo. Indeed, the Greater Flamingo is located at relatively accessible sites near urban towns to which transport could

readily be arranged. This became quite important given the short time frame between ringing operations at each of the sites.

Because the review of criteria with reference to the Greater Flamingo yielded a positive result, it was determined that it could indeed serve as a sentinel, and that it would be worthwhile proceeding with fieldwork and laboratory analysis to determine whether or not antibiotic resistance was readily detectible.

4.5.2.2 Flamingo capture and sampling

Flamingo capture was successfully achieved at each of the five sites. The number of chicks ringed during capture at each of the sites ranged from 400 to 812 chicks, and represented between 7.5 and 98.2 percent of the total number of chicks that fledged that year at the respective sites (See Table 4.6). This variation is due largely to resource limitations; that is, the large number of chicks (8,118) that fledged at Fuente de Piedra, for example, could not all be ringed due to resource limitations; including the fact that the number of rings is limited. In addition, there are usually a few clever flamingos that manage to evade capture and escape!

With the exception of Rio Odiel, the target of 75 samples was achieved. The sample size of 75 represented between 1% and 12% of chicks that fledged, and between 9% and 19% of the chicks that were ringed, at each of the sites in 2010. The five sites at which samples were collected are the only five sites within the Western Mediterranean metapopulation at which ringing operations took place during 2010; thus this sampling effort can be considered as complete as possible.

Table 4.6. Number of samples taken at each of the fieldwork sites

Site	Fledged chicks	Ringed chicks (%)	Samples taken (%)
Camargue	2,400	812 (34%)	75 (3%, 9%)
Comacchio	1,822	422 (23%)	75 (4%, 18%)
Ebre Delta	631	400 (63%)	75 (12%, 19%)
Fuente de Piedra	8,118	611 (8%)	75 (1%, 12%)
Rio Odiel	558	548 (98%)	50 (9%, 9%)

This table describes the total number of fledged chicks in 2010 (when this sampling took place), the total number of chicks ringed as part of the greater ringing operation, and the number of samples taken for the purposes of this research. (Adapted from Flamingo Atlas, 2011).

The samples were successfully transported to the laboratory and were received and placed into the refrigerator at 4°C as planned. Upon completion of all fieldwork, laboratory analysis of the samples commenced.

4.5.2.3 Laboratory analysis

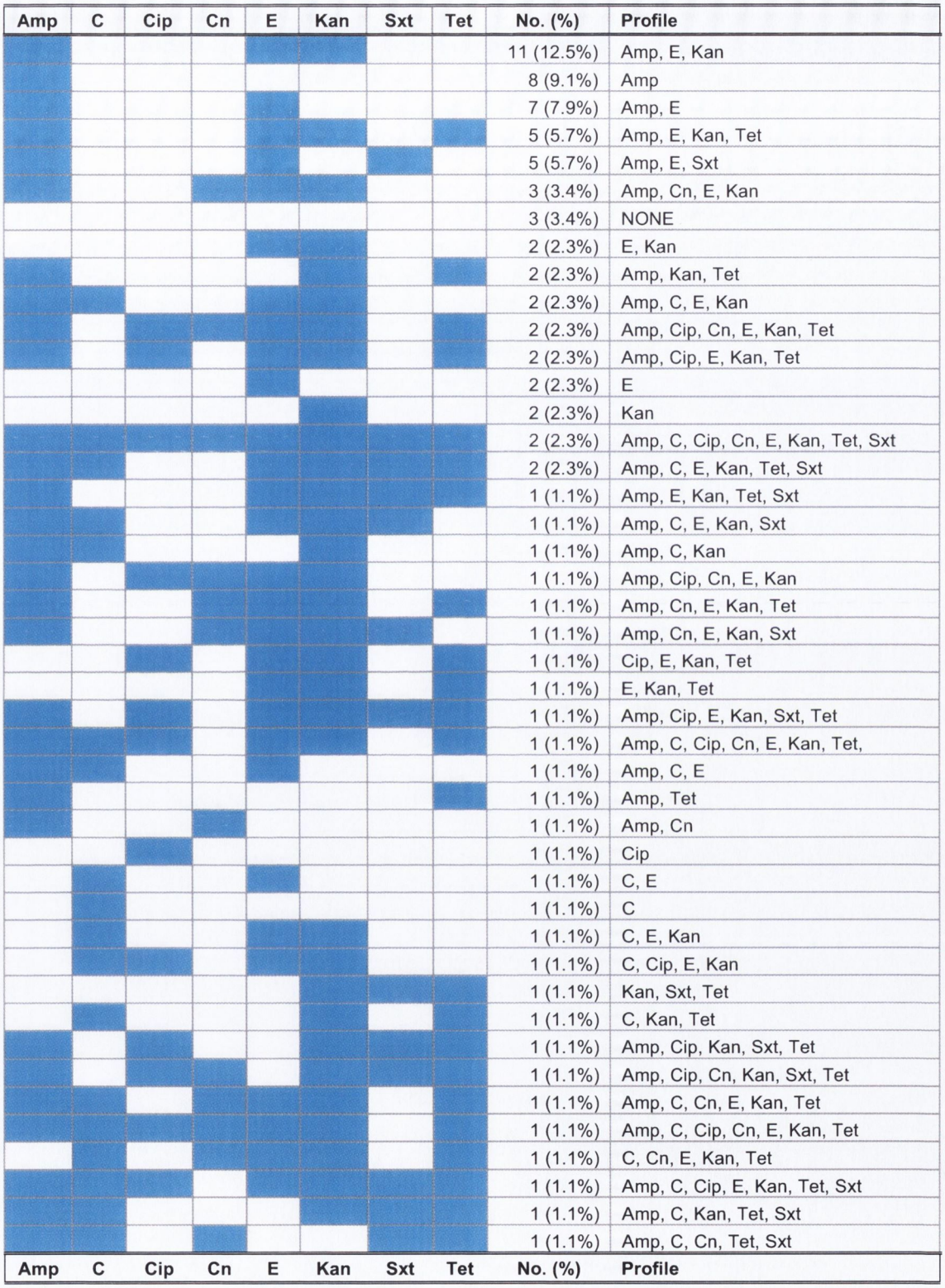
Twenty bacterial isolates from Greater Flamingos at each of the five sites were evaluated for resistance to eight antibiotics using Kirby-Bauer disc diffusion, for a total of one hundred samples (each was randomly selected). Each of the one hundred samples yielded growth on the Mueller Hinton medium, providing evidence that the sampling strategy and transport of samples was successful.

Susceptibility testing

The susceptibility of the bacteria to the antibiotics varied between samples. Some samples, for example, yielded a fainter secondary zone within the primary zone of inhibition, while others yielded dot colonies within a clear zone of inhibition; and others displayed a heavy lawn of growth congruent to the antibiotic disc, suggesting strong resistance. These descriptions were translated into a sensitive/resistant classification for purposes of the preliminary analysis for this investigation, to compare both intra-and inter- site differences. The results of these analyses are presented below.

Resistance profiles

Full resistance profiles were obtained for 88 of the 100 samples. A total of 44 different profiles resulted out of a theoretical 256 profiles (Figure 4.3). There were 28 unique profiles i.e. a profile for which only one sample demonstrated the particular pattern of resistance. The most common profile was that in which resistance to Ampicillin, Erythromycin, and Kanamycin was demonstrated (13% of samples), while the second most common was that in which resistance to only Ampicillin was demonstrated (9% of samples); the third most common was that in which resistance to Ampicillin and Erythromycin was demonstrated (8% of samples).



Amp	C	Cip	Cn	E	Kan	Sxt	Tet	No. (%)	Profile
								11 (12.5%)	Amp, E, Kan
								8 (9.1%)	Amp
								7 (7.9%)	Amp, E
								5 (5.7%)	Amp, E, Kan, Tet
								5 (5.7%)	Amp, E, Sxt
								3 (3.4%)	Amp, Cn, E, Kan
								3 (3.4%)	NONE
								2 (2.3%)	E, Kan
								2 (2.3%)	Amp, Kan, Tet
								2 (2.3%)	Amp, C, E, Kan
								2 (2.3%)	Amp, Cip, Cn, E, Kan, Tet
								2 (2.3%)	Amp, Cip, E, Kan, Tet
								2 (2.3%)	E
								2 (2.3%)	Kan
								2 (2.3%)	Amp, C, Cip, Cn, E, Kan, Tet, Sxt
								2 (2.3%)	Amp, C, E, Kan, Tet, Sxt
								1 (1.1%)	Amp, E, Kan, Tet, Sxt
								1 (1.1%)	Amp, C, E, Kan, Sxt
								1 (1.1%)	Amp, C, Kan
								1 (1.1%)	Amp, Cip, Cn, E, Kan
								1 (1.1%)	Amp, Cn, E, Kan, Tet
								1 (1.1%)	Amp, Cn, E, Kan, Sxt
								1 (1.1%)	Cip, E, Kan, Tet
								1 (1.1%)	E, Kan, Tet
								1 (1.1%)	Amp, Cip, E, Kan, Sxt, Tet
								1 (1.1%)	Amp, C, Cip, Cn, E, Kan, Tet,
								1 (1.1%)	Amp, C, E
								1 (1.1%)	Amp, Tet
								1 (1.1%)	Amp, Cn
								1 (1.1%)	Cip
								1 (1.1%)	C, E
								1 (1.1%)	C
								1 (1.1%)	C, E, Kan
								1 (1.1%)	C, Cip, E, Kan
								1 (1.1%)	Kan, Sxt, Tet
								1 (1.1%)	C, Kan, Tet
								1 (1.1%)	Amp, Cip, Kan, Sxt, Tet
								1 (1.1%)	Amp, Cip, Cn, Kan, Sxt, Tet
								1 (1.1%)	Amp, C, Cn, E, Kan, Tet
								1 (1.1%)	Amp, C, Cip, Cn, E, Kan, Tet
								1 (1.1%)	C, Cn, E, Kan, Tet
								1 (1.1%)	Amp, C, Cip, E, Kan, Tet, Sxt
								1 (1.1%)	Amp, C, Kan, Tet, Sxt
								1 (1.1%)	Amp, C, Cn, Tet, Sxt
Amp	C	Cip	Cn	E	Kan	Sxt	Tet	No. (%)	Profile

Figure 4.3 Frequency of antibiotic resistance profiles demonstrated amongst the 88 samples for which full profiles were determined. The profile in which resistance to Ampicillin, Erythromycin, and Kanamycin was demonstrated was the most frequently observed (12.5% of the 88 samples).

Multidrug resistance

As evidenced in the resistance profiles above, multidrug resistance was common amongst the samples screened (Figure 4.4). Of the 88 samples that yielded a full profile, the resistance(s) ranged from none of the antibiotics (3% of samples) to all eight antibiotics (2% of samples). 96% of the samples were resistant to at least one of the eight antibiotics and of these, 81% demonstrated resistance to multiple antibiotics.

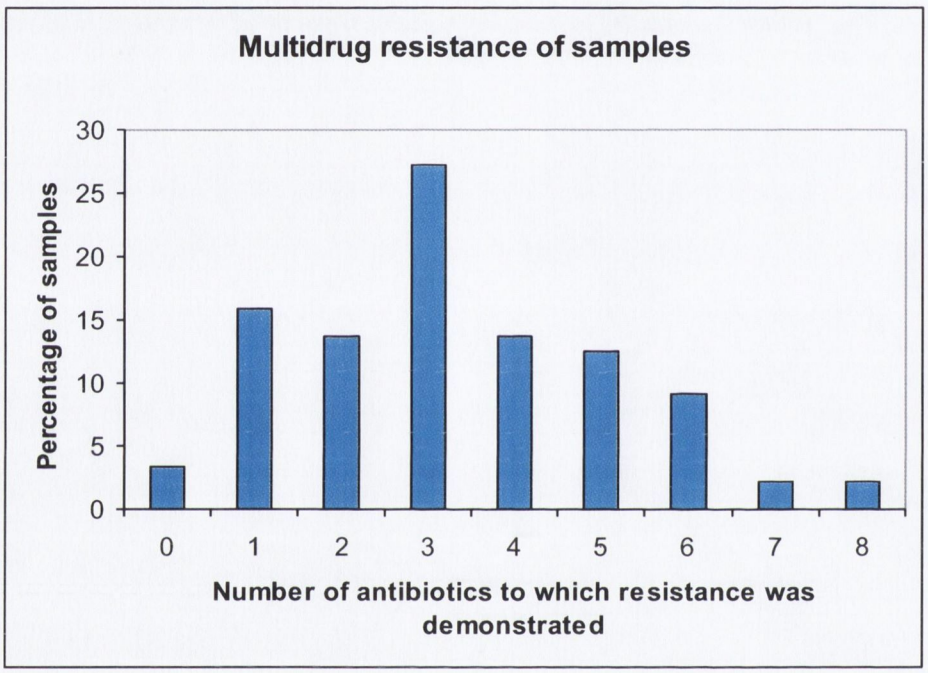


Figure 4.4 Multidrug resistance of samples collected from Greater Flamingo chicks at five sites in the Mediterranean region. 96% of samples were resistant to at least one antibiotic, while 81% demonstrated multidrug resistance.

With reference to the proportion of antibiotics to which samples demonstrated resistance, mean multidrug resistance (MDR) ranged from 35.3% at Comacchio to 52.1% at Fuente de Piedre (Table 4.7). MDR was not statistically different between sites. The MDR of samples from Fuente de Piedre ranged from 17.5% to 100%, while at Comacchio MDR ranged from 17.5% to 50% (with the exception of two outliers) (Figure 4.5).

Table 4.7. Multidrug resistance of samples collected from Greater Flamingo chicks at five sites in the Mediterranean

Site	n	Mean	Std. dev
Camargue	20	.413	.257
Comacchio	17	.353	.161
Ebre Delta	17	.397	.266
Fuente de Piedra	18	.521	.231
Rio Odiel	16	.375	.204

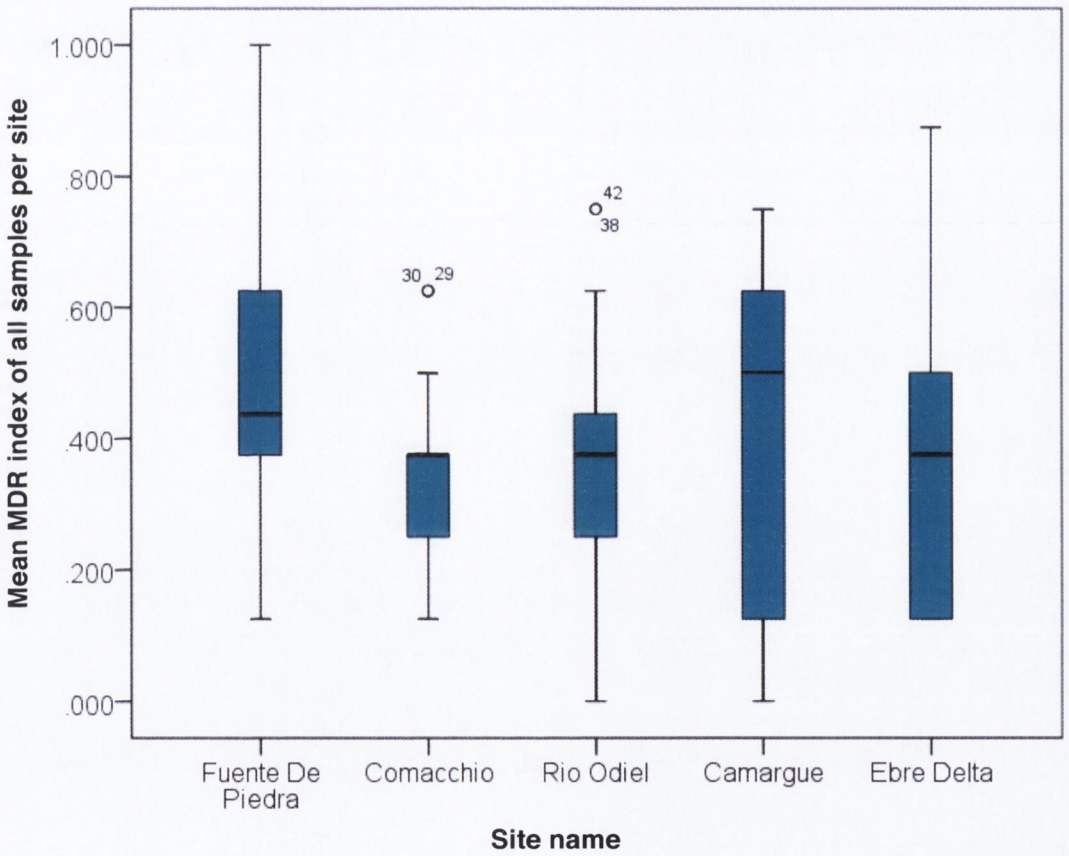


Figure 4.5. Multidrug resistance of samples compared between the five sites. The median MDR index of each site (possible range between 0 and 1) is indicated by the black bar, while the box indicates the interquartile range. Samples from Fuente de Piedre demonstrated a large range of MDR (17.5% to 100%), while samples from Comacchio all demonstrated resistances within a narrow range (17.5 to 47.5%).

Effectiveness of antibiotics

The most effective antibiotics (i.e. those to which the lowest proportion of samples were resistant) were ciprofloxacin and gentamycin, to which 18% of samples demonstrated resistance. Figures for the other antibiotics ranged from this low of 18% up to 78% of samples that demonstrated resistance to ampicillin (Figure 4.6). Two other antibiotics (erythromycin and kanamycin) were ineffective against >50% of samples tested.

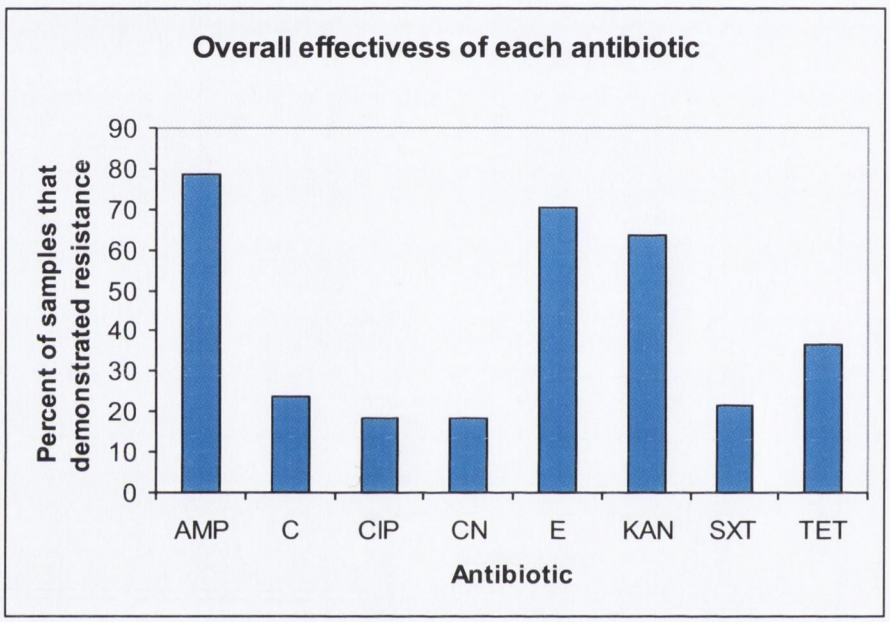


Figure 4.6. Overall effectiveness of each antibiotic tested [ampicillin (AMP), chloramphenicol (C), ciprofloxacin (CIP), gentamycin (CN), erythromycin (E), kanamycin (KAN), sulphamethoxazole/trimethoprim (SXT), and tetracycline (TET)]. The most effective antibiotics were chloramphenicol (C) and gentamycin (CN); the least effective antibiotic was ampicillin (AMP).

Geographical variation in effectiveness of antibiotics & resistance profiles

Overall, the eight antibiotics performed similarly between sites (Figure 4.7). Site-specific differences in antibiotic resistance in flamingos were evaluated using Chi Square tests (Table 4.8). The differences between sites were not significant for most of the antibiotics. However, statistically significant differences were found for ampicillin ($p \leq .01$) and chloramphenicol ($p \leq .05$).

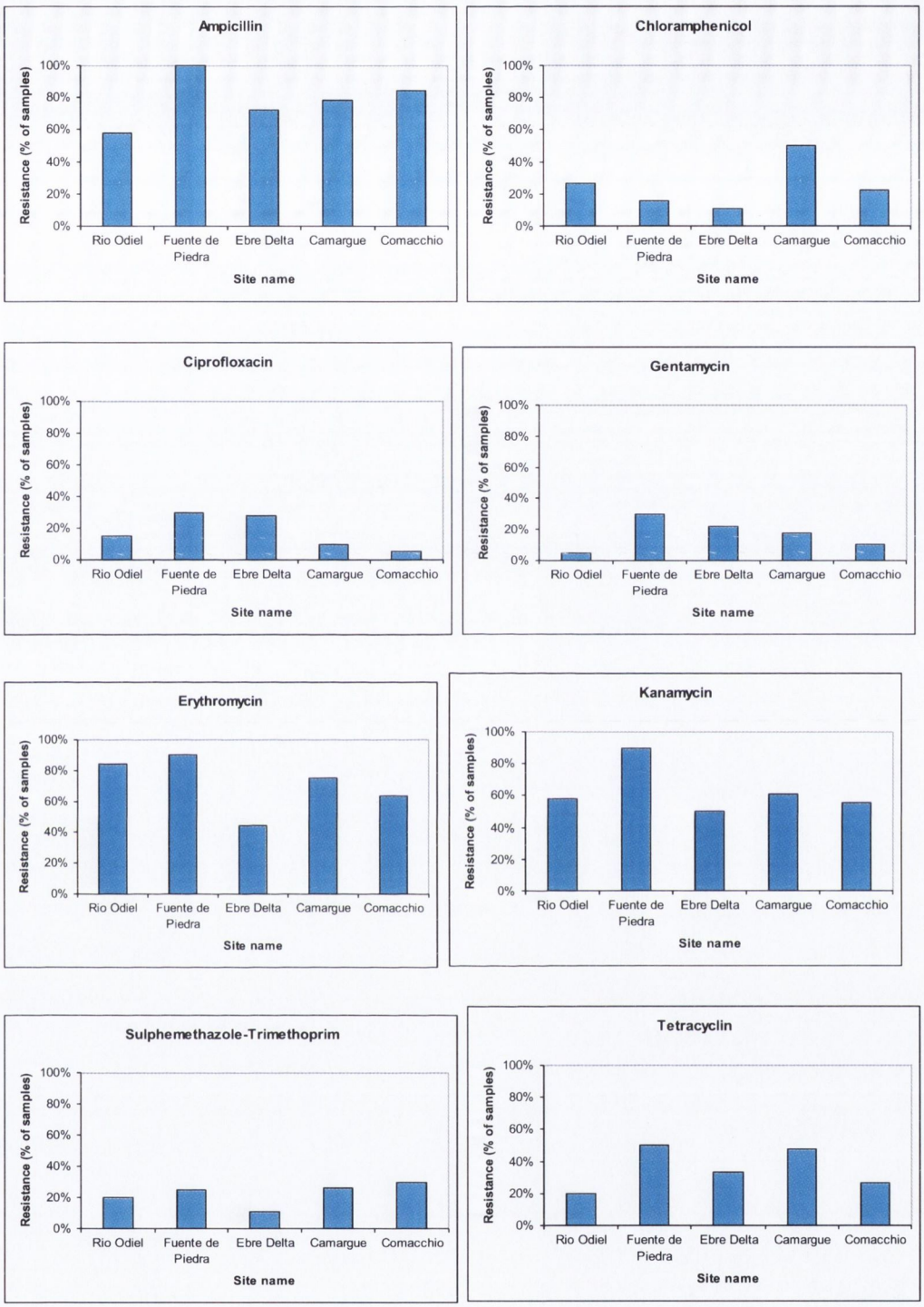


Figure 4.7. Percent of samples that demonstrated resistance to each of the eight antibiotics at the five study sites. In general, most of the antibiotics performed similarly between the different sites.

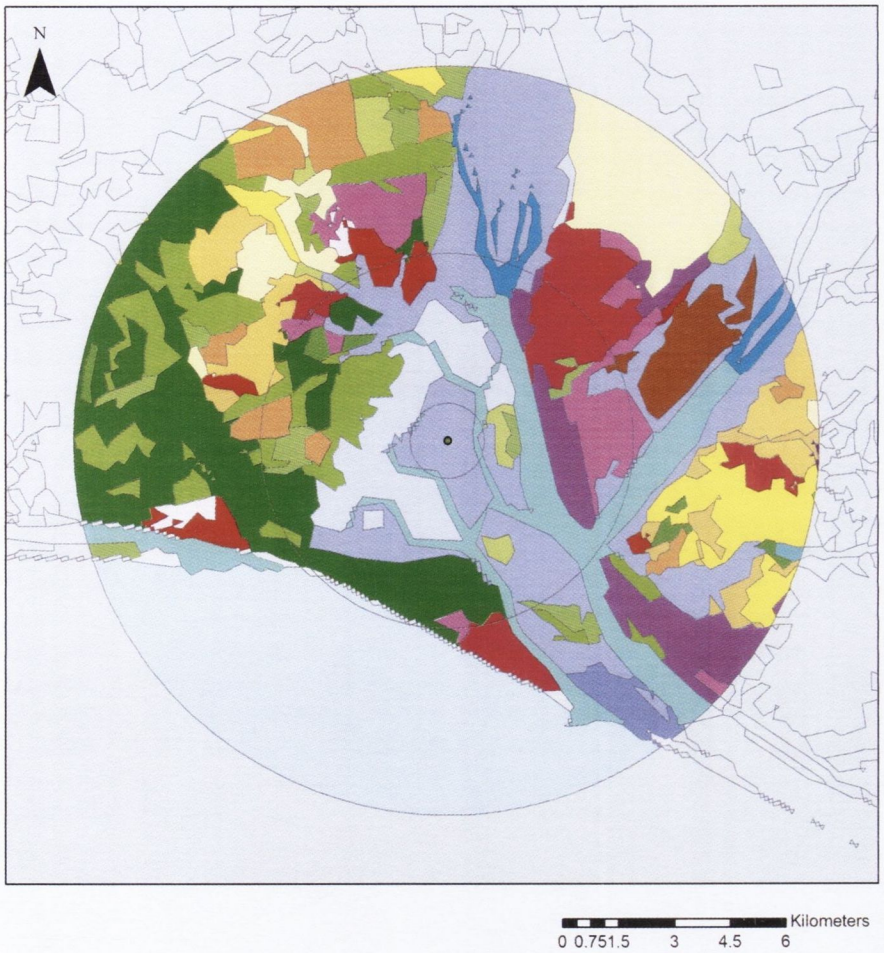
Table 4.8. Results of Chi-Square tests comparing the effectiveness of antibiotics between the five study sites

Antibiotic	Significance (p value)	Chi-square	Degrees of freedom
Ampicillin	.009	13.438	4
Chloramphenicol	.030	10.732	4
Ciprofloxacin	.269	5.188	4
Gentamycin	.480	3.487	4
Erythromycin	.142	6.85	4
Kanamycin	.167	6.462	4
Sulphamethoxazole-Trimethoprim	.868	1.259	4
Tetracycline	.220	5.728	4

4.5.3 Landscape analysis – land cover characterization and relationship to antibiotic resistance

Land cover characterization

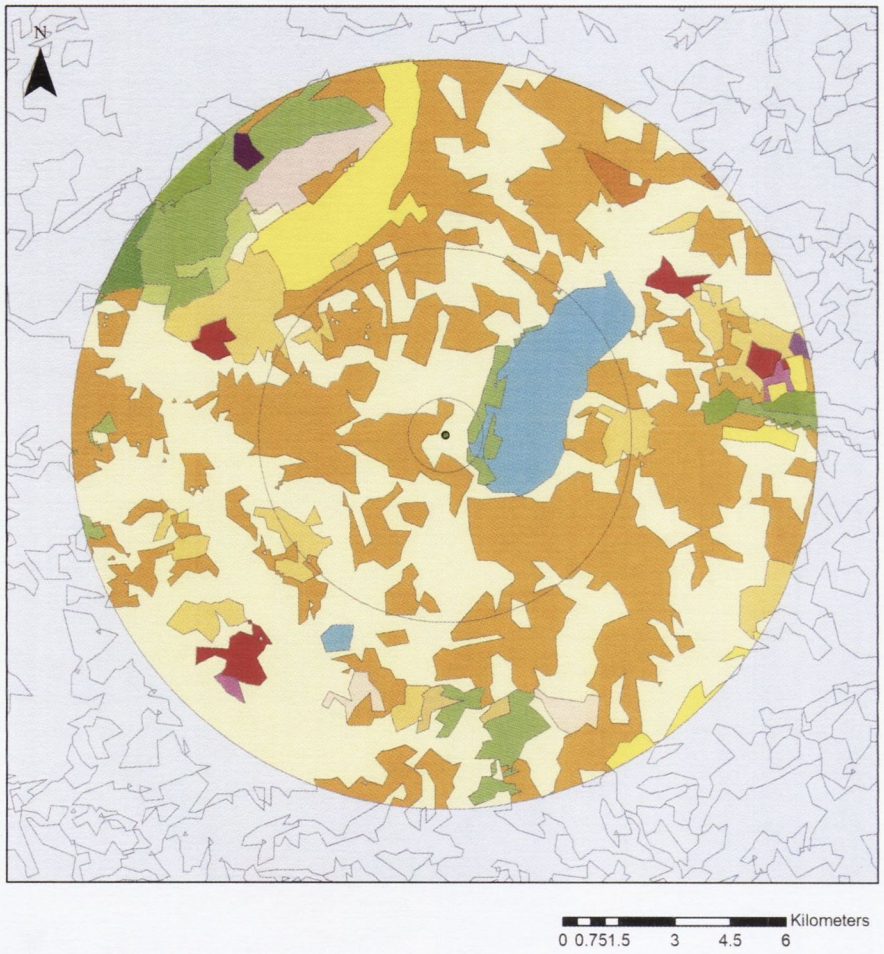
The maps of the five study sites that visualize land cover within the three buffers (1km, 5km, and 10km), are provided on the following pages, along with summary tables that provide details on the proportions of each land cover class at each of the three spatial scales (with reference to Level 1 and Level 3 nomenclature) (Figures 4.8-4.12). Figure 4.13 provides a depiction of the land covers that comprise the buffer zones at each of the spatial scales across the five sites, with reference to Level 1 nomenclature. The statistical analyses that were conducted using these landscape metrics are presented in section that follows.



CORINE Land Cover Classification	1km	5km	10km
Artificial surfaces	**0.00**	**17.81**	**15.12**
Continuous urban fabric	0.00	5.13	4.21
Discontinuous urban fabric	0.00	1.55	1.52
Industrial or commercial units	0.00	5.14	3.68
Mineral extraction sites	0.00	0.00	0.02
Dump sites	0.00	0.09	1.75
Construction sites	0.00	5.90	3.46
Sport and leisure facilities	0.00	0.00	0.39
Agricultural areas	**0.00**	**1.68**	**16.40**
Non-irrigated arable land	0.00	0.00	5.88
Permanently irrigated land	0.00	0.00	3.35
Fruit trees and berry plantations	0.00	1.54	3.02
Complex cultivation patterns	0.00	0.14	2.94
Land principally occupied by agriculture, with significant areas of natural vegetation	0.00	0.00	1.21
Forest and semi-natural areas	**0.00**	**25.56**	**22.08**
Broad-leaved forest	0.00	0.89	0.57
Coniferous forest	0.00	13.83	10.88
Natural grasslands	0.00	1.49	1.27
Sclerophyllous vegetation	0.00	2.26	2.28
Transitional woodland-shrub	0.00	6.82	6.52
Beaches, dunes, sands	0.00	0.27	0.56
Wetlands	**91.47**	**40.05**	**20.18**
Salt marshes	91.29	24.85	15.38
Salines	0.18	15.20	3.93
Intertidal flats	0.00	0.00	0.86
Waterbodies	**8.53**	**14.91**	**26.22**
Water courses	0.00	0.77	1.16
Water bodies	0.00	0.00	0.08
Estuaries	8.53	13.51	7.07
Sea and ocean	0.00	0.63	17.91

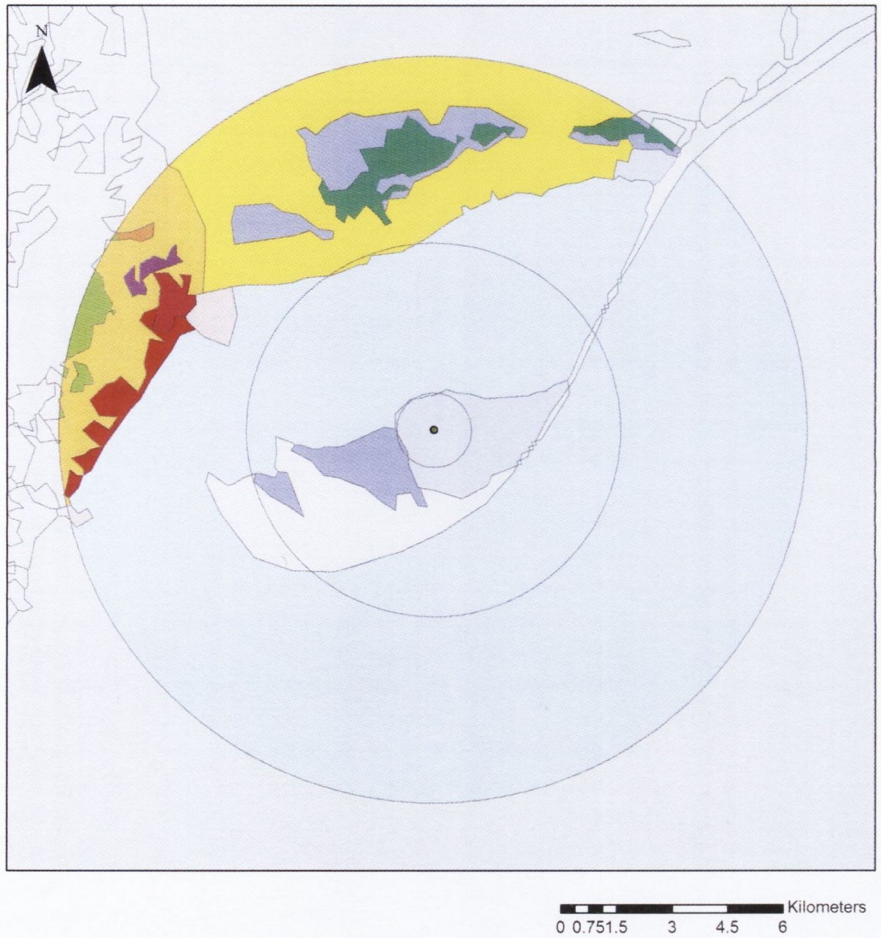
Figure 4.8 Land cover within 1km, 5km, and 10km buffers of the fieldwork site at Rio Odiel, Spain. Land cover classifications used in the map (colour scheme legend in the table on the right) refer to CORINE Level 3 nomenclature, while the categories provided (in bold) in the table use the Level 1 nomenclature.

Fuente de Piedre



Land Class (CORINE Level 3)	1km	5km	10km
Artificial surfaces	**0.00**	**0.00**	**1.65**
Continuous urban fabric	0.00	0.00	1.21
Industrial or commercial units	0.00	0.00	0.07
Mineral extraction sites	0.00	0.00	0.16
Construction sites	0.00	0.00	0.20
Agricultural areas	**87.84**	**85.24**	**87.52**
Non-irrigated arable land	49.98	39.76	41.46
Permanently irrigated land	0.00	0.00	4.05
Vineyards	0.00	0.00	0.37
Olive groves	37.86	42.36	34.04
Annual crops associated with permanent crops	0.00	0.00	0.62
Complex cultivation patterns	0.00	3.12	5.45
Land principally occupied by agriculture, with significant areas of natural vegetation	0.00	0.00	0.27
Agro-forestry areas	0.00	0.00	1.26
Forest and semi-natural areas	**7.62**	**2.18**	**6.97**
Broad-leaved forest	0.00	0.00	0.42
Natural grasslands	0.00	0.00	0.57
Sclerophyllous vegetation	7.62	2.18	5.17
Transitional woodland-shrub	0.00	0.00	0.82
Waterbodies	**4.55**	**12.57**	**3.85**
Water bodies	4.55	12.57	3.85

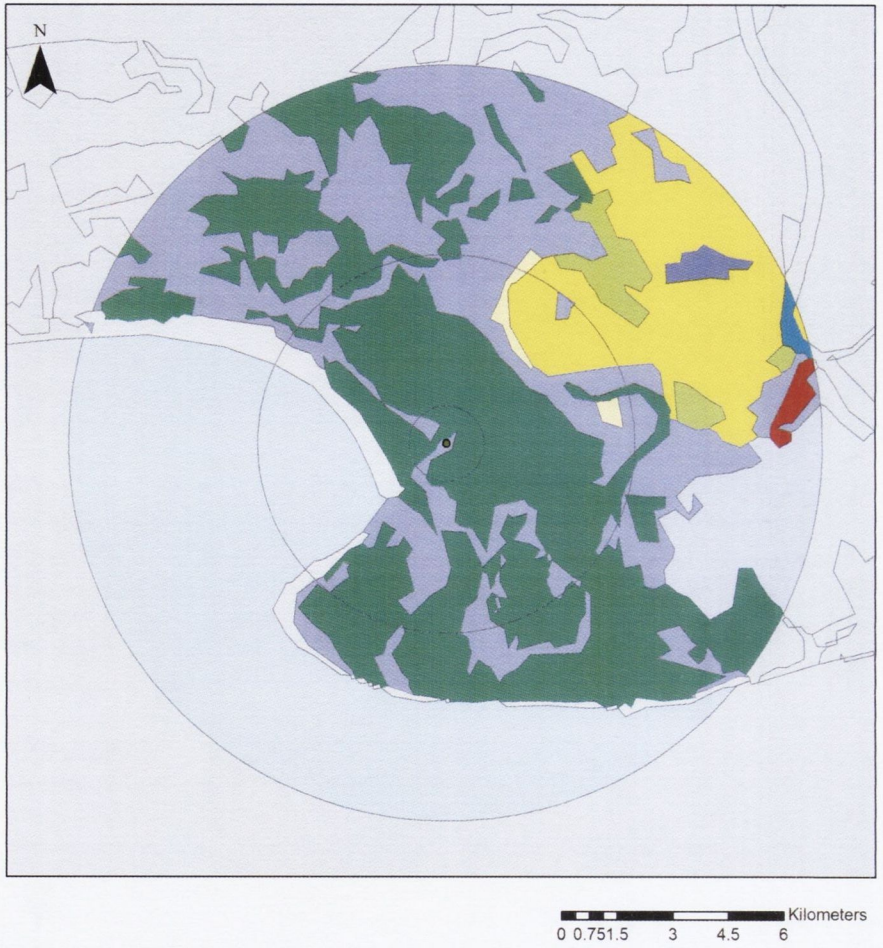
Figure 4.9 Land cover within 1km, 5km, and 10km buffers of the fieldwork site at Fuente de Piedre, Spain. Land cover classifications used in the map (colour scheme legend in the table on the right) refer to CORINE Level 3 nomenclature, while the categories provided (in bold) in the table use the Level 1 nomenclature.



Land Class (CORINE Level 3)	1km	5km	10km
Artificial surfaces	**0.00**	**0.00**	**2.04**
Continuous urban fabric	0.00	0.00	0.37
Discontinuous urban fabric	0.00	0.00	0.93
Industrial or commercial units	0.00	0.00	0.20
Port areas	0.00	0.00	0.53
Agricultural areas	**0.00**	**0.35**	**15.27**
Rice fields	0.00	0.35	11.62
Fruit trees and berry plantations	0.00	0.00	0.08
Complex cultivation patterns	0.00	0.00	3.57
Forest and semi-natural areas	**0.00**	**13.26**	**5.10**
Sclerophyllous vegetation	0.00	0.00	0.52
Beaches, dunes, sands	0.00	13.26	4.57
Wetlands	**98.95**	**16.61**	**7.06**
Salt marshes	1.75	5.55	3.94
Salines	97.20	11.06	3.12
Waterbodies	**1.05**	**69.77**	**70.54**
Coastal lagoons	0.00	0.00	1.65
Sea and ocean	1.05	69.77	68.90

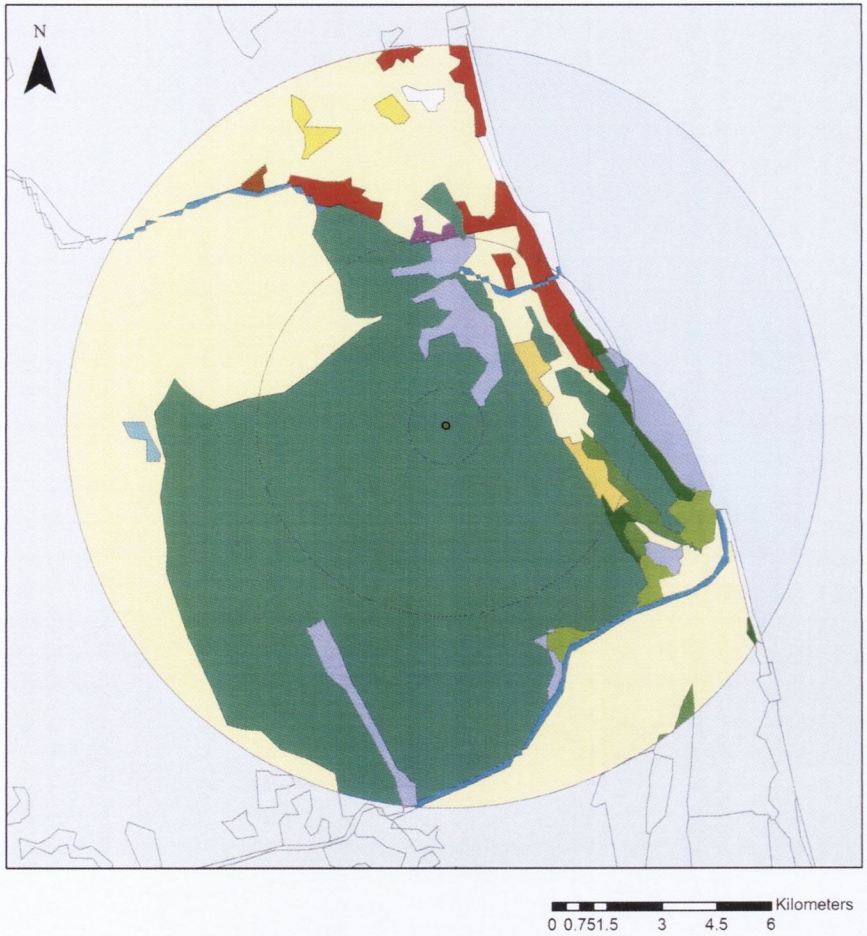
Figure 4.10 Land cover within 1km, 5km, and 10km buffers of the fieldwork site at Ebre Delta, Spain. Land cover classifications used in the map (colour scheme legend in the table on the right) refer to CORINE Level 3 nomenclature, while the categories provided (in bold) in the table use the Level 1 nomenclature.

Camargue



Land Class (CORINE Level 3)	1km	5km	10km
Artificial surfaces	**0.00**	**0.00**	**0.39**
Discontinuous urban fabric	0.00	0.00	0.39
Agricultural areas	**0.00**	**7.11**	**9.94**
Non-irrigated arable land	0.00	1.38	0.41
Permanently irrigated land	0.00	0.00	0.00
Rice fields	0.00	5.73	9.53
Forest and semi-natural areas	**0.00**	**3.88**	**4.35**
Natural grasslands	0.00	0.00	1.60
Beaches, dunes, sands	0.00	3.88	2.75
Wetlands	**17.99**	**23.02**	**27.92**
Inland marshes	0.00	0.00	0.42
Salt marshes	17.99	23.02	23.07
Salines	0.00	0.00	4.42
Waterbodies	**82.01**	**65.99**	**57.41**
Water courses	0.00	0.00	0.24
Coastal lagoons	82.01	51.74	28.21
Sea and ocean	0.00	14.25	28.96

Figure 4.11 Land cover within 1km, 5km, and 10km buffers of the fieldwork site at the Camargue, France. Land cover classifications used in the map (colour scheme legend in the table on the right) refer to CORINE Level 3 nomenclature, while the categories provided (in bold) in the table use the Level 1 nomenclature.



Land Class (CORINE Level 3)	1km	5k m	10k m
Artificial surfaces	**0.00**	**2.88**	**2.46**
Discontinuous urban fabric	0.00	2.73	2.02
Industrial or commercial units	0.00	0.16	0.13
Dump sites	0.00	0.00	0.08
Sport and leisure facilities	0.00	0.00	0.23
Agricultural areas	**0.00**	**13.20**	**32.53**
Non-irrigated arable land	0.00	11.07	31.61
Rice fields	0.00	0.00	0.37
Complex cultivation patterns	0.00	2.13	0.55
Forest and semi-natural areas	**0.00**	**2.64**	**2.89**
Broad-leaved forest	0.00	0.00	0.11
Coniferous forest	0.00	1.11	0.67
Mixed forest	0.00	0.73	0.57
Transitional woodland-shrub	0.00	0.00	0.77
Beaches, dunes, sands	0.00	0.80	0.76
Wetlands	**0.00**	**5.73**	**3.17**
Salt marshes	0.00	5.73	3.17
Waterbodies	**100.00**	**75.54**	**58.95**
Water courses	0.00	0.44	0.83
Water bodies	0.00	0.00	0.13
Coastal lagoons	100.00	74.66	38.61
Sea and ocean	0.00	0.44	19.38

Figure 4.12 Land cover within 1km, 5km, and 10km buffers of the fieldwork site at Comacchio, Italy. Land cover classifications used in the map (colour scheme legend in the table on the right) refer to CORINE Level 3 nomenclature, while the categories provided (in bold) in the table use the Level 1 nomenclature.

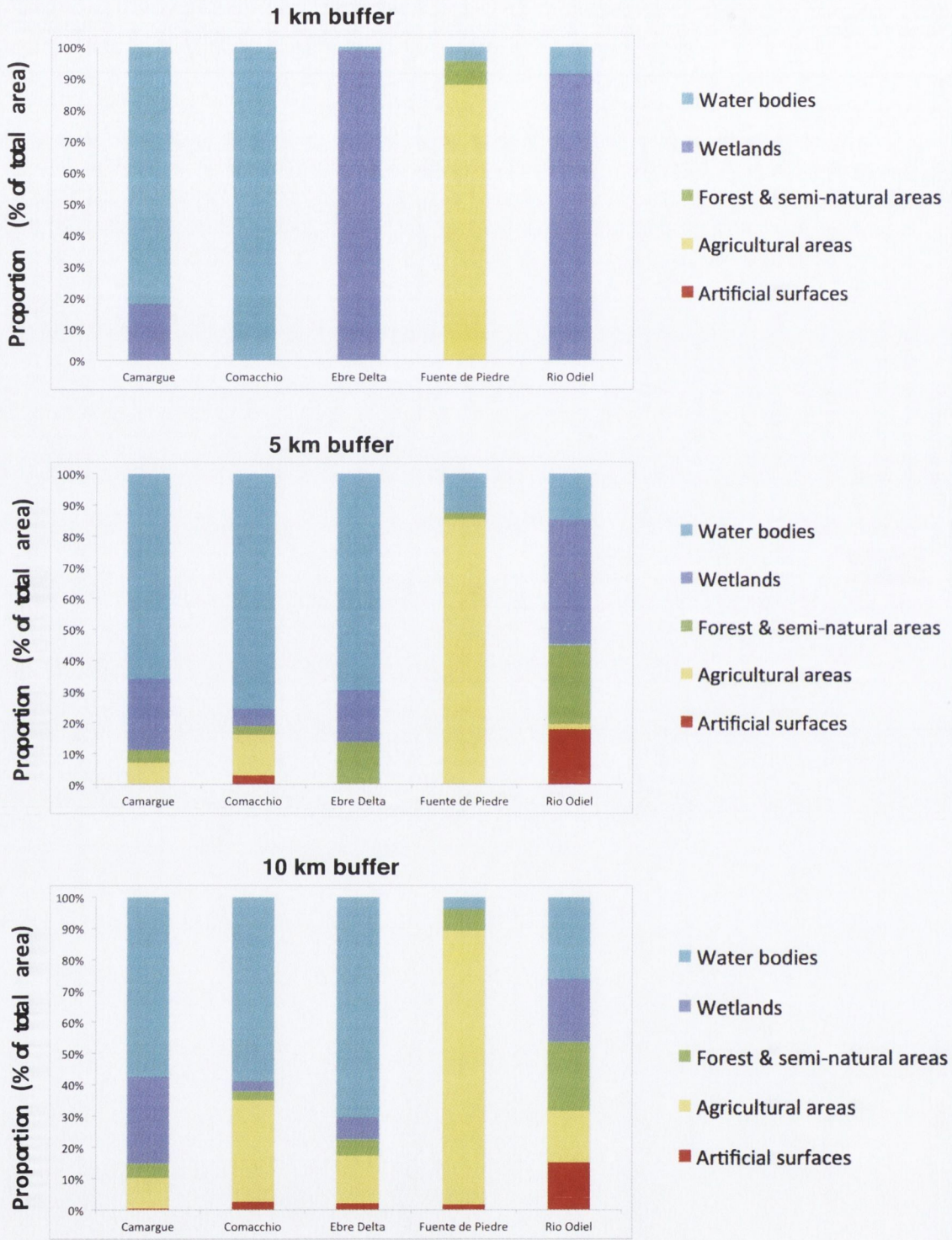


Figure 4.13 The proportions of land cover class at each of the five sites at three spatial scales (buffers of 1km, 5km, and 10km), classed with reference to the CORINE Land Cover database Level 1 classifications. Sites (from left to right) are the Camargue, Comacchio, Ebre Delta, Fuente de Piedre, and Rio Odiel.

Land cover and antibiotic resistance at different spatial scales

The results of stepwise regression in SPSS indicated that there was little significant relationship between the various land cover 'predictors' and antibiotic resistance at any of the three spatial scales (Table 4.9). Indeed, for half of the antibiotics (four of the eight), none of the land covers at any of the spatial scales were found to serve as statistically significant predictors. The only antibiotics for which significant relationships were found were ampicillin, erythromycin, kanamycin, and tetracycline. For ampicillin, wetlands at the 5km spatial scale were found to act a statistically significant predictor for the prevalence of resistance ($p \leq .05$). Water bodies at 5km were found to serve as a predictor for erythromycin resistance ($p \leq .05$), while for kanamycin, agricultural areas 5km and wetlands 10km were found to serve as predictors ($p \leq .01$). Wetlands were also a statistically significant predictor (though at the 1km spatial scale) of resistance to tetracycline ($p \leq .05$).

Table 4.9 Land cover predictors of resistance at three spatial scales as determined via stepwise regression in SPSS.

Antibiotic	Predictors(s) (spatial scale)	Significance
Ampicillin	Wetlands (5km)	$p \leq .05$
Chloramphenicol	All were deleted from model	na
Ciprofloxacin	All were deleted from model	na
Erythromycin	Water bodies (5km)	$p \leq .05$
Gentamycin	All were deleted from model	na
Kanamycin	Agricultural areas (5km)	$p \leq .05$
	Wetlands (10km)	$p \leq .01$
Sulphamethoxazole-Trimethoprim	All were deleted from model	na
Tetracycline	Wetlands (1km)	$p \leq .05$

4.6 Discussion

This study has provided, for the first time, an evaluation of wild Greater Flamingos as sentinels of antibiotic resistance. In doing so, this investigation first developed a list of criteria that can be used in selecting a sentinel of antibiotic resistance. Following on from this, the prevalence of antibiotic resistant bacteria in the Greater Flamingo was assessed. The results of this analysis indicated variation between antibiotics and study sites, and provided evidence of the potential influence of landscape factors on the prevalence of resistance. In the following sections, these results are discussed within the context of relevant research.

4.6.1 Criteria for sentinel selection

In developing a list of criteria for the selection of a sentinel, it became evident that within the literature, there has been little agreement on which criteria are most appropriate to use. This is best exemplified by a well-documented debate focused on the mink; documented by a series of articles and commentaries published between 2007 and 2012 concerning its use as a sentinel of environmental health. Along with providing a list of characteristics of sentinel species, Basu *et al.* (2007) recommended the mink as a sentinel. Bowman and Schulte-Hostedde (2009) challenged this assertion, suggesting that Basu *et al.* (2007) neglected to consider the fact that ideal sentinels should be a continuous resident of the system that is under investigation. In the exchange that followed, it became clear that the residency status of a species is indeed important to consider in selecting a sentinel. This is an important consideration in particular for selecting a sentinel of antibiotic resistance, given that an animal that is a resident of multiple sites, or visits multiple sites on a regular basis (e.g. for foraging), will be exposed to bacteria at each of these sites. Thus, it can be difficult to determine the site at which resistant bacteria were acquired. Along these lines, the mobility of birds has been cited as a shortcoming in using them as environmental health sentinels (Chambers, 2008).

This is interesting to consider with specific reference to the use of the Greater Flamingo in the current investigation. Given its ability to fly, the Greater Flamingo is a relatively mobile animal that can, and does, move frequently from between sites within its range. The ongoing tracking of Greater Flamingos (which serves as the basis for the annual ringing operations) continues to provide insights into the extent of these movements, including details of the extent of wetland connectivity within its range, and the extent and nature of its metapopulation (Amat *et al.*, 2005; Balkız *et al.* 2007). Such long-term monitoring programmes can be of particular relevance in understanding the potential for transfer of disease between sites, and the analysis of disease networks and modeling of disease spread has been studied extensively in a variety of wildlife species (Hess, 1996; Johnson, 1997; Fèvre *et al.*, 2006; Keeling *et al.*, 2010; Saldaña, 2010). The Greater Flamingo is interesting in that many of its breeding sites are under significant anthropogenic influence. However, species that demonstrate frequent movements can make assessing the source(s) of infectious disease difficult. This potential

problem was overcome in the current investigation with the use of fledgling flamingos, which are not yet able to fly. Fledgling birds have similarly been used to determine the extent of local contamination with reference to trace metals (Amiard-Triquet *et al.* 1991). However, this didn't entirely remove uncertainty about the source of resistant bacteria in the flamingos, given questions about the potential transfer of resistant bacteria from adult birds to chicks – either directly (e.g. feeding) or indirectly, through the environment (Lombardo *et al.*, 1996).

Challenges such as this highlight the complexity and range of issues that need to be taken into account when selecting a sentinel species, and makes clear the need for having a comprehensive list of criteria with which to evaluate a candidate sentinel species. The list of criteria developed in this investigation proved useful in assessing the Greater Flamingo as a sentinel. Here, there was the advantage that there was only one species proposed for evaluation. In a study where several candidate species are proposed, it would likely be useful to further develop the list of criteria, to further assist researchers in selecting the best of the proposed species. This could be achieved by developing a more refined system, perhaps one that is quantitative and/or a selection process that is sequential in nature (with multiple stages at which candidate species are excluded), as opposed to using a straightforward list of criteria such as that developed here (Chambers, 2008).

4.6.2 Determination of antibiotic resistant bacteria in the Greater Flamingo

In this first study to evaluate the Greater Flamingo as a sentinel of antibiotic resistance, the prevalence of antibiotic resistance was determined via laboratory analysis of bacterial samples collected from five sites in the Mediterranean region. In accordance with previous studies that have found antibiotic resistant bacteria in a variety of wild bird species, the Greater Flamingo was found to serve as a reservoir of antibiotic resistance at each of the five study sites (Cole *et al.*, 2005; Blanco *et al.*, 2007; Dolejska *et al.*, 2007, 2008; Costa *et al.*, 2008; Guenther *et al.*, 2010). The specific resistances to antibiotics demonstrated in this study are similar to those found in other research efforts in wild birds. In the present study, resistance to ampicillin was the most prevalent, and this mirrors the results of similar research. For example, in assessing resistance in yellow-legged gulls in Southern France, Bonnedahl *et al.* (2009) noted high levels of resistance to ampicillin; and interestingly, their research location corresponds to one of the sites from which samples were taken in the present study (the Camargue). In their study, Bonnedahl *et al.* (2009) suggested the specific resistances they noted were likely due to the gulls having contact with human activity. The Camargue, and indeed much of the Mediterranean region, is an area for which the interactions between human populations and the environment is extensive, and the impacts of human activity on a variety of environmental systems are well documented (Haas, 1989; Béthoux *et al.*, 2002; Zalidis *et al.*, 2002; Blondel, 2006; Geri *et al.*, 2010).

This close association between human and wildlife populations may also explain one of the particularly notable findings of the present investigation, which was the widespread prevalence of multidrug resistance (noted for 81% of samples). The occurrence of multidrug resistance has been documented in a variety of avian species. An investigation of resistance in faecal samples from yellow-legged gulls in the south of France, for example, found multidrug resistance in one third of isolates (Bonnedahl *et al.*, 2009). Their study used a panel of six antibiotics, three of which were used in the present study (tetracycline, ampicillin, and chloramphenicol). An investigation by Rose *et al.* (2009) found a similar overall prevalence of multidrug resistance (43%) in their analysis of samples from several animals (including seabirds). The disparity in MDR indices (i.e. proportion of isolates resistant to two or more antibiotics) between the current study and these other studies might be the result of the fact that previous studies have used a range of antibiotics (both in total number assessed and specific antibiotics themselves), so comparing the specific multidrug resistance indices is difficult. Similarly, although Rose *et al.* (2009) found an overall MDR of 43%, their study included samples from a variety of animals, and they noted a higher prevalence of resistant amongst bird isolates than in marine mammals. Thus, although direct comparisons are complicated, what is apparent from this investigation within the context of similar research – is that multidrug resistance is prevalent amongst a variety of wild bird species. Multidrug resistance presents a serious problem in a clinical context (i.e. patients in hospitals), and although a large number of clinically available antibiotics exist, increasingly, bacteria are demonstrating resistances to multiple classes of antibiotics (Cohen, 1992; Levy and Marshall, 2004). The spread and persistence of antibiotic resistant bacteria in the Greater Flamingo is also interesting considering that it lives in large colonies, and raises questions about the extent to which this aspect of its life history might facilitate the spread of bacteria throughout all of the individuals in a given colony. The evidence from this investigation and others like it, of multidrug resistance in environmental systems, is indeed alarming (Sayeh *et al.*, 2005; Jones *et al.*, 2008; Allen *et al.*, 2010). Thus, this remains an important topic for investigation, and understanding the factors that affect the prevalence of antibiotic resistance are an important topic for more detailed investigation.

4.6.3 Landscape analysis of antibiotic resistance in the Greater Flamingo

Many studies have endeavoured to determine the extent to which human activity affects the prevalence of antibiotic resistance in the environment, by attempting to link landscape factors and human activity to specific resistances. The use of a multi-site methodology (i.e. taking samples from several sites within a geographic region) can be a useful approach, because it allows for comparisons between sites to be made, while keeping other aspects of research (e.g. species used, methodologies, etc.) constant. The present investigation has offered

insights into patterns of resistance through use such an approach, allowing for the investigation of factors that have previously been suggested to affect resistance.

The range of specific resistances found in the present investigation is likely indicative of a variety of influences on the environmental systems in which the study sites were located, and in particular, the anthropogenic influences that affect the Greater Flamingo's breeding sites. This hypothesis was tested through use of landscape analysis using data provided by the EU CORINE programme, which offers an inventory of land cover data for Europe. The results of landscape analysis in ArcGIS indicated that land cover might predict the prevalence of resistance in certain antibiotics, but only at certain spatial scales (1km, 5km, and/or 10km). For most of the antibiotics, no statistically significant predictors of resistance were found. Land covers that were found to significantly predict resistance for a given antibiotic were wetlands (at a variety of spatial scales for ampicillin, kanamycin, and tetracycline), water bodies (for erythromycin), and agricultural areas (kanamycin). Neither the 'forest & semi-natural areas' nor 'artificial surfaces' land cover classes were found to predict resistance, which is interesting given that antibiotics and antibiotic resistance have previously been suggested to derive from urban areas (which are included in the 'artificial surfaces' class) (Carroll *et al.*, 2009; Laroche *et al.*, 2009; Watkinson *et al.*, 2009; Ham *et al.*, 2012). However, the results in the current investigation might have been affected by the fact that the relative proportion of these land covers was smaller than others, thus, their influence would be likely to be less than those that were more abundant. Aquatic land covers (including 'water bodies' and 'wetlands'), for example, comprised a relatively higher proportion of the overall land cover, which is unsurprising given the location of four of the five field sites on the Mediterranean Sea. That aquatic systems were found to predict resistance confirms the results of similar research efforts, which have found antibiotics and antibiotic resistant bacteria to be prevalent in aquatic systems (Sevaño *et al.*, 2002; Schwarz *et al.*, 2003; Laroche *et al.*, 2009).

A somewhat surprising finding was that agricultural areas were only found to serve as a significant predictor for one antibiotic (gentamycin), given that the landscape analyses failed to find significant links between 'agricultural areas' as defined by the CORINE land cover inventory, and the prevalence of resistance to the different antibiotics. This is in contrast to many other investigations, which have linked antibiotic resistance to agriculture. For example, Blanco *et al.* (2009) linked antibiotic resistance in red-billed choughs in several different regions of Spain to the agricultural practice of spreading manure from livestock and/or sewage sludge onto agricultural land as a fertilizer. Several of the resistances they found were similar to those found here, including ciprofloxacin and gentamycin (in areas where sewage sludge was used) and ampicillin (where livestock manure was used). Heuer *et al.* (2011) cited the spread of antibiotic resistant genes as the driver behind an increased prevalence of antibiotic resistance, as a result of manure application in agricultural contexts. The extent to which agricultural manure contributes to antibiotic resistance in environmental systems is

unsurprising, given that up to 75% of antibiotics given to livestock are excreted as waste by animals (Chee-Sanford *et al.*, 2009). The antibiotics can persist in the environment for a long time, thus allowing for organisms living within the system to develop resistances (Kemper, 2008). Further to this, the antibiotics, once applied to the soil, can seep into the soil and can ultimately end up in groundwater (Kümmerer, 2003). This spread of antibiotics throughout the system is in addition to the antibiotic resistant bacteria themselves, which are also excreted by the livestock, and can survive in the environment for several weeks (Chee-Sanford *et al.*, 2009).

Although in many cases links between land use and/or human activity and antibiotic resistance in environmental systems are very clear, it is extremely important to be cautious in making such links and drawing conclusions regarding the impact of human activity. In examining the high levels of antibiotic resistant bacteria found in this study and other similar investigations, and considering the differences between sites with reference to the role of human activity, it is important to consider research that has provided evidence of high levels of antibiotic resistance where human activity is lacking. In 2012, Bhullar *et al.* published evidence of bacteria resistant to fourteen antibiotics that were taken from a cave that had been isolated from humans for over four million years. This naturally arising resistance, they assert, provides evidence that resistance can and does arise in the absence of human activity. This was not the first time that research from isolated systems has yielded isolates that displayed resistance; similar work has been conducted in both deep-sea and deep-terrestrial (i.e. up to 300 metres below the Earth's surface) systems (Brown and Balkwill, 2009; Toth *et al.*, 2010). This is not to say that all similarly isolated sites yield either resistant bacteria or resistance genes. For example, a study conducted in the Galapagos Islands (at a site where human activity is minimal) indicated that in the absence of human activity, no resistance genes had been acquired (Thaller *et al.*, 2010) – thus indicating that naturally arising resistance does not, in all cases, arise. These inconsistencies highlight the need for intensive, large-scale monitoring regimes, in which system-wide samples are taken. By taking a variety of samples (i.e. from both biotic and abiotic sources) across a large geographic area, it is possible to identify sources of antibiotics and antibiotic resistant bacteria, and with this, the pathways for transport of resistant bacteria and antibiotics can be better understood (Chee-Sanford *et al.*, 2009). The use of sentinels can serve not only as an evaluation of the environmental status, but also, can be important tool for use as an early warning for potential threats to human health (King *et al.*, 2004; Stuart *et al.*, 2008).

The prevalence of resistant bacteria is problematic not only with regards to the bacteria themselves (and potential uptake by wildlife living within the environment), but also for the resultant opportunity for the transfer of antibiotic resistant genes between environmental systems, wildlife, and humans (Catry, 2001; Chee-Sanford *et al.*, 2001; O'Brien, 2002). However, it was beyond the scope of this chapter to describe the resistances found here in a

human health context, though it is deemed critical to do so under the One Health framework, the integrated approach to wildlife, environmental, and human health which has become increasingly accepted (Daszak *et al.*, 2007). To accurately predict consequences for human health, refined analyses are required to quantify antibiotic resistance in such a way that they are relevant to human health (i.e. clinical concentrations of antibiotics, and/or identification of resistant bacterial species); collaboration with those working in the area of human health, either in research or clinical contexts, may thus offer significant potential for advancing our knowledge base. Stahl (1997) noted that linking sentinel data and human health effects can be problematic, suggesting that difficulties often arise as a result of uncertain data. Vassilev *et al.* (1999) also cited the problem of linking environmental indicators and human health, and highlighted the importance of using public health criteria in addition to environmental indicators as a means of assessing the impact on human health. This is a process which ideally is dynamic and goes both ways; Rabinowitz *et al* (2005) discussed the importance of clinicians and human healthcare professionals accessing and reading articles that relate to disease in animals, given the potential for shared diseases between humans and animals. In these papers and others, the need for integration between what are traditionally separate fields becomes clear, and the continued assessment of the relevance of disease outbreaks and events across the disciplines is of importance (King *et al.*, 2004). To achieve this integration, Rabinowitz *et al.* (2005) proposed 'linkage points' as a means by which animal sentinels and human health can be linked, including, for example, the monitoring of diseases to which both humans and animals are susceptible, and is a useful approach with reference to the One Health initiative in which these are inextricably linked.

This research was limited in several ways. Although samples were collected from fledgling flamingos (unable to fly), there is the potential for transfer from adult flamingos. Indeed, Eraud *et al.* 2008 suggested that crop milk can act as a mechanism with which environmental condition can be transferred. Given that foraging distances can be as large as 180km, it is possible that bacteria acquired at other sites could be transferred to a chick at the study site (Rendos-Martin and Johnson, 1996). The Fuente de Piedra colony in Spain has been frequently recorded foraging at marshes over 100km away, and the mobility of waterbirds is often cited as a complicating factor for elucidating prevalence of disease between sites (Amat and Green, 2005). Perhaps the Greater Flamingo best exemplifies the complexity of investigating disease between sites, given that much remains to be discovered about the nature of its population(s) in the Mediterranean region. The continued efforts of ringing operations – and more recently, genetic analysis – will contribute a lot to the study of infectious disease transfer of the Greater Flamingo. In regards to research on the Greater Flamingo, problems with data that are too general can be overcome through use of more refined laboratory analyses. This includes the use of a more precise measure of resistance, such as minimum inhibitory concentration (as this allows for a translation of relevance to clinical doses

of antibiotics). As well, identification of resistant bacterial species is required, to determine which species are of relevance to human health. Despite these limitations, as a preliminary investigation, this study was worthwhile in that it validated the Greater Flamingo as a sentinel (which can be used in future, more refined analyses), and served to provide evidence of the prevalence of antibiotic resistance in Greater Flamingos at the five study sites. This will also serve to help address the impact on human health, and there exist well-defined criteria that will help make a clearer link to public health.

4.7 Conclusions and recommendations for future research

4.7.1 Conclusions

The development and use of criteria for sentinel selection proved to be critically important to ensure than an appropriate species was selected. The Greater Flamingo is a valid sentinel of antibiotic resistance, as it meets the majority of criteria for a sentinel and antibiotic resistant bacteria were readily detected in bacterial samples at each of the five sites.

Antibiotic resistance is prevalent in Rio Odiel, Ebre Delta, and Fuente de Piedra, Spain, the Camargue, France, and Comacchio, Italy. At each of these sites, resistance to all eight antibiotics was identified, although the levels of resistance to the eight antibiotics varied between sites. Fuente de Piedra, in Spain, was the site at which the highest proportion of samples were resistant, while Comacchio in Italy had the smallest proportion of resistant samples. Across the five sites, ampicillin was the antibiotic to which the most samples were resistant, while ciprofloxacin was the antibiotic to which the fewest samples were resistant.

In general, landscape analyses did not identify many significant predictors of antibiotic resistance, though several that were identified (in particular, the 'water bodies' and 'wetlands' land cover classes) suggest that aquatic systems may be robust predictors of resistance.

4.7.2 Recommendations for future research

A more refined analysis of antibiotic resistance is needed, beyond the methods used to conduct this preliminary screening. This will not only provide more detailed information of relevance to the environmental health status of each of the five sites, but also will make it easier to draw conclusions regarding relevance to human health. The identification of bacterial samples should be undertaken – to extend the direct plating technique used here, DNA techniques should be used to determine the bacteria that are indicating resistances.

Given the high levels of resistance found here, and the potential implications for human health, it is clear that antibiotic resistance samples should ideally be collected and evaluated on a regular basis. With reference to this, I recommend the establishment of a regular monitoring programme.

Although not conducted here due to resource limitations, a comprehensive analysis of environmental samples (e.g. water, sediment, etc.) should be undertaken, as part of the monitoring programme (as suggested above). This should occur at both a small-scale (the site at which the flamingo breeding population is found), as well as at a larger scale (including the surrounding landscape), to determine potential sources of resistance. This would align with the One Health narrative, in which a holistic approach is advised.

4.8 References

Alanis, A.J. 2005. Resistance to antibiotics: are we in the post-antibiotic era? Archives of Medical Research **36,** 697-705.

Albanese, G., Arveda, G., Baccetti, N., Dall'Antonia, P., & Morelli, F. 2009. The Flamingo Project in mainland Italy: an update of the situation at the end of 2007. Flamingo **1**, 52.

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J. and Handelsman, J. 2010. Call of the wild: antibiotic resistance genes in natural environments. Nature Reviews Microbiology **8,** 251-259.

Amat, J.A., Rendón, M.A., Rendón-Martos, M., Garrido, A. and Ramírez. J.M. 2005. Ranging behaviour of greater flamingos during the breeding and post-breeding periods: linking connectivity to biological processes. Biological Conservation **125,** 183-192.

Amat, J.A., Rendón, M.A., Garrido-Fernández, J., Garrido, A., Rendón-Martos, M. and Pérez-Gálvez, A. 2011. Greater flamingos *Phoenicopterus roseus* use uropygial secretions as make-up. Behavioral Ecology and Sociobiology **65,** 665-673.

Aminov, R.I. 2009. The role of antibiotics and antibiotic resistance in nature. Environmental Microbiology **11,** 2970-2988.

Arnqvist, G. and Wooster, D. 1995. Meta-analysis: synthesizing research findings in ecology and evolution. Trends in Ecology and Evoluton **10,** 236-240.

Azcárate, M.C. 2010. Contentious hotspots: ecotourism and the restructuring of place at the biosphere reserve Ria Celestun (Yucatan, Mexico). Tourist Studies **99,** 99-116.

Baccetti, N., Panzarin, L., Cianchi, F., Puglisi, L., Basso, M., & Arcamone, E. 2008. Two new Greater Flamingo (Phoenicopterus roseus) breeding sites in Italy. Flamingo **16**, 24-27.

Baldassarre, G.A., and Arengo, F. 2000. A review of the ecology and conservation of Caribbean flamingos in Yucatan, Mexico. Waterbirds, **23**, 70-79.

Balkız, Ö., Özesmi, U., Pradel, R., Germain, C., Sıkı, M., Amat, J. A., Rendon-Martos, M., Bacetti, N. and Béchet, A. 2007. Range of the Greater Flamingo Phoenicopterus roseus metapopulation in the Mediterranean: new insights from Turkey. Journal of Ornithology **148**, 347-355.

Barba-Brioso, C., Fernández-Caliani, J. C., Miras, A., Cornejo, J. and Galán, E. 2010. Multi-source water pollution in a highly anthropized wetland system associated with the estuary of Huelva (SW Spain). Marine Pollution Bulletin **60**, 1259-1269.

Basu, N., Scheuhammer, A. M., Bursian, S. J., Elliott, J., Rouvinen-Watt, K., & Chan, H. M. 2007. Mink as a sentinel species in environmental health. *Environmental Research* **103**, 130-144.

Béchet, A. and Johnson, A. R. 2008. Anthropogenic and environmental determinants of Greater Flamingo Phoenicopterus roseus breeding numbers and productivity in the Camargue (Rhone delta, southern France). Ibis **150**, 69-79.

Béchet, A., Rendón-Martos, M., Rendón, M. A., Amat, J.A., Johnson, A.R. and Gauthier-Clerc, M. 2012. Global economy interacts with climate change to jeopardize species conservation: a case study in the Greater flamingo in the Mediterranean and West Africa. Environmental Conservation **39**, 1-3.

Beeby, A. 2001. What do sentinels stand for? Environmental Pollution **112,** 285-298.

Berthet, B. 2013. Sentinel Species. In Amiard-Triquet, C., Amiard, J.-C. and Rainbow, P.S. (eds) Ecological Biomarkers: Indicators of Ecotoxicological Effects. CRC Press, Boca Raton, Florida, USA.

Béthoux, J. P., Morin, P. and Ruiz-Pino, D. P. 2002. Temporal trends in nutrient ratios: chemical evidence of Mediterranean ecosystem changes driven by human activity. Deep Sea Research Part II: Topical Studies in Oceanography **49**, 2007-2016.

BiodiversityInOurLives. 2013. Our Beermats. Available at: biodiversityinourlives.com, Accessed 29 September 2013.

Bird Life International, 2012. BirdLife Species factsheet: *Phoenicopterus roseus*. Downloaded from http://www.birdlife.org on 20/09/2012.

Blackburn, J.K., Mitchell, M.A., Blackburn, M-C.H., Curtis, A. and Thompson, B.A. 2010. Evidence of antibiotic resistance in free-swimming, top-level marine predatory fishes. Journal of Zoo and Wildlife Medicine **41,** 7-16.

Blanco, G., Lemus, J.A., Grande, J., Gangoso, L., Grande, J.M., Donázar, J.A., Frías, O. and Hiraldo, F. 2007. Geographical variation in cloacal microflora and bacterial antibiotic resistance in a threatened avian scavenger in relation to diet and livestock farming practices. Environmental Microbiology **9,** 1738-1749.

Blondel, J. 2006. The 'design' of Mediterranean landscapes: a millennial story of humans and ecological systems during the historic period. Human Ecology **34**, 713-729.

Bonnedahl, J., Drobni, M., Gauthier-Clerc, M., Hernandez, J., Granholm, S., Kayser, Y., ... & Olsen, B. 2009. Dissemination of Escherichia coli with CTX-M type ESBL between humans and yellow-legged gulls in the south of France. PLoS One **4**, e5958.

Borghesi, F., Andreotti, A., Baccetti, N., Bianchi, N., Birke, M., Migani, F. and Dinelli, E. 2011. Flamingo feathers to monitor metal contamination of coastal wetlands: methods and initial results concerning the presence of mercury at six Mediterranean sites. Chemistry and Ecology **27**, 137-151.

Boucheker, A., Samraoui, B., Prodon, R., Amat, J.A., Rendón-Martos, M., Baccetti, N., Esquerre, F.V.I., Nissardi, S., Balkız, Ö., Germain, C., Boulkhssaim, M. and Béchet, A. 2011. Connectivity between the Algerian population of Greater Flamingo Phoenicopterus roseus and those of the Mediterranean basin. Ostrich **82**, 167-174.

Bowman, J. and Schulte-Hostedde, A.I. 2009. The mink is not a reliable sentinel species. Environmental research **109**, 937-939.

Britton, R.H. and Podlejski, V.D. 1981. Inventory and classification of the wetlands of the Camargue (France). Aquatic Botany **10**, 195-228.

Britton, R.H. and Johnson, A.R. 1987. An ecological account of a Mediterranean salina: the Salin de Giraud, Camargue (S. France). Biological Conservation **42**, 185-230.

Brown, M. G., & Balkwill, D. L. 2009. Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. Microbial Ecology **57**, 484-493.

Burrell, G.A. and Seibert, F.M. 1914. Gases found in coal mines. Government Printing Office, Washington D.C., 24pp.

Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., Jacoby, G.A., Kishony, R. *et al.* 2011. Tackling antibiotic resistance. Nature Reviews Microbiology **9,** 894-896.

Calvo, C., Gómez, M.A. and González-López, J. 1998. Fecal coliform-related bacterial and coliphage populations in five lakes of southeastern spain. Microbiological Research **153,** 283-288.

Carroll, S. P., Dawes, L., Hargreaves, M. and Goonetilleke, A. 2009. Faecal pollution source identification in an urbanising catchment using antibiotic resistance profiling, discriminant analysis and partial least squares regression. *Water research*, *43*(5), 1237-1246.

Cars, O., Hedin, A. and Heddini, A. 2011. *The global need for effective antibiotics—moving towards concerted action.* Drug Resistance Updates **14,** 68-69.

Carson, R. 1962. Silent spring. Houghton Mifflin Harcourt.

Cawley, K. 2013. Latin Dictionary and Grammar Aid. University of Notre Dame Archives. Available online at http://archives.nd.edu/latgramm.htm. Accessed 23 August 2013.

Cézilly, F., Tourenq, C. & Johnson, A. R. 1994. Variation in parental care with offspring age in the Greater Flamingo. Condor **96**, 809–812.

Cézilly, F. and Johnson, A.R. 1995. Re-mating between and within breeding seasons in the Greater Flamingo *Phoenicopterus ruber roseus.* Ibis **137,** 543-546.

Cézilly, F., Vallefont, A., Boy, V. and Johnson, A.R. 1996. Annual variation in the survival rates and breeding probabilities of the greater flamingos, *Phoenicopterus ruber roseus.* Ecology **77,** 1143-1150.

Chambers, S. A. 2008. *Birds as environmental indicators: Review of literature.* Parks Victoria. Available online at: http://parkweb.vic.gov.au/__data/assets/pdf_file/0018/314523/19_2346.pdf

Chauvelon, P. 1998. A wetland managed for agriculture as an interface between the Rhône river and the Vaccarès lagoon (Camargue, France): transfers of water and nutrients. Hydrobiologia **373**, 181-191.

Chee-Sanford, J. C., Mackie, R. I., Koike, S., Krapac, I. G., Lin, Y. F., Yannarell, A. C., ... & Aminov, R. I. 2009. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. Journal of Environmental Quality **38**, 1086-1108.

Childress, B. and Jarrett, N. 2005. Guidelines for capturing and handling wild Lesser Flamingos for research. Afring News **34,** 2-4.

Childress, B., Harper, D., Hughes, B. and Ferris, C. 2006. Adaptive benefits of differential post-fledging development patterns in the Lesser Flamingo (*Phoenoicopterus minor*). Ostrich: Journal of African Ornithology **77,** 84-89.

Clinical and Laboratory Standards Institute / NCCLS 2006. Performance standards for Antimicrobial disc diffusion tests; Approved standards. 9th ed. CLSI Document M2-M9. Wayne Pa: Clinical and Laboratory Standards Institute.

Cohen, M.L. 1992. Epidemiology of drug resistance: implications for a post-antimicrobial era. Science **257,** 1050-1055.

Cole, D., Drum, D.J.V., Stallknecht, D.E., White, D.G., Lee, M.D., Ayers, S., Sobsey, M. and Maurer, J.J. 2005. Free-living Canada Geese and antimicrobial resistance. Emerging Infectious Diseases **11,** 935-938.

Conly, J.M. and Johnston, B.L. 2005. Where are all the new antibiotics?The new antibiotic paradox. Canadian Journal of Infectious Diseases and Medical Microbiology **16,** 159-160.

Cooper, H.M. 1998. Synthesizing Research: A Guide for Literature Review. Sage Publications, Thousand Oaks, California, 205pp.

Costa, D., Poeta, P., Sáenz, Y., Vinué, L., Coelho, A. C., Matos, M., ... & Torres, C. 2008. Mechanisms of antibiotic resistance in Escherichia coli isolates recovered from wild animals. Microbial Drug Resistance **14**, 71-77.

Côté, I.M. and Reynolds, J.D. 2012. Meta-analysis at the intersection of evolutionary ecology and conservation. Evolutionary Ecology **26,** 1237-1252

Cotin, J., García-Tarrasón, M., Sanpera, C., Jover, L. and Ruiz, X. 2011. Sea, freshwater or saltpans? Foraging ecology of terns to assess mercury inputs in a wetland landscape: The Ebro Delta. Estuarine, Coastal and Shelf Science **92**, 188-194.

Curcó, A., Vidal, F., and Piccardo, J. 2009. Conservation and management of the Greater Flamingo Phoenicopterus roseus at the Ebre delta. Flamingo, Special Publication **1**, 37-43.

Daszak, P., Epstein, J.H., Kilpatrick, A.M., Aguirre, A.A., Karesh, W.B. and Cunningham, A.A. 2007. Collaborative research approaches to the role of wildlife in zoonotic disease emergence. Current Topics in Microbiology and Immunology **315,** 463-475.

de Kraker M.E.A., Davey P.G., Grundmann H., on behalf of the BURDEN study group. 2011. Mortality and Hospital Stay Associated with Resistant Staphylococcus aureus and Escherichia coli Bacteremia: Estimating the Burden of Antibiotic Resistance in Europe. PLoS Med 8(10): e1001104. doi:10.1371/journal.pmed.1001104

Deville, A.-S., Grémillet, D., Gauthier-Clerc, M., Guillemain, M., Von Houwald, F., Gardelli, B. and Béchet, A. 2013. Non-linear feeding functional responses in the Greater Flamingo (*Phoenicopterus roseus*) predict immediate negative impact of wetland degradation on this flagship species. Ecology and Evolution **3,** 1413-1425.

Diawara, Y., Arnaud, A., Mohamed, C. and Béchet, A. 2009. Monitoring of the Greater flamingo *Phoenicopterus roseus* in Mauritania. Flamingo, Special Publication 1, January 2009, 34-36.

Dolejska, M., Cizek, A. & Literak, I. 2007. *High prevalence of antimicrobial-resistant genes and integrons in* Escherichia coli *isolates from black-headed gulls in the Czech Republic.* Journal of Applied Microbiology **103,** 11-19.

Dolejska, M., Šenk, D., Čížek, A., Rybaříková, J., Sychra, O., & Literak, I. 2008. Antimicrobial resistant *Escherichia coli* isolates in cattle and house sparrows on two Czech dairy farms. *Research in veterinary science* **85**, 491-494.

ECDC/EMEA Joint Technical Report. 2009. The bacterial challenge: time to react. EMEA/576176/2009

Eraud, C., Dorie, A., Jacque, A. and Faivre, B. 2008. The crop milk: a potential new route for carotenoid-mediated parental effects. Journal of Avian Biology **39,** 247-251.

Ernoul, L., Mesléard, F., Gaubert, P. and Béchet, A. 2013. Limits to agri-environmental schemes uptake to mitigate human–wildlife conflict: lessons learned from Flamingos in the Camargue, southern France. International Journal of Agricultural Sustainability, (ahead-of-print), 1-14.

Eysenck, H. J. 1952. The effects of psychotherapy: an evaluation. Journal of Consulting Psychology **16**, 319-324.

Eysenck, H.J. 1978. An exercise in mega-silliness. American Psychologist **33**, 517.

FAO. 2007. *Wild Birds and Avian Influenza: an introduction to applied field research and disease sampling techniques.* Edited by D. Whitworth, S.H. Newman, T. Mundkur and P. Harris. FAO Animal Production and Health Manual, No. 5. Rome. Available online at www.fao.org/avianflu

Fèvre, E.M., Bronsvoort, B.M.C., Hamilton, K.A. and Cleaveland, S. 2006. *Animal movements and the spread of infectious disease.* Trends in Microbiology **14,** 125-131.

Flamingo Resource Centre, 2008. Species. Available at: www.flamingoresources.org/species.html. Accessed 20 September 2013.

Fox, D.L. 1962. Metabolic fractionation, storage and display of carotenoid pigments by flamingoes. Comparative Biochemistry and Physiology **6,** 1-24.

Frascari, F., Matteucci, G. and Giordano, P. 2002. Evaluation of a eutrophic coastal lagoon ecosystem from the study of bottom sediments. Hydrobiologia **475,** 387-401.

Galicia, E., and G.A. Baldassarre. 1997. Effects of motorized tourboats on the behavior of nonbreeding American Flamingos (Phoenicopterus ruber ruber) in Yucatan, Mexico. Conservation Biology **11**, 1159-1165.

García, C. M and Niell, F. X. 1993. Seasonal change in a saline temporary lake (Fuente de Piedra, southern Spain). Hydrobiologia **267**, 211-223.

García, C. M., Echevarría, F. and Niell, F. X. 1995. Size structure of plankton in a temporary, saline inland lake. Journal of Plankton Research **17**, 1803-1817.

García, C. M., García-Ruiz, R., Rendón, M., Niell, F. X. and Lucena, J. 1997. Hydrological cycle and interannual variability of the aquatic community in a temporary saline lake (Fuente de Piedra, Southern Spain). Hydrobiologia **345**, 131-141.

Geraci, J., Béchet, A., Cézilly, F., Ficheuz, S., Baccetti, N., Samraoui, B. and Wattier, R. 2012. Greater flamingo colonies around the Mediterranean form a single interbreeding population and share a common history. Journal of Avian Biology **43,** 341-354.

Geri, F., Amici, V. and Rocchini, D. 2010. Human activity impact on the heterogeneity of a Mediterranean landscape. Applied Geography **30**, 370-379.

Gilliver, M., Bennett, M., Begon, M., Hazel, S., and Hart, C. 1999. Antibiotic resistance found in wild rodents. Nature **401,** 233-234.

Glass, G.V. and Smith, M.L. 1978. Reply to Eysenck. American Psychologist **33**, 517-519.

GlaxoSmithKline. 2012. Bacteria battle: the struggle to find new antibiotics. Available at: <http://www.gsk.com/infocus/bacteria-battle.htm>. Accessed 1 October 2012.

González-Solís, J., Bernadí, X., & Ruiz, X. (1996). Seasonal variation of waterbird prey in the Ebro Delta rice fields. *Colonial Waterbirds*, 135-142.

Gould, I.M. 2009. Controversies in infection: infection control or antibiotic stewardship to control healthcare-acquired infection? Journal of Hospital Infection **73,** 386-391.

Gould, I.M. 2012. The antibiotic paradox. In: Antibiotic Policies. Eds Gould, I.M. and Meer, J.W.M. Springer New York. 15-25.

Grande, J. A., Borrego, J., Morales, J. A. and De la Torre, M. L. 2003. A description of how metal pollution occurs in the Tinto–Odiel rias (Huelva-Spain) through the application of cluster analysis. Marine Pollution Bulletin **46**, 475-480.

Guenther, S., Grobbel, M., Lübke-Becker, A., Goedecke, A., Friedrich, N.D., Wieler, L.H. and Ewers, C. 2010. Antimicrobial resistance profiles of *Escherichia coli* from common European wild bird species. Veterinary Microbiology **144,** 219-225.

Haas, P. M. 1989. Do regimes matter? Epistemic communities and Mediterranean pollution control. International organization **43**, 377-403.

Haggbloom, S. J., Warnick, R., Warnick, J.E., Jones, V.K., Yarbrough, G.L., Russell, T.M., Borecky, C.M., GcGahhey, R., Powell J.L. III, Beavers, J. and Monte, E. 2002. The 100 most eminent psychologists of the 20th century. Review of General Psychology, **6**, 139–152.

Halliday, J.E.B., Meredith, A.L., Knobel, D.L., Shaw, D.J., Bronsvoort, B.M.C. and Cleaveland, S. 2007. A sentinel for evaluating animals as sentinels for infectious disease surveillance. Journal of the Royal Society Interface **4,** 973-984.

Halpern, M.T., Schmier, J.K., Snyder, L.M., Asche, C., Sarocco, P.W., Lavin, B., Nieman, R. and Mandell, L.A. 2005. Meta-analysis of bacterial resistance to macrolides. Journal of Antimicrobial Chemotherapy **55,** 748-757.

Ham, Y. S., Kobori, H., Kang, J. H., Matsuzaki, T., Iino, M. and Nomura, H. 2012. Distribution of antibiotic resistance in urban watershed in Japan. *Environmental Pollution, 162*, 98-103.

Hancock, R.E.W. 2007. The end of an era? Nature Reviews Drug Discovery **6,** 28.

Heckel, D.G. Insecticide resistance after Silent Spring. Science **337,** 1612-1614.

Hess, G. 1996. Disease in metapopulation models: implications for conservation. Ecology **77,** 1617-1632.

Heuer, H., Schmitt, H., & Smalla, K. 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. Current Opinion in Microbiology **14**, 236-243.

Ibàñez, C., Canicio, A., Day, J. W. and Curcó, A. 1997. Morphologic development, relative sea level rise and sustainable management of water and sediment in the Ebre Delta, Spain. Journal of Coastal Conservation **3,** 191-202.

Ioannidis, J.P.A. 2005. Why Most Published Research Findings Are False. PLoS Med 2(8): e124. doi:10.1371/journal.pmed.0020124

IUCN. 2007. Habitat Classification Scheme. Available online at: http://www.iucnredlist.org /documents/June_2012_Guidance_Habitats_Classification_Scheme.pdf. Accessed 22 July 2012.

IUCN. 2012. Summary Statistics, The IUCN Red List of Threatened Species. Available online at: http://www.iucnredlist.org/about/summary-statistics. Accessed 5 August 2012.

Javed, S., Khan, S.B., Al Mansouri, R. and Al Hosani, E.A. 2006. Satellite tracking of Greater Flamingos *Phoenicopterus roseus* from the United Arab Emirates. Tribulus **16.1.** Available online at http://www.enhg.org/trib/v16n1/TribulusV16N1P16-17.pdf. Accessed 20 September 2013.

Johnson, A. R., F. Czilly, and V. Boy. 1993. Plumage de- velopment and maturation in the Greater Flamingo Phoenicopterus ruber roseus. Ardea 81: 25-34.

Johnson, A.R. 1997. Long-term studies and conservation of Greater Flamingos in the Camaruge and Mediterranean. Colonial Waterbirds **20,** 306-315.

Johnson, A. 2000. An overview of the Greater Flamingo ringing program in the Camargue (Southern France) and some aspects of the species' breeding biolgy studied using marked individuals. Waterbirds: The International Journal of Waterbird Biology **23,** 2-8.

Johnson, A. and Arengo, F. 2003. Annual Report 2001. IUCN-SSC/Wetlands International Flaminog Specialist Group Newsletter No. 11, December 2003. Station Biologique, Tour du Valat, Arles, France.

Johnson, A. and Cézilly, A. 2007. The Greater Flamingo. Bloomsbury Publishing.

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. Nature **451**, 990-993.

Keeling, M.J., Danon, L., Vernon, M.C. and House, T.A. Individual identity and movement networks for disease metapopulations. Proceedings of the National Academy of Sciences of the United States of America **107,** 8866-6670.

Kemper, N. 2008. Veterinary antibiotics in the aquatic and terrestrial environment. Ecological Indicators **8**, 1-13.

King, C. E. and van Weeren, L. 2005. Captive flamingo management on a European level. Rotterdam Zoo, Rotterdam. Available online at: http://www.flamingoresources.org /docs/literature/eaza_king_2005.pdf. Accessed 20 September 2013.

Landau, K. I., & van Leeuwen, W. J. 2012. Fine scale spatial urban land cover factors associated with adult mosquito abundance and risk in Tucson, Arizona. Journal of Vector Ecology **37**, 407-418.

Landres, P.B., Verner, J. and Thomas, J.W. 1988. Ecological uses of vertebrate indicator species: a critique. Conservation Biology **2,** 316-328.

Laroche, E., Pawlak, B., Berthe, T., Skurnik, D. and Petit, F. 2009. Occurrence of antibiotic resistance and class 1, 2 and 3 integrons in Escherichia coli isolated from a densely populated estuary (Seine, France). FEMS microbiology ecology, **68**, 118-130.

Larsen, P.O. and von Ins, M. 2010. The rate of growth in scientific publication and the decline in coverage provided by Science Citation Index. Scientometrics **84,** 575-603.

Lee, R., Arengo, F. & Bechet, A. (eds.). 2011. Flamingo, Bulletin of the IUCN-SSC/Wetlands International Flamingo Specialist Group, No. 18. Wildfowl & Wetlands Trust, Slimbridge, UK.

Levy, S. B., & Marshall, B. 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nature medicine **10**, S122-S129.

Liu, D., Xiao, W., Lu, J. and Zhang, Z. Review of research progress of infectious diseases in wild birds. Shengtai Xuebao/Acta Ecologica Sinica **31,** 6959-6966.

Lloyd, D.H. 2007. Reservoirs of antimicrobial resistance in pet animals. Clinical Infectious Diseases **45,** S148-S152.

Luborsky, L. 1954. A note on Eysenck's article "The effects of psychotherapy: an evaluation." British Journal of Psychology **45**, 129-131.

MacNally, R. 2000. Regression and model-building in conservation biology, biogeography and ecology: the distinction between – and reconciliation of – 'predictive' and 'explanatory' models. Biodiversity and Conservation **9,** 655-671.

Mañosa, S., Mateo, R. and Guitart, R. 2001. A review of the effects of agricultural and industrial contamination on the Ebro delta biota and wildlife. Environmental Monitoring and Assessment **71**, 187-205.

Martin, G.R., Jarrett, N., Tovey, P. and White, C.R. 2005. Visual fields in flamingos: chick-feeding versus filter-feeding. *Naturwissenschaften* **92**: 351–354.

Martinez, J.L. 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environmental Pollution **157,** 2893-2902.

Martos M.R. and Johnson A.R. 1996. Management of nesting sites for greater flamingos. Colonial Waterbirds,167-183

Mascarelli, A. 2012. Environment: toxic effects. Nature **483,** 363-365.

Mateo, R., Martínez-Vilalta, A. and Guitart, R. 1997. Lead shot pellets in the Ebro Delta, Spain: Densities in sediments and prevalence of exposure in waterfowl. Environmental Pollution **96**, 335-341.

Mateo, R., Gámez, A. and Guitart, R. 1998. Organochlorine residues in hunted wild mallards in the Ebro Delta, Spain. *Bulletin of environmental contamination and toxicology*, *60*(1), 134-141.

McEwen, S.A. 2006. Antibiotic use in animal agriculture: what have we learned and where are we going? Animal Biotechnology **17,** 239-250.

McGraw, K.J. 1996. Mechanics of carotenoid-based coloration. In: Hill, G.E. and McGraw, K.J. (eds). Bird Coloration: Function and Evolution. Harvard University Press, Cambridge, USA>

Miller, R.V., Gammon, K. and Day, M.J. 2009. Antibiotic resistance among bacteria isolated from seawater and penguin fecal samples collected near Palmer Station, Antarctica. Canadian Journal of Microbiology **55,** 37-45.

Moher, D., Cook, D.J., Eastwood, S., Olkin, I., Rennie, D., Stroup, D.F. *et al.* 1999. Improving the quality of reports of meta-analyses of randomized controlled trials: the QUOROM statement. The Lancet **354,** 1896-1900.

Munari, C., Rossi, R., & Mistri, M. 2005. Temporal trends in macrobenthos community structure and redundancy in a shallow coastal lagoon (Valli di Comacchio, Northern Adriatic Sea). Hydrobiologia **550**, 95-104.

Munari, C. 2011. Effects of the 2003 European heatwave on the benthic community of a severe transitional ecosystem (Comacchio Saltworks, Italy). Marine pollution bulletin **62**, 2761-2770.

Munari, C. and Mistri, M. 2012. Ecological status assessment and response of benthic communities to environmental variability: The Valli di Comacchio (Italy) as a study case. Marine environmental research 2012.

Nager, R. G., Johnson, A. R., Boy, V., Rendon-Martos, M., Calderon, J. and Cézilly, F. 1996. Temporal and spatial variation in dispersal in the greater flamingo (Phoenicopterus ruber roseus). Oecologia **107**, 204-211.

Nager, R.G., Hafner, H., Johnson, A.R. and Cézilly, F. 2010. Environmental impacts on wetland birds: long-term monitoring programmes in the Camargue, France. Ardea **98,** 309-318.

National Research Council Commission on Life Sciences. 1991. *Animals as Sentinels of Environmental Health Hazards.* National Academy Press, Washington D.C. 160pp.

O'Brien D.J., Kaneene J.B. and Poppenga R.H.1993. The use of mammals as sentinels for human exposure to toxic contaminants in the environment. *Environmental Health Perspectives* **99**, 351–368.

Ochoa, V., Riva, C., Faria, M., de Alda, M. L., Barceló, D., Fernandez Tejedor, M., *et al* and Barata, C. 2012. Are pesticide residues associated to rice production affecting oyster production in Delta del Ebro, NE Spain?. Science of the Total Environment **437**, 209-218.

Ochoa, V., Barata, C., & Riva, M. C. 2013. Heavy metal content in oysters (Crassostrea gigas) cultured in the Ebro Delta in Catalonia, Spain. Environmental Monitoring and Assessment, 1-10.

Olıas, M., Nieto, J. M., Sarmiento, A. M., Cerón, J. C., and Cánovas, C. R. 2004. Seasonal water quality variations in a river affected by acid mine drainage: the Odiel River (South West Spain). Science of the total environment **333**, 267-281.

Oro, D., & Pradel, R. 1999. Recruitment of Audouin's gull to the Ebro Delta colony at metapopulation level in the western Mediterranean. Marine Ecology Progress Series **180**, 267-273.

Palmer, J.A., Suggate, J., Bajd, B., Hart K.P., P., Ho, R.K.P., Ofwono-Orecho, J.K.W., Peries, M., Robottom, I., Tsaliki, E. and Van Staden, C. 1998. An Overview of Significant Influences and Formative Experiences on the Development of Adults' Environmental Awareness in Nine Countries. Environmental Education Research, **4**, 445-464.

Pappas, G., Kiriaze, I.J. and Falagas, M.E. 2008. Insights into infectious disease in the era of Hippocrates. International Journal of Infectious Diseases **12,** 347-350.

Paracuellos, M., Castro, H., Nevado, J.C., Oña, J.A., Matamala, J.J., García, G. and Salas, G. 2002. Repercussions of the abandonment of Mediterranean saltpans on waterbird communities. Waterbirds **25,** 492-498.

Patz, J.A., Daszak, P., Tabor, G.M., Aguirre, A.A., Pearl, M., Epstein, J., Wolfe, N.D., Kilpatrick, A.M., Foufopoulos, J., Molyenux, D. and Bradley, D.J. 2004. Unhealthy landscapes: policy recommendations on land use change and infectious disease emergence. Environmental Health Perspectives **112,** 1092-1098.

Phillips, D.J.H. 1970. The use of biological indicator organisms to monitor trace metal pollution in marine and estuarine environments – a review. Environmental Pollution **13,** 281-317.

Piddock, L.J.V. 2011. The crisis of no new antibiotics—what is the way forward? The Lancet Infectious Diseases **12,** 249-253.

Pullin, A.S. and Knight, T.M. 2001. Effectiveness in conservation practice: pointers from medicine and public health. Conservation Biology **15,** 50-54.

Rabinowitz, P.M., Gordon, Z., Holmes, R., Taylor, B., Wilcox, M., Chudnov, D., Nadkarni, P. and Dein, F.J. 2005. Animals as sentinels of human environmental health hazards: an evidence-based analysis. EcoHealth **2,** 26-37.

Rabinowitz, P., Schotch, M. and Conti, L. 2009. Humans and animal sentinels for shared health risks. Veterinaria Italiana **45,** 23-24.

Rainbow, P.S. 1995. Biomonitoring of heavy metal availability in the marine environment. Marine Pollution Bulletin **31,** 183-192.

Rendon-Martos, M. and Johnson, A.R. 1996. Management of nesting sites for Greater Flamingos. Colonial Waterbirds **19,** 167-183.

Rendón-Martos, M., Garrido, A., Chávez, J., Méndez, J.M. and Sayago, J.M. 2008. Odiel marshes: a new breeding site for Greater Flamingos (*Phoenicopterus roseus*) in Spain. Flamingo **16,** 23-24.

Rendón-Martos, M., Garrido, A., Rendón, M. Á. and Ramírez, J. M. 2009. Greater Flamingo Phoenicopterus roseus monitoring and conservation at Fuente de Piedra Lake. Flamingo **1**, 1.

Rodríguez-Pérez, H., Green, A.J. and Figuerola, J. 2007. Effects of Greater flamingo *Phoenicopterus ruber* on macrophytes, chironomids and turbidty in natural marshes in Doñana, SW Spain. Fundamental and pplied Limnology **170,** 167-175.

Rose, S. 2010. Hans Eysenck' controversial career. The Lancet **376,** 407-408.

Rosenblatt-Farrell, N. 2009. The landscape of antibiotic resistance. Environmental health Perspectives **117,** A244-A250.

Rosenzweig, S. A transvaluation of psychotherapy: a reply to Hans Eysenck. The Journal of Abnormal and Social Psychology **49**, 298-304.

Sainz, A., Grande, J. A. and De la Torre, M. L. 2004. Characterisation of heavy metal discharge into the Ria of Huelva. Environment International **30**, 557-566.

Saldaña, J. 2010. Modelling the spread of infectious diseases in complex metapopulations. Mathematical Modelling of Natural Phenomena **5,** 22-37.

Samraoui B, Boulkhssaïm M, Bouzid A, Bensaci E, Germain C, Béchet A, Samraoui F. 2009. Current research and conservation of the Greater Flamingo *Phoenicopterus roseus* in Algeria. In: Béchet A, Rendón-Martos M, Amat JA, Baccetti N and Childress B (eds), Flamingo, Bulletin of the IUCN-SSC/Wetlands International Flamingo Specialist Group, Special Publication 1: Proceedings of the IVth international workshop on the Greater Flamingo in the Mediterranean region and northwest Africa, Antequera, Spain, 5–6 November 2007. Slimbridge: Wildfowl and Wetlands Trust. pp 20–25.

Sánchez, M.I., Green, A.J. and Castellanos, E.M. 2006. Spatial and temporal fluctuations in presence and use of chironomid prey by shorebirds in the Odiel saltpans, south–west Spain. Hydrobiologia **567** 329–340

Sánchez, M.I., Varo, N., Matesanz, C., Ramo, C., Amat, J.A., Green, A.J. 2013. Cestodes change the isotopic signature of brine shrimp, Artemia, hosts: Implications for aquatic food webs. International Journal for Parasitology **43**, 73-80, http://dx.doi.org/10.1016/j.ijpara.2012.11.003.

Sanz-Aguilar, A., Béchet, A., Germain, C., Johnson, A.R. and Pradel, R. 2012. To leave or not to leave: survival trade-offs between different migratory strategies in the greater flamingo. Journal of American Ecology **81,** 1171-1182.

Sayah, R. S., Kaneene, J. B., Johnson, Y., and Miller, R. 2005. Patterns of antimicrobial resistance observed in Escherichia coli isolates obtained from domestic-and wild-animal fecal samples, human septage, and surface water. Applied and Environmental Microbiology **71**, 1394-1404.

Schaefer, A.M., Goldstein, J.D., Reif, J.S., Fair, P.A. and Bossart, G.D. 2009. Antibiotic-resistant organisms cultured from Atlantic bottlenose dolphins (*Tursiops truncates*) inhabiting estuarine waters of Charleston, SC and Indian River Lagoon, FL. Ecohealth **6,** 33-41.

Schmitz, R.A., Aguirre, A.A, Cook, R.S., and Baldassarre, G.A. 1990. Lead poisoning of Caribbean Flamingos in Yucatan, Mexico. Wildlife Society Bulletin **18**, 399-404.

Schultz, K.F., Altman, D.G., Moher, D. 2010. *CONSORT 2010 statement: updated guidelines for reporting parallel group randomised trials.* BMJ **340,** 698-702.

Schwartz, T., Kohnen, W., Jansen, B., & Obst, U. 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiology Ecology **43**, 325-335.

Séveno, N. A., Kallifidas, D., Smalla, K.; van Elsas, J. D., Collard, J.-M.; Karagouni, A. D. and Wellington, E. M. H. 2002. Occurrence and reservoirs of antibiotic resistance genes in the environment. Reviews in Medical Microbiology **13,** 15-27.

Shead, S. 2012. Want to persuade flamingos to mate? Play them Barry White and Marvin Gaye. MailOnline, available at http://www.dailymail.co.uk/sciencetech/article-2186523/Flamingos-mate-Barry-White-Marvin-Gaye-CDs.html. Accessed 30 September 2013.

Singer, R.S., Ward, M.P and Maldonado, G. 2006. *Can landscape ecology untange the complexity* Smith, P., Hiney, M.P. & Samuelson, O.B. 1994. *Bacterial resistance to antimicrobial agents used in fish farming: a critical evaluation of method and meaning.* Annual Review of Fish Diseases **4,** 273-313.

Sjölund, M., Bonnedahl, J., Hernandez, J., Bengtsson, S., Cederbrant, G., Pinhassi, J., Kahlmeter, G. and Olsen, B. 2008. Dissemination of multidrug-resistant bacteria into the Arctic. International Polar Year **14,** 70-72.

Smith, M.L. and Glass, G.V. 1977. Meta-analysis of psychotherapy outcome studies. American Psychologist **32,** 752-760.

Sorokin, Y., Sorokin, P. and Gnes, A. 1996. Structure and functioning of the anthropogenically transformed Comacchio lagoonal ecosystem(Ferrara, Italy). Marine ecology progress series. Oldendorf **133**, 57-71

Stewart, G. 2009. Meta-analysis in applied ecology. Biology Letters **6,** 78-81.

Stroup, D.F., Berlin, J.A., Morton, S.C., Olkin, I., Williamson, G.D., Rennie, D., Moher, D., Becker, B.J., Sipe, T.A., and Thacker, S.B. 2000. Meta-analysis of observational studies in epidemiology – A proposal for reporting. Journal of the American Medical Association **283,** 2008-2012.

Tamisier, A. 1991. The Camargue: in search of a new equilibrium between man and nature. Landscape and Urban Planning **20**, 263-267.

Taylor, L.H., Latham, S.M. and Woolhouse, M.E. 2001. *Risk factors for human disease emergence.* Philosophical Transactions of the Royal Society B **29,** 983-989.

Thaller, M. C., Migliore, L., Marquez, C., Tapia, W., Cedeño, V., Rossolini, G. M., & Gentile, G. 2010. Tracking acquired antibiotic resistance in commensal bacteria of Galapagos land iguanas: no man, no resistance. PloS One, **5**(2), e8989.

Toth, A., Damjanova, I., Puskás, E., Jánvári, L., Farkas, M., Dobák, A., ... & Pászti, J. 2010. Emergence of a colistin-resistant KPC-2-producing Klebsiella pneumoniae ST258 clone in Hungary. European Journal of Clinical Microbiology & Infectious Diseases **29**, 765-769.

Tour du Valat. 2013. Greater Flamingos. Available at: http://en.tourduvalat.org/dossiers/flamants_roses. Accessed 29 September 2013.

Tourenq C., Johnson, A.R. and Gallo, A. 1995. Adult aggressiveness and crèching behavior in the greater flamingo, *Phoenicopterus ruber roseus.* Colonial Waterbird **18**, 216–221

Tourenq, C., Aulagnier, S., Mesléard, F., Durieux, L., Johnson, A., Gonzalez, G., and Lek, S. 1999. Use of artificial neural networks for predicting rice crop damage by greater flamingos in the Camargue, France. Ecological Modelling, **120**, 349-358.

Tourenq, C., Aulagnier, S., Durieux, L., Lek, S., Mesléard, F., Johnson, A. and Martin, J.-L. 2001. Identifying rice fields at risk from damage by the greater flamingo. Journal of Applied Ecology **38,** 170-179.

van der Schalie, W.H., Gardner, H.S. Jr., Bantle, J.A., De Rosa, C.T., Finch, R.A., Reif, J.S., Reuter, R.H., Backer, L.C., Burger, J., Folmar, L.C. and Stokes, W.S. 1999. Animals as sentinels of human health hazards of environmental chemicals. Environmental Health Perspectives **107,** 309-315.

Vassilev, Z. P., Robson, M. G., & Kaplan, M. B. 1999. Health-based environmental indicators: approaches, potential and limitations. Journal of Environmental Medicine **1**(4), 201-206.

Waldvogel, F.A. 2004. *Infectious diseases in the 21st century: old challenges and new opportunities.* International Journal of Infectious Disease **8,** 5-12.

Wampold, B.E. 2001. The Great Psychotherapy Debate: Models, Methods, and Findings. Lawrence Erlbaum Associates, Inc. Mahwah, New Jersey.

Watkinson, A. J., Murby, E. J., Kolpin, D. W. and Costanzo, S. D. 2009. The occurrence of antibiotics in an urban watershed: From wastewater to drinking water. Science of the total environment **407**, 2711-2723.

Wilson, D.B. and Lipsey, M.W. Practical Meta-Analysis. Applied Social Research Methods Series **49,** 251pp.

World Health Organization. 2000. Overcoming antimicrobial resistance. World Health Report on Infectious Diseases. Available online at: http://www.who.int/infectious-disease-report/2000/other_versions/index-rpt2000_text.html. Accessed 8 June 2012.

World Health Organisation. 2007. Guidance on regulations for the transport of infectious substances 2007-2008. Available online at: http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2cc.pdf. Accessed 29 September 2013.

World Health Organisation 2011. List of critically important antimicrobials for human medicine. Available online at: http://www.who.int/foodborne_disease/resistance/cia/en/. Accessed 29 September 2013.

Wright, G.D. 2010. Antibiotic resistance in the environment: a link to the clinic? Current Opinion in Microbiology **13**, 589-594, doi: 10.1016/j.mib.2010.08.005.

Zacharias, M.A. and Roff, J.C. 2001. Use of focal species in marine conservation and management: a review and critique. Aquatic Conservation: marine and Freshwater Ecosystems **11,** 59-76.

Zalidis, G., Stamatiadis, S., Takavakoglou, V., Eskridge, K. and Misopolinos, N. 2002. Impacts of agricultural practices on soil and water quality in the Mediterranean region and proposed assessment methodology. Agriculture, Ecosystems & Environment **88**, 137-146.

Zweers, G., de Jong, F. and Berkhoudt, H. 1995. Filter feeding in flamingos (*Phoenicopterus ruber*). The Condor **97,** 297-324.

5 MIC analysis and genotypic characterization of antibiotic resistant bacteria isolated from wild Greater Flamingos

5.1 Introduction

5.1.1 Microbial flora of wild birds

The microbial flora of wild birds is traditionally considered with reference to the external (e.g. those found on feathers) and internal (e.g. those found in the gastronintestinal tract) bacterial communities that comprise it (Shawkey *et al.*, 2005; van Dongen *et al.*, 2013). Here, specific consideration is given to the internal microbial flora of wild birds, though this is an area for which comprehensive information is sparse. There exists significantly more information on bacteria in poultry (e.g. broiler chickens) than in wild birds (Lombardo *et al.*, 1996; Benskin *et al.*, 2009; Santos *et al.*, 2012; van Dongen *et al.*, 2013). Much of the information that does exist on bacteria in wild birds comes from targeted investigations of sick or diseased birds; less information exists on bacteria in healthy individuals and/or populations and even within this, much of the research has focused on pathogenic bacteria, which can be present in a bird (at an individual or population level) with no adverse effects, despite posing a risk to human health (Brittingham *et al.*, 1988; Dobbin *et al.*, 2005; Benskin *et al.*, 2009; Santos *et al.*, 2012; van Dongen *et al.*, 2013). Technological advances, including the use of molecular tools for DNA analysis, have made it easier to rapidly identify numerous bacterial species and as a result, several recent efforts have been undertaken to identify the total microbial flora with reference to both pathogenic and non-pathogenic species (Kohl, 2012; Santos *et al.*, 2012; van Dongen *et al.*, 2013). Such research has provided interesting insights into the microbial flora of wild birds, including details on the factors that determine what bacteria are found, as well as details on the diversity of specific bacterial species that comprise it.

5.1.2 Factors that determine the microbial flora of wild birds

A variety of factors determine the bacteria that are found in wild birds, though until recently very little research had focused on investigating and describing exact processes by which wild birds acquire bacteria (Lucas and Heeb, 2005). A review of the relevant research indicates that the factors shaping bacterial assemblages of wild birds can be broadly classed with reference to those which are intrinsic to the bird itself (i.e. physiology and life history) and those which are extrinsic (i.e. the environment). Intrinsic factors such as internal pH, temperature, and anatomy serve to determine what bacteria are able to survive inside of a bird, while elements of its life history including its diet, feeding ecology, habitat and migratory patterns also play a role in affecting the bacteria a bird can be exposed to and therefore able to acquire (Lombardo, 1996; Soler *et al.*, 2010; Kohl, 2012; Santos *et al.*, 2012). Extrinsic factors – i.e. the environment (or environments, in the case of migratory or otherwise mobile birds) in which a bird lives – dictate what bacteria a bird is exposed to. Differences in bacteria found in aquatic versus terrestrial environments and urban versus rural environments affect

the bacteria found in a given environment, and factors including salinity and temperature can also affect it (Rollin and Baylet, 1983). Given that birds acquire certain components of their microbial flora from the environments in which they live, human activity (e.g. land use) has also been suggested to affect the internal microbial flora of wild birds (Klomp *et al.*, 2008; Allen *et al.*, 2010). There exists significant overlap between the intrinsic and extrinsic factors, given that a bird's interaction with its environment determines which bacteria it is exposed to, and the intrinsic factors dictate if the bird will acquire the bacteria and whether or not the bacteria will survive. Given the numerous factors that can affect the microbial flora of wild birds, it is unsurprising that variation exists on a number of scales, both inter- and intra- species. Within a species found across a large geographic range, variation is likely given the different environments that a bird encounters. Variation even exists within an individual animal; recent evidence suggests that the bacterial community within a bird changes a considerable amount over time as the bird ages (van Dongen, 2013).

Unlike mammals, which inherit much of their microbial flora via the birthing process, birds, which hatch from sterile eggs, can acquire the specific bacteria that comprise their microbial flora via a variety of pathways, and these mechanisms differ with reference to the age of the bird (Kohl, 2012). Some research has suggested the role of genetics in determining which bacteria can be acquired (Lombardo, 1996; Banks *et al.*, 2009). After hatching, bird chicks acquire bacteria from environmental sources including, early on, those transferred from the adult to the chick during feeding both the food itself as well as the adult's saliva (Rollin and Baylet, 1983; Mills, 1999; van Dongen *et al.*, 2013). Interestingly, however, the number of shared species between chicks and adults is relatively low, and the bacterial assemblages found in chicks may be more diverse than those present in adults (van Dongen *et al.*, 2013). It has been found that within several days, environmental influences within the nest are the strongest determinant of microbial communities, during which the chick also begins to acquire bacteria either from other chicks in the nest, ingesting materials that comprise the nest, and/or consumption of faeces (Singleton and Harper, 1998; Mills, 1999). As the chick matures and is able to feed itself, it acquires bacteria more directly from its environment via its own feeding, and because the mechanism of feeding has changed, the bacteria it acquires differ from those it acquired as a chick (Benskin *et* al., 2009; van Dongen, 2013). The diversity of food a bird species consumes (i.e. herbivorous versus omnivorous diets) affects the composition of bacterial communities (Lombardo *et al.*, 1996; Berger *et al.*, 2003). Beyond the changes in its diet as it ages and the amount, diversity, and type of food it consumes, other suggestions for the variety of microbial communities that exists within (and between) species of adult birds include increased mobility (i.e. the bird leaving the nest), changes in internal chemistry and temperature, and changes in the immune system (van Dongen *et al.*, 2013). Another mechanism by which birds acquire bacteria includes the sexual transmission of bacteria

(White *et al.*, 2010). In a hypothesis described in 1999 by Lombardo *et al.*, it was suggested that it was advantageous for female birds to be promiscuous (i.e. those birds that exhibit more frequent copulation, with either the same or multiple partners) because the potential for acquiring beneficial microbes outweighs the likelihood of acquiring harmful ones. However, more recent evidence has suggested both that such a strategy is not always effective and that any changes to the diversity of microbial flora after copulation may only persist in the short-term (Hupton *et al.*, 2003; White *et al.*, 2010).

5.1.3 Diversity of species of bacteria found in wild birds

The microbial flora of wild birds is characterised by the occurrence of both Gram-positive and Gram-negative organisms, which serve a range of critical functions (Kohl, 2012). Given the diversity of mechanisms by which they acquire bacteria, it is unsurprising that there is an incredible diversity of species found in wild birds. For example, in their analysis of 240 bacterial isolates taken from samples of shorebirds in Portugal, Santos *et al.* (2012) identified 42 genera of bacteria belonging to four phyla (*Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Deinococci-Thermus*). They found that amongst the samples they analysed, several genera dominated within their respective phyla: *Corynebacterium* within the phylum *Actinobacteria*, *Enterococcus* within the *Firmicutes* phylum, and *Escherichia* within the *Proteobacteria* phylum. These four phyla were also found to be common amongst isolates identified in similar research as described in a review paper by Kohl (2012). Of the three investigations that focused on wild birds reviewed in his study, all found these four phyla; additional phyla identified included *Tenericutes*, *Spirochaetes*, *Fusobacteria*, *Planctomycetes*, *Cyanobacteria*, and the candidate phylum *TM7*. Even more recent empirical work by van Dongen (2013) confirmed the results of these previous experimental and review works; in characterising cloacal bacteria from kittiwakes (*Rissa tridactyla*), the most commonly represented phyla were *Firmicutes*, *Actinobacteria*, and *Proteobacteria*. Although most of the bacteria with these phyla are normally non-pathogenic, amongst these commonly identified phyla are several that are important to consider within the context of human health, and as stated previously, it is these potentially pathogenic organisms that are often the focus of research (Oliveira *et al.*, Dobbin *et al.*, 2005; Kohl, 2012). Commonly investigated pathogenic organisms include *E. coli*, *Pseudomonas* spp., *Salmonella*, and *Campylobacter* (Brittingham *et al.*, 1988; Abulreesh *et al.*, 2007).

The mobile nature of most bird species has led to the suggestion that they have the potential to serve as vectors in transferring pathogenic bacteria, including those described above (Sellin, 2000; Hubálek, 2004). Such transfer has been widely reported, and can occur either through daily movement (e.g. foraging within a small geographic range) or through much larger-scale migratory movements (Palmgren *et al.*, 1997; Hubálek, 2004; Blanco *et al.*, 2007;

Jordain *et al.*, 2007; Boyce *et al.*, 2009; Santos *et al.*, 2012). This means that pathogenic organisms can be transferred from a site where they are present to a site where they have not yet been established. One grouping of pathogenic organisms that have received a particularly large amount of attention in recent years is antibiotic resistant bacteria. As with other pathogenic bacteria described above, these pose a threat to human health and have the potential to be transferred across borders. As a result, antibiotic resistant bacteria have been investigated in a variety of bird species (e.g. Literak 2007, 2010; see Chapter 2 for critical review and meta-analysis). Many of these efforts have investigated antibiotic resistance with reference to established standards. Several international organisations exist which set guidelines for conducting these antibiotic susceptibility analyses, including details on interpreting the results – making use of clinical reference points ('breakpoints') as a standard to which results can be compared with which a classification of 'susceptible' or 'resistant' can be assigned. Two of the more widely used protocols have been established in the United States by the Clinical Laboratory Standards Institute (CLSI) and in Europe by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Both the CLSI and EUCAST frequently update and publish revisions to their methodological guidelines and interpretive criteria, to encompass newly emerging data as well as evolving paradigms on the development, function, and significance of resistance in general in addition to changes in specific methodologies and protocols (Kahlmeter *et al.*, 2003; van der Bij *et al.*, 2012; Hombach *et al.*, 2013)

Currently, there is little knowledge about the microbial flora of the Greater Flamingo as it relates to bacterial species that demonstrate antibiotic resistance. As a highly mobile avian species that frequently moves within a network of sites in its range, the Greater Flamingo is important to consider as a reservoir of antibiotic resistant bacteria (Balkiz *et al.*, 2009; Geraci *et al.*, 2012). Many of the Greater Flamingo's breeding and non-breeding sites in the Mediterranean region are located in the vicinity of human activity, meaning that the potential for acquiring and transferring antibiotic resistant bacteria, antibiotics, and antibiotic resistant genes (either directly or indirectly) is likely to be high (e.g. Rendon *et al.* 2008; Bechet *et al.*, 2009; Borghesi *et al.*, 2011). It is therefore critical to understand the diversity of bacterial species present in the Greater Flamingo and assess the extent of resistance of these microbes to a range of clinically important antibiotics.

5.2 Aims

The aim of this study was to further investigate antibiotic resistance that was found in preliminary screening of bacterial samples taken from Greater Flamingo chicks at five of its breeding sites in the Mediterranean region (Chapter 4). The specific objectives of this investigation were to:

1. Identify bacteria isolates from the Greater Flamingo that demonstrated resistance to antibiotics in preliminary screening
2. Quantify antibiotic resistance with reference to established standards

Together, the two components of this research will serve to provide insights into the extent to which the Greater Flamingo serves as a reservoir for antibiotic resistant bacteria in the Mediterranean region. In extending previous research (i.e. the preliminary screening of bacterial samples), the methodologies used here will allow for an assessment of the diversity of bacteria species that demonstrate resistance to antibiotics and consideration of the potential threat to human health. By considering the results of this investigation within the context of other research conducted on antibiotic resistant bacteria in wild birds, a more comprehensive understanding of the variety, extent, and potential implications for humans of antibiotic resistant bacteria commonly found in wild birds can be achieved.

5.3 Methods

Ethical approval for sample collection was requested and subsequently granted by the Trinity College Dublin Bioresources Unit. The importation of samples into Ireland was authorised by the Product Import Section, Food Safety Liaison Division, in the Department of Agriculture, Fisheries and Food (DAFF), who granted an importation license, '*Importation of Carcases and Animal Products (Prohibition) Orders, 1967 and 1992, Poultry, Poultry Carcases, Poultry Eggs and Poultry Products Restriction on Importation Order, 1971*'. Full details of these approvals/licenses are provided in Chapter 4 along with details on sample collection, an overview of which is given in the following section.

5.3.1 Sample collection & bacteria isolation

Sample collection

To obtain bacterial samples, cloacal swabs were taken from wild Greater Flamingos at five sites in Southern Europe using sterile culture swabs in Amies Transport Medium Without Charcoal (Copan Innovation Ltd., supplied by Sarstedt Ireland). Bacterial samples were recovered from the following sites: Rio Odiel, Spain; Fuente de Piedre, Spain; Ebre Delta, Spain; Camargue, France; and Comacchio, Italy (as described in Section 3 of Chapter 4). Fieldwork for this investigation was conducted in July and August 2010 as part of annual flamingo ringing operations at each of the sites, which are timed to follow the birth of flamingo chicks. The ringing operations are typically organised by local research institutions, and involve the capture, leg-banding, measuring, and sampling (including bacteria, blood, and feather samples) of hundreds of Greater Flamingo chicks, the results of which feed into a variety of research projects.

Bacterial samples for this investigation were recovered from birds that would only have been exposed to a single site due to their inability to fly (flamingo chicks were between eight and ten weeks old). In addition, the capture and subsequent processing of flamingo chicks was logistically easier than it would have been with adults. Seventy-five samples were recovered from flamingos at each site (one sample was taken per bird), with the exception of Rio Odiel, at which 50 samples were collected. Samples were refrigerated at 4°C within 3-4 hours at most, and were shipped within 24 hours to the Microbiology Research Unit of the Dublin Dental University Hospital, Trinity College, Dublin, Ireland, where they were refrigerated at 4°C until analysis.

Bacteria isolation and confirmation of resistance

In the preliminary screening of 20 samples per site to determine the antibiotic resistance of total bacteria to a panel of eight antibiotics (using Kirby-Bauer disc diffusion as described in

Chapter 4), colonies were identified as resistant to an antibacterial agent if growth inside a zone of inhibition was observed. For the purposes of this investigation, single colonies (where possible) representative of a bacterium of interest were selected and streaked out onto Mueller-Hinton Agar (MHA) ready prepared medium 90mm plates using a sterile inoculating loop to obtain single colonies (Oxoid, supplied by Fisher Scientific, Ireland). Plates were incubated at 30°C for 24 hours. Resistance (as determined in initial screening in Chapter 3) was then confirmed with antibiotic discs by streaking out a lawn from a single colony on a half-plate of MHA, and applying the relevant antibiotic disc. Resistance was evaluated after incubating the plates for 24 hours at 30°C by assessing whether or not bacterial growth occurred within the zone of inhibition and/or adjacent to the antibiotic disc.

5.3.2 MIC determination and genotypic identification of isolates

5.3.2.1 MIC determination

Minimum Inhibitory Concentration (MIC) is a technique that allows for the determination of an antibiotic's ability to prevent the growth of bacteria (Wiegand *et al.*, 2008). It was developed as a way to provide a standardised method at a time in which there was little consistency in undertaking and reporting antibiotic resistance testing, which at the time was mostly done via antibiotic disc diffusion testing (Piddock, 1990). While antibiotic disc diffusion testing provided (and still provides) a relatively simple technique for describing a bacterial isolate as 'susceptible', 'intermediate', or 'resistant' to a given antibiotic, MIC offers the benefit of quantifying resistance by testing the response of the isolate to a range of concentrations of the antibiotic. By assigning a numerical value to the lowest concentration at which an antibiotic is effective at preventing bacterial growth – the minimum inhibitory concentration, or MIC, – the technique allows for quantification of the level of resistance (Bala *et al.*, 2005). As a result, although antibiotic disc diffusion continues to be used to detect the presence/absence of resistance, MIC analysis has become the preferred methodology when investigating or considering the implications of antibiotic resistance within a clinical context, where the concentrations of an antibiotic are of critical importance (Jorgensen and Ferraro, 2009).

In this investigation, MIC analysis was undertaken with reference to the guidelines provided by the National MRSA Reference Laboratory at St James Hospital, Dublin for MIC determination by broth microdilution. The methodology involves the use of 96 well microtitre trays, each of which holds eleven concentrations of a given antibiotic (concentrations decrease from left to right across a row) in addition to a control well. Each tray has eight rows, meaning that up to eight different bacterial samples can be tested per tray. The procedure involves several stages: 1) preparation of microtitre trays, 2) preparation of standardised inoculum, 3)

inoculation of microtitre tray with standardised inoculum, 4) incubation and 5) determination of MIC. The methodology is summarised as follows:

Preparation of microtitre trays

Antimicrobial stock solutions were prepared for each of the eight antibiotics. The various concentrations of antibiotic were prepared in bijou using antibiotic stock solutions to prepare doubling dilutions of the following concentrations: 0.125µg/ml, 0.25µg/ml, 0.5µg/ml, 1µg/ml, 2µg/ml, 4µg/ml, 8µg/ml, 16µg/ml, 32µg/ml, 64µg/ml, and 128µg/ml. 50µl volumes of each of the antibiotic dilutions were dispensed into the wells #1 to #11 in the microtitre tray, from high to low concentration of antibiotic (across the tray from left to right). Well #12 was filled with 50µl CAMHB with no antibiotic added to serve as the control. The trays were frozen at -20°C until susceptibility testing of the bacterial samples was undertaken.

Preparation of standardised inoculum

Colonies were selected with a wire loop and inoculated in a bijou in 3mls saline solution to a density of 0.5 MacFarland turbidity (typically several small colonies or one large colony were necessary to reach the correct density). Turbidity of the suspension was assessed by visually comparing the sample to reference standards. The bacterial suspension was then diluted by placing 10µl of the suspension into 990µl cation-adjusted Mueller-Hinton broth (CAMHB) in a sterile Eppendorf tube.

Inoculation of microtitre tray with standardised inoculum

50µl of the first isolate was dispensed into each of the twelve wells in the first row. This was repeated for each of the isolates in the remaining rows (i.e. one isolate per row). Once complete, each tray was covered with a sticky plastic film 'lid', gently shaken, placed into plastic bags in groups of 3 that were then secured with autoclave tape. The trays were inverted and incubated at 30° for 24 hours.

Determination of MIC

Following incubation, the microtitre trays were visually inspected for bacterial growth to determine the MIC. In broth microdilution testing, MIC is defined as the lowest concentration of antimicrobial at which visible bacterial growth is prevented. There was a clear distinction between growth (the liquid in the well appeared cloudy) and no growth (the liquid in the well appeared clear). Technically, the exact MIC exists between these two wells – that is, the wells at which the change from no growth to growth occurs – though it is recorded as the higher of the two concentrations.

5.3.2.2 Genotypic identification of isolates

Isolates were identified via 16S rDNA sequencing. When first introduced in the 1990s, 16S rDNA sequencing was highly anticipated. In the 1998 edition of the microbiology textbook, Brock Biology of Microorganisms, the authors proclaimed, "A new golden age of microbiology is upon us!" (Macrae, 2000). The methodology revolutionised the field of microbiology, as it allowed for the rapid identification of a range of microorganisms to genus or even species level (Sacchi *et al.*, 2002). Whereas previously, identification of microorganisms ordinarily relied on a phenotypic approach, the molecular basis of 16S rDNA sequencing provided a faster, more accurate and transferable methodology that could be used in the identification of all bacteria – including those which are slow-growing, have unusual phenotypes, are relatively rare, or are otherwise difficult to identify via the traditional approach (Drancourt *et* al., 2000, Patel *et al.*, 2000). As a result of these advantages, in the decades that have followed its introduction, 16S rDNA sequencing has become a popular phylogenetic tool for the identification of bacteria, including samples derived from environmental, veterinary, and clinical sources (Drancourt *et al.*, 2000, Woo *et al.*, 2008).

In this investigation, 16S rDNA sequencing was used to identify bacteria from wild Greater Flamingos that had been determined to be resistant to one or more antibiotics (via Kirby Bauer disc diffusion and MIC testing). The methodology underlying 16S rDNA sequencing involved four primary stages 1) DNA isolation, 2) PCR amplification, 3) Purification of PCR products, and 4) DNA sequencing and analysis.

DNA isolation

DNA was isolated from bacterial isolates using the Qiagen DNeasy kit, following the protocol provided by the manufacturer. Mueller Hinton agar plates were divided in half, and each half-plate was inoculated with a single colony. The plates were incubated at 30°C for 24 hours. Lysis buffer (comprised of 4.75ml TE, 250µg mg.ml lysostaphin and 0.1g lysozyme) was then prepared in a falcon tube.

Following the 24 hour incubation period, a sterile inoculating loop was used to take a 2.5cm x 2.5cm square patch from the bacterial lawn (previously inoculated on half-plates), which was suspended in a sterile Eppendorf containing 250µl of lysis buffer. The Eppendorf was vortexed and incubated in a waterbath for 2-3 hours at 30°C, and was vortexed every 30 minutes, for cell lysis. Once lysis occurred, 25µl proteinase K was added to each Eppendorf, followed by 250µl of buffer AL (both of which were supplied with the kit). The Eppendorfs were vortexed and incubated at 70°C for 30 minutes to digest proteins and remove contamination from the nucleic acids. Following this, 200µl of EtOH (taken from a -20°C freezer) was added. The Eppendorfs were inverted, at which point a white precipitate was observed. The contents of each Eppendorf were transferred to minicolumns (in collection tubes), which were centrifuged

at 11000rpm for 1 minute. The collection tubes were discarded and the minicolumns were placed into new collection tubes. 500µl of buffer AW1 was added and the minicolumns were centrifuged at 11000rpm for 1 minute. The contents of the collection tubes were discarded and the minicolumns were placed back in the collection tubes. 500µl of buffer AW2 was added to the minicolumns, which were centrifuged at 14,000rpm for 3 minutes. The collection tubes were discarded and the minicolumns were placed into sterile Eppendorf tubes. 100µl of buffer AE was added onto the sample in the minicolumns, which were left at room temperature for 5 minutes. The minicolumns (now in Eppendorfs) were centrifuged at 11,000rpm for 1 minute. The minicolumns were discarded, and the Eppendorf (now containing the DNA sample) were retained. The DNA quality was assessed following electrophoresis, by running 3µl of each DNA sample (with 5µl loading buffer) on 0.8% agarose gels using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific Inc. USA). Samples were stored at 4°C.

PCR amplification

From the DNA samples, 16S rDNA gene fragments were amplified with universal 16S rDNA primers 142R (5′-CGGC/TTACCTTGTTACGA-3′) and 533F (5′-AGAGTTTGATC/TA/CTGGCTCAG-3′). The conditions used for PCR amplification, previously described by Singh *et al.* (2003), were as follows. Reaction Master Mix was prepared to ensure there was a sufficient amount for the number of samples (i.e. reactions). The Reaction Master Mix was comprised of the components listed in Table 5.1 (amounts listed pertain to that needed for one reaction). Preparation details for the Reaction Master Mix and the reaction are also provided in Table 5.1.

Table 5.1 Preparation of reaction master mix

Component	Amount (for 1 rxn)	Preparation details
Sterile Distilled Water (SDW)	31.5µl	1. SDW was measured into an Eppendorf, and the four other components were added.
Mg-free PCR buffer	5µl	
2.5mM $MgCl_2$	5µl	
200µM dNTPs (dATP, dCTP, dTTP, and dGTP)	5µl	
300nM F & R primers (details above)	5µl	
Taq DNA polymerase	.25µl	2. Taq DNA polymerase was added to the Eppendorf.
DNA	1µl	3. The DNA sample was then added.

PCR amplifications were performed in a G-storm GS1 thermocycler (Applied Biosystems, Foster City, CA). The reaction conditions were as described in Table 5.2:

Table 5.2 Reaction conditions for PCR amplifications

Temperature	Time	No. of cycles	Purpose
94°C	2min	1	Initial denaturation
94°C	30sec		Denaturation
50°C	30sec	35	Annealing
72°C	10sec		Extension
72°C	10min	1	Final elongation
4°C	HOLD	---	

PCR products were visualised by conventional agarose gel electrophoresis as follows. 4µl of the product was run on 1.7% agarose gel, and was visualised under UV light. The expected product (i.e. region that it is expected the primers will amplify) was between 900bp and 1.5kb.

Purification of Polymerase Chain Reaction (PCR) products

PCR products were purified using a GenElute™ PCR Clean-Up Kit (Sigma) following the manufacturers instructions, provided with the kit. In brief, the procedure was as detailed in Table 5.3:

Table 5.3 Procedure for PCR purification

Addition of component/reagent and/or step(s) to follow	Centrifuge speed (rpm)	Time
Add 500µl binding solution to 100µl PCR reaction & mix. Transfer into a binding column and centrifuge.	14,000	1 min
Discard eluate and replace binding column into the collection tube. Add 0.5ml wash solution (diluted with ethanol) to the column and centrifuge.	14,000	1 min
Discard eluate and replace binding column into the collection tube and centrifuge	14,000	2 min
Discard eluate and the collection tube. Transfer column to a new collection tube and add 50µl elution solution in the centre of the column. Leave for 1 minute and centrifuge.	14,000	1 min

The purified DNA (in the eluate) was stored at -20°C until sequencing was undertaken.

DNA sequencing and analysis

DNA sequencing was carried out commercially by Geneservice Limited (SourceBioscience, Guinness Enterprise Centre, Dublin, Ireland) using an ABI 3730xl Sanger sequencing

platform. The sequence chromatograms that were received were analysed using the BioNumerics 'Sequence Assembler' software version 7.1 (Applied Maths, Ghent, Belgium). Sequences (forward and reverse) were imported and assembled. Using *alignment overview*, the sequences were checked for quality via both the chromatogram and sequence files. Where necessary, sequences were trimmed to delete zones (particularly those at the ends) that were of low quality. Where discrepancies were found in the sequences, modifications were made. The resolved sequences were exported and saved.

The sequences were then analysed using the 'Basic Local Alignment Search Tool' (BLAST) programme (BLASTN 2.2.28+) to determine the bacterial species. This online programme is available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. For the purposes of this investigation, the programme was used as follows. From the website homepage, 'nucleotide blast' was selected as the *Blast programme*. On the following page, the query (sequence) was entered, and under *Choose a search set*, 'others' was selected. The bacterial isolates were identified based on the results returned, which provided a description (i.e. genus and species names) of nucleotide sequences that match, along with a 'Max ident' which provided an indication of how good the alignments matched between the 'hit' and the query sequence (the sample).

5.3.3 Establishment of antibiotic-resistance phenotypes

A subset of 16 of the 44 bacterial isolates that were identified in 16S rDNA testing was evaluated for resistance against a panel of fifteen antibiotics using Kirby Bauer disc diffusion. The full methodologies for Kirby Bauer disc diffusion are provided in Chapter 3. Further to the methods as described there, where disc diffusion was used as a preliminary screening of bacterial samples, several additional steps were taken for the purposes of this investigation. To begin, the isolates (which had been stored on ceramic beads at -20°C) were streaked out on Trypticase Soy Agar (TSA) plates to isolate individual colonies. The plates were incubated at 30°C for 24 hours, after which colonies were suspended in 2ml Phosphate Buffered Saline solution (PBS) in a bijou to a turbidity of 0.5 McFarland standard. The inoculated solution was plated out on TSA using a wire loop to create a lawn; each isolate was plated onto two plates, as there were 16 antibiotic discs to be tested (eight discs can be accommodated per plate). The antibiotics used in this analysis were selected with reference to the list of antibiotics generated in Chapter 3, i.e. those that are clinically relevant and have been used in similar research. In this investigation, multiple concentrations of several of the antibiotics (chloramphenicol, cefoxitin, gentamycin, and kanamycin) were used to validate methodologies (as a stronger concentration should yield a larger zone of inhibition). Similarly, one disc (gentamycin 30µg) was tested twice (once per plate) to validate methodologies (the zone sizes should be the same). The antibiotics (and their concentrations) used were:

Table 5.4 Antibiotics used in disc diffusion testing

Antibiotic	Dosage (μg)	Abbreviation
Amoxicillin-Clavulanic acid	30	AMC
Ampicillin	10	AMP
Chloramphenicol	30 & 50	C
Ciprofloxacin	5	CIP
Erythromycin	15	E
Cefoxitin	10 & 30	FOX
Gentamycin	10 & 30*	CN
Imipenem	10	IPM
Kanamycin	5 & 30	KAN
Tetracycline	30	TET
Trimethoprim-sulfamethoxazole	25	SXT

*Gentamycin 30(μg) was tested twice per sample (i.e. using two discs).

Plates were incubated for 24 hours at 30°C. Zone sizes were measured using a ruler (the diameter of the zone of inhibition) and recommendations provided by EUCAST were used to classify isolates as sensitive or resistant, where breakpoints existed. In most cases (where they existed), breakpoints were described with reference to genera, and not species. Breakpoints do not exist for all antibiotics for all species of bacteria, particularly where an antibiotic has not been used sufficiently in a clinical context to establish a breakpoint (although the antibiotic might be approved for use) (EUCAST, 2013).

5.4 Results

5.4.1 MIC determination and genotypic identification of isolates

DNA extracted from 54.5% of the 44 isolates exhibited 100% similarity with a single species to submitted sequences on the GenBank database. Of the remaining isolates that were queried, identification to a single species was not possible because either a) isolates had 100% similarity to multiple sequences, or b) sequences that were 100% hits were identified only at the genus level. Nine genera, six families, and three phyla were detected among the 44 isolates investigated. The most frequently identified bacteria were species in the genera *Pseudomonas* (17 isolates, 39% of total), *Escherichia* (10 isolates, 23% of total), and *Enterococcus* (7 isolates, 16% of total). Families represented included Alcaligenaceae, Enterobacteriaceae, Pseudomonadaceae, Sphingobacteriaceae, Streptococcaceae, and Xanthomonadaceae. With the exception of the *Enterococcus* and *Sphingobacteriaceae* genera, which belonged to the Firmicutes and Bacteriodetes phyla, respectively, all genera belonged to the Proteobacteria phylum. The following section provides descriptions of the species that were detected by 16S rDNA sequencing, including details of their respective MICs as determined via broth microdilution analysis (species are presented with reference to their genus), while Figure 5.1 provides a visualisation of variation in the bacteria species identified across the five sites. These results are compiled in Table 5.5, which provides, for each of the isolates, the species identified via DNA analysis, as well as the results of MIC determination. The values presented indicate the MIC of the isolate, which can range from 'all negative' (where all concentrations of the antibiotic were effective in inhibiting bacterial growth) to 'all positive' (where even the highest concentration of antibiotic did not inhibit bacterial growth. Between these, values can range from 0.25 to 128, and indicate the lowest concentration of antibiotic (measured in µg/ml) at which bacterial growth was not able to occur – i.e. the minimum concentration at which it was effective.

Alcaligenes

One isolate, from the Camargue in France, was identified as *Alcaligenes faecalis* (Figure 5.1). *A. faecalis* is a gram-negative bacterium, and is classed in the family Alcaligenaceae. This isolate was determined to have an MIC of 16µg/ml to ciprofloxacin (Table 5.5).

Cronobacter or Escherichia

Two isolates, taken from a single sample from the Ebre Delta, were found to be resistant to ciprofloxacin and chloramphenicol, respectively, in disc diffusion analysis in preliminary screening (Figure 5.1). Both queried sequences exhibited DNA sequence similarity to *Cronobacter sakazakii* and *Escherichia coli*. Both *C. sakazakii* and *E. coli* belong to the Gram-negative family Enterobacteriaceae. *Cronobacter sakazakii* was formerly known as *Enterobacter sakazakii*, having been reclassified in 2007 (Iverson *et al.*, 2007). In MIC

analysis, the ciprofloxacin resistant isolate had an MIC of 8μg/ml while the chloramphenicol resistant isolate had an MIC of 128μg/ml (Table 5.5).

Enterococcus

Seven isolates were determined to belong to the *Enterococcus* genus, which consists of Gram-positive bacteria in the family Streptococcaceae. These included two *E. faecalis* isolates (from the Camargue) and three *E. faecium* isolates (two from Comacchio and one from Ebre Delta) (Figure 5.1). The remaining two isolates could not be definitively characterised because they exhibited 100% DNA sequence similarity with two species – one isolate recovered from Rio Odiel was 100% with both *E. faecium* and *E. faecalis*, and the isolate recovered from Comacchio exhibited 100% DNA sequence similarity with both *E. faecium* and *E. durans*. The isolates were resistant to a variety of the antibiotics, including three resistant to kanamycin (MICs of 8μg/ml and 16μg/ml), one resistant to each of erythromycin (MIC of 128μg/ml), ciprofloxacin (MIC of 2μg/ml), tetracycline (MIC of 32μg/ml), and gentamycin (MIC of 16μg/ml) (Table 5.5).

Escherichia

Ten isolates were identified as belonging to the genus *Escherichia*. Of these, all ten were determined to be *E. coli,* a Gram-negative species in the family Enterobacteriaceae. The ten isolates were from samples collected at the Camargue (two isolates), Fuente de Piedre (three isolates), Ebre Delta (four isolates), and Rio Odiel (one isolate) (Figure 5.1). The isolates demonstrated a variety of resistances, including one resistant to kanamycin (MIC of 0.25μg/ml), two resistant to tetracycline (both had MICs of 128μg/ml), one resistant to gentamycin (MIC of 16μg/ml), one resistant to ciprofloxacin (MIC of 1μg/ml), and three resistant to erythromycin (two with MICs of 4μg/ml and one that did not demonstrate any antibiotic resistance, as all concentrations of antibiotic were effective at inhibiting bacterial growth). There were two isolates, one resistant to Trimethoprim-sulfamethoxazole and one resistant to ampicillin in pre-screening, for which no concentration of antibiotic was effective – that is, bacterial growth was present in all of the wells.

Hafnia

Three isolates were identified as *Hafnia alvei*, which is a Gram-negative species in the family Enterobacteriaceae. All three isolates were taken from the same original sample from Fuente de Piedre, Spain (Figure 5.1). One was resistant to tetracycline and had an MIC of 16μg/ml, while the other two were resistant to erythromycin and had MICs of 8μg/ml and 16μg/ml.

Pantoea

One erythromycin-resistant isolate from the Camargue was identified as *Pantoea agglomerans*, which is a Gram-negative bacterium in family Enterobacteriaceae (Figure 5.1). The isolate was determined to have an MIC of 0.25µg/ml.

Pseudomonas

Seventeen isolates were identified as belonging to the Gram-negative genus *Pseudomonas* (in the family Pseudomonadaceae). Of these, four isolates were identified to species level: *P. aeruginosa* and *P. fluorescens* from Fuente de Piedre, Spain, and *P. putida*, which was found in samples from both the Camargue, France and Comacchio, Italy (Figure 5.1). The other thirteen isolates were identified to genus level as *Pseudomonas*, but were found to be 100% homologous with multiple species on the GenBank database. Putative species included *P. azotoformans, P. fluorescens, P. koreensis, P. marginalis*, *P. moraviensis*, *P. putida, P. reactans*, and *P.* rhodesiae. Four isolates returned a *"Pseudomonas* spp*"* result as 100% homologous. Isolates identified as *Pseudomonas* were derived from samples collected at all five of the study sites, and demonstrated resistances to five of the eight antibiotics, with MICs ranging between 0.5 and 128µg/ml (Table 5.5). The specific resistances (and their respective MICs) are as follows: erythromycin [MICs of 8 and 32µg/ml (two isolates), and two isolates for which no concentration of erythromycin inhibited bacterial growth], SXT (MICs ranging between 16 and 64µg/ml), tetracycline (32µg/ml), gentamycin (MICs of 0.5 and 32µg/ml), and chloramphenicol (MICs of 16 and 64µg/ml). The isolate that demonstrated resistance to kanamycin in disc diffusion testing in pre-screening was found not to be resistant in MIC broth microdilution, as all wells were negative for bacterial growth. Put otherwise, larger numbers mean that the concentration of antibiotic required to prevent bacterial growth is higher.

Sphingobacterium

One kanamycin-resistant isolate was identified as belonging to the *Sphingobacterium* genus, from the family Sphingobacteriaceae. The queried sequence indicated a 100% match to both *Sphingobacterium kitahiroshimense* and *Sphingobacterium faecium.* The isolate, from the Camargue, France (Figure 5.1) was determined to have an MIC of 64µg/ml.

Stenotrophomonas

Two isolates, both kanamycin resistant, were identified as belonging to the *Stenotrophomonas* genus. The *Stenotrophomonas* genus is comprised of Gram-negative bacteria and is classified in the family Xanthomonadaceae. One of the isolates, from a sample taken in Comacchio, France, was 100% homologous to *S. maltophilia* (Figure 5.1). The resistance of this isolate, though verified in the confirmation test, was not apparent in the MIC analysis, in which bacterial growth was inhibited in all wells. The second *Stenotrophomonas* isolate was taken

from a sample from Fuente de Piedre (Figure 5.1), and was also identified as *S. maltophilia*. The MIC for this isolate could not be quantified, as bacterial growth was present in each of the microtitre wells.

Table 5.5 Bacterial identification and Minimum Inhibitory Concentration (MIC) determination of 44 isolates from wild Greater Flamingo chicks

Sample code	16S rDNA sequencing	Antibiotic	MIC (µg/ml)	Site
CG065	Alcaligenes faecalis	CIP	16	Camargue
EB009	Cronobacter sakazakii or Escherichia coli	CIP	8	Ebre Delta
EB009	Cronobacter sakazakii or Escherichia coli	C	128	Ebre Delta
CG054	Enterococcus faecalis	KAN	16	Camargue
CG020	Enterococcus faecalis	E	128	Camargue
CO014	Enterococcus faecium	CIP	2	Comacchio
CO042	Enterococcus faecium	TET	32	Comacchio
EB037	Enterococcus faecium	KAN	8	Ebre Delta
CO010	Enterococcus faecium or durans	KAN	8	Comacchio
OD075	Enterococcus faecium or faecalis	CN	16	Rio Odiel
CG035	Escherichia coli	KAN	0.25	Camargue
CG035	Escherichia coli	TET	128	Camargue
FP073	Escherichia coli	TET	128	Fuente de Piedre
EB032	Escherichia coli	E	4	Ebre Delta
EB043	Escherichia coli	CN	16	Ebre Delta
EB007	Escherichia coli	SXT	all pos.	Ebre Delta
FP073	Escherichia coli	CIP	1	Fuente de Piedre
FP073	Escherichia coli	AMP	all pos.	Fuente de Piedre
OD059	Escherichia coli	E	all neg.	Rio Odiel
EB004	Escherichia coli	E	4	Ebre Delta
FP059	Hafnia alvei	TET	16	Fuente de Piedre
FP059	Hafnia alvei	E	8	Fuente de Piedre
FP059	Hafnia alvei	E	16	Fuente de Piedre
CG024	Pantoea agglomerans	E	0.25	Camargue
FP049	Pseudomonas aeruginosa	KAN	all neg.	Fuente de Piedre
FP001	Pseudomonas fluorescens	E	8	Fuente de Piedre
OD056	Pseudomonas fluorescens or reactans	C	16	Rio Odiel
CO017	Pseudomonas fluorescens, azotoformans, or reactans	E	128	Comacchio
OD006	Pseudomonas fluorescens, reactans, azotoformans	SXT	32	Rio Odiel
OD061	Pseudomonas fluorscens or reactans	E	32	Rio Odiel
OD018	Pseudomonas fluorescens or reactans	TET	32	Rio Odiel
FP013	Pseudomonas moraviensis or koreensis	CN	32	Fuente de Piedre
CG002	Pseudomonas putida	SXT	64	Camargue
CO068	Pseudomonas putida	SXT	16	Comacchio

Sample code	16S rDNA sequencing	Antibiotic	MIC (µg/ml)	Site
EB017	Pseudomonas rhodesiae, fluorescens or spp	E	all pos.	Ebre Delta
EB017	Pseudomonas rhodesiae, fluorescens, or marginalis	E	all pos.	Ebre Delta
CO032	Pseudomonas sp	SXT	16	Comacchio
CO045	Pseudomonas sp	E	32	Comacchio
CG065	Pseudomonas sp.	C	64	Camargue
CO032	Pseudomonas sp.	CN	0.5	Comacchio
OD007	Pseudomonas fluorescens or P. reactans	SXT	32	Rio Odiel
CG037	Sphingobacterium kitahiroshimense or faecium	KAN	64	Camargue
CO013	Stenotrophomonas maltophilia	KAN	all neg.	Comacchio
FP020	Stenotrophomonas maltophilia	KAN	all pos.	Fuente de Piedre

*Indicates that the MIC is at or over the breakpoint, as determined with reference to EUCAST and CLSI guidelines. Antibiotics are abbreviated as follows: AMP (ampicillin), C (chloramphenicol), CIP (ciprofloxacin), CN (gentamycin), E (erythromycin), K (kanamycin), SXT (Trimethoprim-sulfamethoxazole), and TET (tetracycline). Isolates identified as resistant (with reference to EUCAST guidelines) are in red, those susceptible are in green.

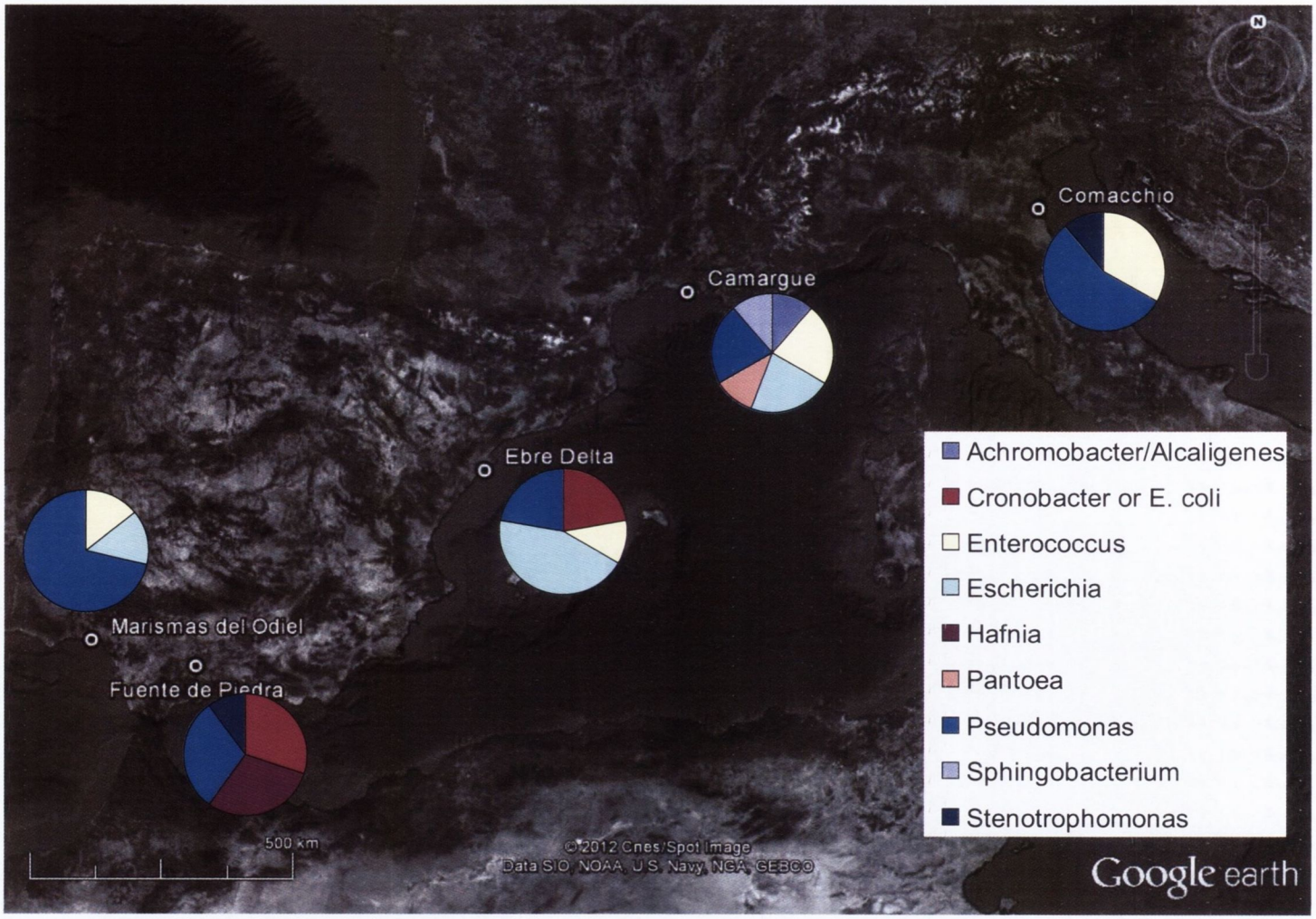


Figure 5.1 Species identified via 16S rDNA sequencing at each of the five sites: Rio Odiel, Spain; Fuente de Piedre, Spain; Ebre Delta, Spain; Camargue, France, and Comacchio, Italy.

5.4.2 Antibiotic resistance phenotypes

The results of disc diffusion analysis of the subset of 16 isolates on which additional antibiotic resistance analysis are given in Table 5.6, which provides the measurements of the zones of inhibition. The values in this table correspond to the measured zones of inhibition, which are reported in mm. These numbers can range from 0mm (where bacterial growth occurred adjacent to the antibiotic disc – i.e. the bacteria was resistant to the antibiotic) and upwards. Smaller values correspond to a bacterial isolate that was more resistant to a given antibiotic, whereas larger values mean that the antibiotic was effective at inhibiting bacterial growth. The relative importance of these values was assessed via examination of published breakpoints; however, very little data existed to determine these breakpoints for the range of bacterial species and antibiotics tested, because where antibiotics have not been used sufficiently in a clinical context, breakpoints have not yet been established. Where data did exist, the results revealed that the isolates demonstrated a range of additional resistances. Of the 16 isolates tested, 100% were resistant to at least one of the fifteen antibiotics, while 94% of isolates (15 out of 16) exhibited multidrug resistant phenotypes (that is, the isolate was resistant to two or more of the antibiotics).

Amongst the antibiotics for which data on breakpoints existed, high rates of resistance to ampicillin (10μg), erythromycin (15μg), and cefoxitin (both 10μg and 30μg) were observed, while resistances to gentamycin (10μg), imipenem (10μg), and ciprofloxacin (5μg) were less prevalent. Concurrent resistances, when a bacterial isolate is resistant to two similar antibiotics, are easy to interpret/visualise in Table 5.6, in which antibiotics are grouped with reference to their class (as described by the World Health Organisation). Four of the six *Pseudomonas* isolates, for example, were resistant to both amoxicillin-clavulanic acid and ampicillin, which are both grouped within the 'Beta lactams, Penicillins' class of antibiotics. Two other isolates – the *Enterococcus faecalis* isolate and one of the Enterococcus faecium isolates – were also resistant to both amoxicillin-clavulanic acid and ampicillin. The same two isolates were also resistant to cefoxitin and imipenem, both of which are within the 'Other Beta-Lactam Antibacterials' class of antibiotics. In contrast to this, the three *E. coli* isolates were sensitive to both cefoxitin and imipenem, providing evidence of concurrent sensitivities.

Five of the antibiotics were tested at multiple concentrations. Gentamycin, for example, was tested at both 10μg and 30μg. As would be expected, all of the isolates had a larger zone of inhibition at the higher concentration. Cefoxitin was also tested at multiple concentrations, of 10μg and 30μg, and in all cases, the zone of inhibition was larger at the higher concentration. For chloramphenicol, which was tested at 30μg and 50μg, five of the sixteen isolates had zones of inhibition that were the same (two isolates) or smaller (three isolates) at the higher concentration. The zones of inhibition of the isolates for kanamycin, which was tested at 5μg

and 30µg, were larger at the higher concentration of antibiotic in all but one of the cases. One of the antibiotics, gentamycin, was tested twice at the same concentration – two 30µg discs were tested. The variation in the measured zone of inhibition between the replicates ranged from 0mm to 10mm, with twelve of the sixteen isolates within 2mm.

Table 5.6 Susceptibility of isolates as determined using Kirby Bauer disc diffusion techniques. Zones of inhibition are reported in mm.

			A					B		C		D	E			F	G	H
Sample	Original resist.	Bacterial species	CN 10µg	CN 30µg	CN 30µg	K 5µg	K 30µg	C 30µg	C 50µg	AMC 30µg	AMP 10µg	E 15µg	FOX 10µg	FOX 30µg	IPM 10µg	CIP 5µg	SXT 25µg	TET 30µg
CG065	CIP	*Alcaligenes faecalis*	16	22	22	11	19	36	30	24	18	17	0	0	34	16	38	34
EB009	CIP	*Cronobacter sakazakii* or *Escherichia coli*	20	26	25	16	24	26	28	24	0	0	19	24	34	12	16	0
CG020	E	*Enterococcus faecalis*	23	27	28	0	13	12	15	0	0	0	0	0	16	31	20	25
CO014	CIP	*Enterococcus faecium*	16	20	20	0	16	36	36	24	0	22	0	16	26	20	30	28
CO014	CIP	*Enterococcus faecium*	19	34	24	10	20	32	32	14	0	0	0	0	11	24	34	21
EB037	KAN	*Enterococcus faecium*	16	21	20	0	13	36	34	32	15	15	0	11	24	26	34	10
CG035	TET	*Escherichia coli*	21	24	30	14	21	16	15	28	0	0	19	26	34	36	19	20
EB043	CN	*Escherichia coli*	0	0	0	8	16	26	31	22	0	0	16	26	36	8	38	0
FP073	TET	*Escherichia coli*	24	26	28	26	24	30	34	22	0	0	18	26	34	23	42	0
FP059	TET	*Hafnia alvei*	24	28	26	15	23	24	30	0	12	0	20	24	30	32	27	21
CG002	SXT	*Pseudomonas putida*	28	30	30	14	32	16	18	12	0	0	0	0	36	36	19	20
CO032	SXT	*Pseudomonas sp*	26	34	34	22	31	27	30	13	0	17	0	0	28	36	32	22
CG065	C	*Pseudomonas sp.*	28	34	30	14	29	0	13	0	0	0	0	0	30	38	19	14
OD056	C	*Pseudomonas fluorescens or reactans*	26	28	32	18	26	14	17	0	0	0	0	0	13	44	36	30
OD006	SXT	*Pseudomonas fluorescens, reactans, azotoformans*	24	28	28	16	26	14	20	0	0	0	0	0	12	36	21	24
EB017	AMP	*Pseudomonas rhodesiae, P. fluorescens, or P. sp*	28	32	30	16	32	14	18	0	0	0	0	0	21	38	22	28

Resistance at or over established breakpoints (determined with reference to EUCAST and CLSI guidelines) is indicated by red shading, sensitivity by green, while isolates classed as 'intermediate' are shaded orange. Bacteria/antibiotic combinations for which no data existed are not shaded. Isolates are listed in alphabetical order by genus; antibiotics are grouped by class of antibiotic (as described in Chapter 3), which are presented in alphabetical order. Classes are: A (Aminoglycoside antibacterials); B (Amphenicols); C (Beta-lactams, Penicillins); D (Macrolides); E (Other Beta-Lactam Antibacterials); F (Quinolone antibacterials); G (Sulfonamides and trimethoprims); H (Tetracyclines). Antibiotic abbreviations are as follows: CN (gentamycin), K (kanamycin), C (chloramphenicol), AMC (amoxicillin-clavulanic acid), AMP (ampicillin), E (erythromycin), FOX (cefoxitin), IPM (imipenem), CIP (ciprofloxacin), SXT (Trimethoprim-sulfamethoxazole), and TET (tetracycline). Note that C, CN, and FOX were tested in multiple concentrations; CN 30µg was tested twice.

Analysis of resistance via disc diffusion indicated that with the exception of one isolate, multidrug resistance was found in all isolates (Figure 5.2).

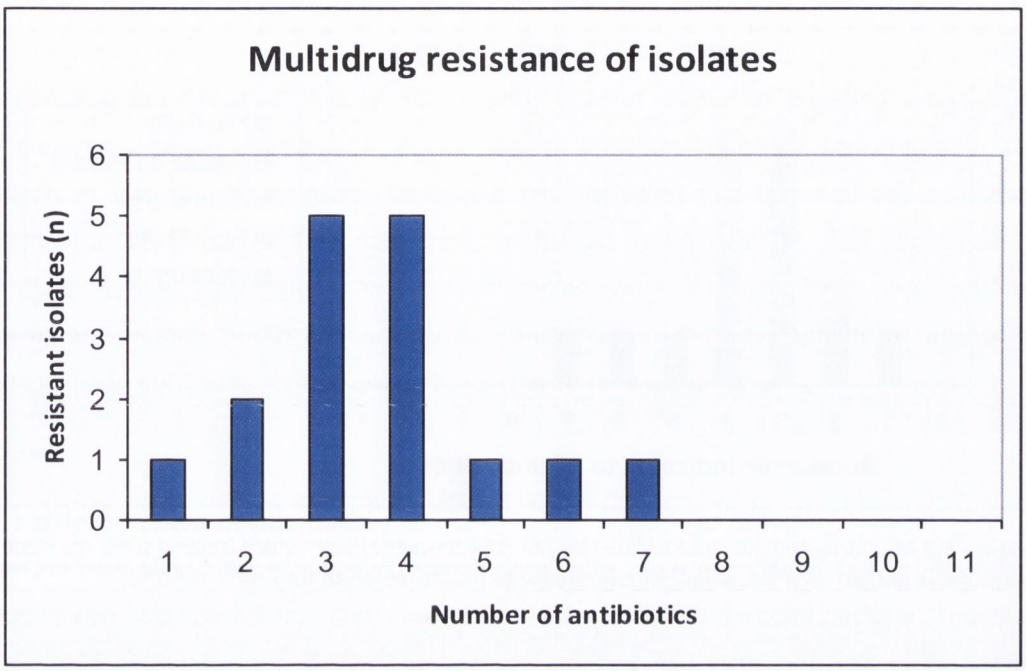


Figure 5.2 Multidrug resistance of isolates. Antibiotics tested at more than one concentration were considered only once. Isolates were most frequently resistant to three or four antibiotics. No isolate was resistant to eight or more of the eleven antibiotics on the panel.

Multidrug resistance is further visualised by site and by bacterial genera in Figures 5.3 and 5.4, respectively. With the exception of one isolate that was resistant to only one antibiotic, (the isolate from the Camargue determined to be *Alcaligenes faecalis*), all isolates demonstrated multidrug resistance. Variation in the number of multidrug resistant isolates between sites is evident; for example, there was more variation in the multidrug resistance of isolates taken from flamingos at the Camargue (isolates were resistant to between one and seven antibiotics) than at Comacchio (isolates were resistant to either three or four antibiotics). Patterns of resistance between bacterial species is also evident; for example, *Pseudomonas* isolates were most frequently resistant to four antibiotics, as were *Enterococci* isolates, while *Escherichia* isolates were most frequently resistant to three antibiotics.

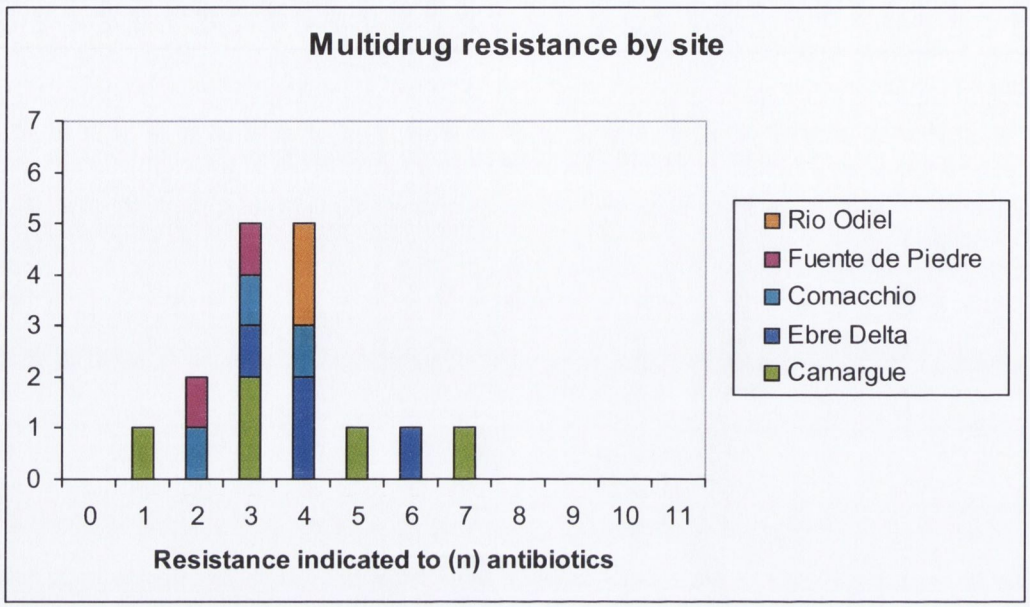


Figure 5.3 Multidrug resistance of isolates by site. The frequency with which isolates were resistant to multiple antibiotics is visualised, with reference to the site at which they were collected.

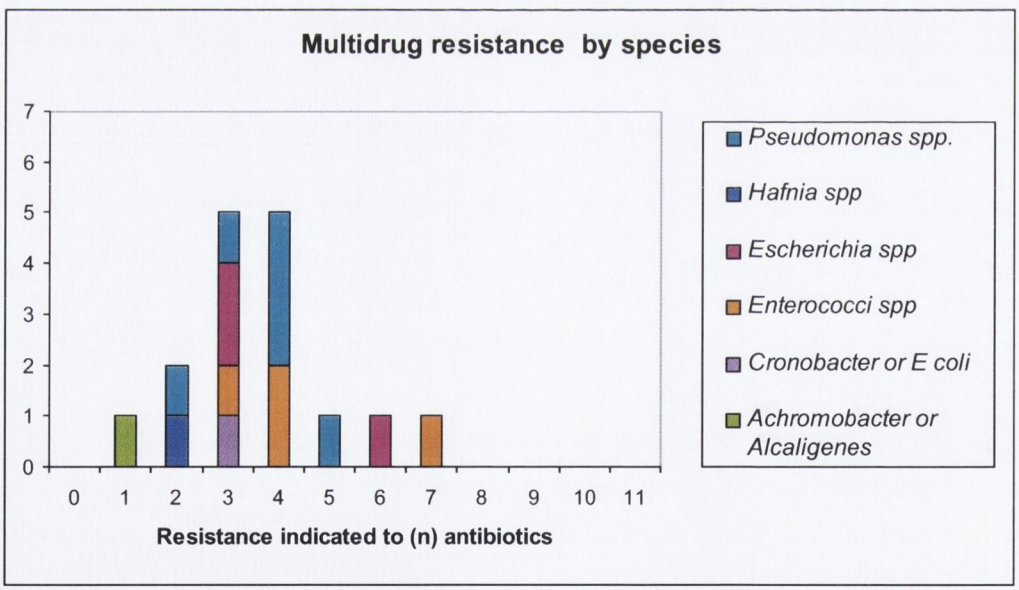


Figure 5.4 Multidrug resistance of isolates by bacterial species. The frequency with which the various bacterial species were resistant to multiple antibiotics is visualised.

5.5 Discussion

This study reports on bacterial species found in Greater Flamingo chicks, with a focus on identifying isolates that had, in preliminary screening using disc diffusion, demonstrated antibiotic resistance. Several novel insights have arisen from this study. In accordance with previous studies, the data presented here provide evidence of the prevalence of wild birds as reservoirs for a variety of species of antibiotic resistant bacteria, though this is the first study to do so using the Greater Flamingo as a study species within its breeding range in the Mediterranean region. Further to this, the use of minimum inhibitory concentration (MIC) analysis to quantify the antibiotic resistance has indicated that some of the resistances demonstrated are important to consider in a human health context. The results of this investigation have implications for both environmental, animal (domestic, livestock, and wildlife) and human health and should be considered in the development of appropriate strategies and policies to combat antibiotic resistance on a global scale.

5.5.1 Microbial flora of wild birds

Over the past two to three decades, numerous studies have provided evidence indicating a range of wild bird species that serve as reservoirs for antibiotic resistant bacteria (Tsubokura *et al.*, 1995; Sjölund *et al.*, 2008). The bacterial species that were identified in the present research correspond to those found in many of these other previous research efforts on a variety of bird species, and particularly research conducted in a European context. The bacteria identified in the present study belonged to three phyla including Proteobacteria, Bacteroidetes and Firmicutes, with the majority of isolates belonging to the Proteobacteria phylum. This corresponds to phyla found in similar research on wild bird species conducted worldwide that – like the Greater Flamingo – are found in aquatic environments, including Black-legged Kittiwake (*Rissa tridactyla*) chicks in Alaska, USA; various shorebirds at the Tagus Estuary in Portugal, including the Black-winged Stilt (*Himantopus himantopus*), Black-tailed Godwit (*Limosa limosa limosa*), and Common Redshank (*Tringa tetanus*); Adelie Penguins (*Pygoscelis adeliae)* in Antarctica; and the Californian Gull (*Larus californicus)* in the United States (Lu *et al.*, 2008; Banks *et* al., 2009; Kohl, 2012; Santos *et al.*, 2012; van Dongen *et al.*, 2013). Interestingly, although several similar investigations have found species belonging to the *Actinobacteria* phylum, it was not found here, despite the fact it has previously been reported in aquatic and/or marine environments (Maldonado, 2005; Kohl, 2012; Santos *et al.*, 2012; van Dongen *et al.*, 2013). This might be at least in part explained by the work conducted by van Dongen *et al.*, (2013) on age-related differences in the bacterial assemblages of black kittiwakes (*Rissa tridactyla*). These authors determined that the genus they found to be most common in adults – *Corynebacterium* (which belongs to the *Actinobacteria* phylum), was much less abundant (in fact, they were nearly absent) in chicks.

In total, they found that genera in the *Actinobacteria* phylum occurred nearly 50% more often in adults than they were in chicks. If this is consistent between different species of birds, the lack of bacterial species from the *Actinobacteria* phylum in this investigation might be explained by the fact that the samples used in the present study were taken from flamingo chicks, where they would be less common than if the samples had been taken from adult birds. One of the more relevant genera within this phylum with reference to human health is *Mycobacterium*, which includes several opportunistic bacteria species in both wildlife and in humans (Good, 1985). Porter (1998) noted that due to its long incubation period, *M. avium* is more commonly diagnosed in adult birds. This was supported by the research conducted by van Dongen *et al.* (2013), who found species belonging to the *Mycobacterium* genus only in adult birds. Taken together, these two investigations provide evidence that it is perhaps unsurprising that the *Actinobacteria* phylum was not represented in the present study of bird chicks.

The most commonly identified genera here, *Pseudomonas*, is commonly found in aqueous environments (Spiers *et al.*, 2000). It is therefore not surprising that it is commonly found in the microbial flora of birds that live in aquatic habitats, like the Greater Flamingo. Although their focus was microbial communities found on feathers of the eastern bluebird (*Sialis sialis*) in the United States, Shawkey *et al.* (2005) found large numbers of *Pseudomonas* isolates and attributed it to their high prevalence in soil and water. *P. aeruginosa* in particular is recognised as a common avian pathogen (Brittingham *et al.*, 1988; Walker *et al.*, 2002). Its occurrence in the Greater Flamingo in the present study is therefore not surprising. Amongst the other isolates that were less common amongst samples identified in the present study, most are also known to occur in environmental contexts (i.e. soil and/or water samples). For example, *Sphingobacterium*, *Escherichia* and *Stenotrophomonas* isolates have all previously been reported in soil and in aqueous environments (Matsumaya *et al.*, 2008; Brooke, 2012; Pereira *et al.*, 2013). *Achromobacter* and *Alcaligenes* are also typically described as occurring in aqueous environments (Busse and Stolz, 2006). In the present research, one isolate could not be definitively characterised (it was determined to be one of these two species), though this is not unusual; difficulty differentiating the *Achromobacter* and *Alcalgines* species has previously been noted (Coeyne *et al.*, 2003; Spilker *et al.*, 2012). Several studies have investigated bacteria found in the Greater Flamingo, and the species found in the present research confirm several of the findings. Rollin and Baylet (1983) classified bacteria from flamingo chicks in the Camargue, France. Commonly occurring species they found in cloacal samples included *E. coli*, *Enterobacter* spp., *Pseudomonas maltophila*, *Edwardsiella* spp. and *Streptococcus* spp. Sato *et al.* (2009) also investigated bacteria in the Lesser Flamingo. Although they analysed samples from wild flamingos in Tanzania, they found similar species to Rollin and Bayet

(1983); common species between the two investigations included *E. coli*, *Edwardsiella* spp., and *Enterobacter* spp.

5.5.2 Antibiotic resistances demonstrated and Implications for human health

Many recent investigations on environmental reservoirs of antibiotic resistant bacteria have suggested a potential threat to human populations, given the potential for transfer from environmental reservoirs to humans via direct and/or indirect mechanisms (O'Brien, 2002; Allen *et al.*, 2010; da Costa *et al.*, 2013). Several of the specific antibiotic resistant isolates that were identified in this investigation have previously been suggested to pose a threat to human health, and the use of MIC breakpoints has provided an indication of whether or not resistance is at a level that has implications for human health. In a clinical context, the MIC breakpoints that are used to class a bacterial isolate as 'susceptible' or 'resistant' are of relevance with reference to the use of antibiotics that are prescribed therapeutically to treat infections. The '90-60 rule' is a commonly used maxim that describes the relationship between the classification of 'susceptible or 'resistant' and patient outcome (Kuper *et al.* 2012). Originally proposed by Rex and Phaller (2002), the 90-60 rule states that an infection caused by a susceptible bacterial isolate will exhibit sensitivity to an antibiotic 90% of the time, while an isolate classed as resistant will exhibit sensitivity to antibiotic therapy only 60% of the time.

There were several isolates in the present study that exceeded their relevant breakpoints (with reference to MIC determination) including the four *Escherichia coli* isolates that were resistant to ampicillin, ciprofloxacin, gentamycin, and trimethoprim-sulfamethoxazole, and a *Pseudomonas* spp isolate that was resistant to gentamycin. This is comparable to similar research efforts that have identified the same resistances amongst similar bacterial species. Resistance to ampicillin amongst *E. coli* isolates appears to be prevalent in bird species worldwide. For example, in separate investigations both Cole (2005) and Milton and Ambrose (2005) found *E. coli* resistant to ampicillin in samples taken from Canada Geese (*Branta canadensis*) in the United States. Guenther *et al.* (2009) investigated resistance amongst a variety of bird species in Europe, and also found ampicillin-resistant *E. coli* isolates. Another study conducted in Europe by Dolejska *et* al., (2007) of Black-Headed Gulls (*Larus ridibundus*) in the Czech Republic provided evidence of *E. coli* resistant to ampicillin. In other studies conducted in Europe, ampicillin resistance has also been found in Rock Doves (*Columba livia*), Caspian Gulls (*Larus cachinnans*), and Common Buzzards (*Buteo buteo*) (Radimersky *et al*, 2010; Radhouani 2011, 2012). In addition to ampicillin, resistance to ciprofloxacin and gentamycin has also been detected in *E. coli* in many of these investigations, though interestingly, none of these studies found resistance to trimethoprim-sulfamethoxazole as was found here. The epidemiological significance of these resistances as demonstrated in the present study, are important to consider with reference to human health. *E. coli*, for example,

is frequently used in assessing faecal contamination, given its significance as a potential pathogen along with the numerous pathways that exist that can facilitate its transfer to humans from wildlife (Guenther *et al.,* 2011; Pesapane *et al.*, 2013). In addition to the transfer of resistant bacteria, the ability of *E. coli* to transfer resistance genes has also been suggested as a threat to human health (Sayah *et al.*, 2005; Singer *et al.*, 2006; Allen *et al.*, 2010). The threat to human health is highlighted in a review of antibiotic resistance in wildlife and the environment with reference to human health by Radhouani *et al.* (2014), in which they assert, "antimicrobial resistance evolving and spreading among bacterial pathogens is a public health problem of increasing magnitude."

Evidence for multidrug resistance was detected across a range of bacterial genera, including *Pseudomonas*, *Enterococci,* and *Escherichia*. This is in agreement with previous research on antibiotic resistant bacteria in wild birds, in which similar genera have demonstrated multidrug resistance (reviewed in Chapter Three). Multidrug resistant isolates and opportunistic species in particular pose a particular threat for immunocompromised individuals (Brooke, 2012). For example, although relatively rare as an infectious-causing species in humans, species belonging to the *Alcaligenes* and *Stenotrophomonas* genera (both of which were included amongst the species identified in the present study) are problematic in immunocompromised patients (Aisenberg *et al.*, 2004; Brooke, 2012; Spilker *et al.*, 2012). In the present study, for some isolates, multidrug resistance was demonstrated across multiple classes of antibiotic in disc diffusion testing (Table 5.6). This resistance of isolates to different classes of bacteria has previously been suggested to provide an indication of the severity of the problem; Livermore (2002) posits, "[is this] our worst nightmare?" As highlighted in the second chapter of this dissertation, the lack of development of new antibiotics – coinciding with the increased resistance to existing ones – means that the availability of robust antibiotics is shrinking, meaning that multidrug resistance looks to set to continue on an upwards trajectory. While no isolate demonstrated resistance to eight or more of the eleven antibiotics on the panel, as noted previously, it is important to take into account the fact that resistance breakpoints did not exist for all species/antibiotics combinations. Thus, multidrug resistance is in reality likely to be higher than what was determined. This, along with other shortcomings of MIC breakpoints, has previously been acknowledged as a challenge within the field that must be addressed, in order to develop a comprehensive understanding of the prevalence of resistance to a range of antibiotics used in a clinical context (Ferraro, 2001; Kahlmeter *et al.*, 2003; Turnidge and Paterson, 2007).

What the results of this investigation do provide is evidence that environmental systems (and the wildlife within them) serve as reservoirs for antibiotic resistant bacteria, which have the potential to be transferred to human populations. Indeed, the circulation of microbes between

environmental, human, and wildlife hosts is acknowledged (Levy, 1997; O'Brien *et al.*, 2002; da Costa *et al.*, 2013). However, the specific pathways and mechanisms of transfer to human populations are less well understood (Schwartz *et al.*, 2003; Martinez, 2009). In regards to its potential to disseminate antibiotic resistant bacteria, the Greater Flamingo is interesting to consider, particularly in light of the evidence that has emerged in the past few years about its movements within the Mediterranean region. Previously believed to exist as several distinct metapopulations, data from ringing operations and genetic analyses have provided evidence that the Greater Flamingo in the Mediterranean region actually persists as a single metapopulation that is much more interconnected than previously believed (Boucheker *et al.*, 2011; Geraci *et al.*, 2012). This is critical to consider in making projections about how antibiotic resistance might be disseminated by the Greater Flamingo, given that the dispersal of pathogenic microorganisms has previously been associated with bird movements (Hubálek *et al.*, 2004; Jourdain *et al.*, 2007). Although the samples taken in this investigation were from fledgling birds that are unable to fly, as adults the Greater Flamingo exhibits frequent movements from site to site (Nager *et al.*, 1996; Amat *et al.*, 2005; Balkiz *et al.*, 2007). These movements may provide pathways by which resistant bacteria and resistance genes can be disseminated throughout the Mediterranean region. Previous research has evaluated the threat of emerging zoonotic disease in this region, describing factors that make the region particularly vulnerable. These factors include high levels of pollution and environmental degradation, which, together with diverse land uses and high human population, result in land matrices that increase the interaction between people and animals (Seimensis, 2008; Vittecoq *et al.*, 2012). Land use change in particular may serve as a factor that increases the potential for transmission, and an increased occurrence of land use change has been noted in the Mediterranean region (Mathevet *et al.*, 2002; Falcucci *et al.*, 2007). One example of how this has served to increase interaction between wildlife and humans is provided by the Greater Flamingo, whose incursions into rice fields has been a source of human-wildlife conflict in the Mediterranean region (Tourenq *et al.*, 2001; Ernoul *et al.*, 2013).

The threat to humans of antibiotic resistance in the Greater Flamingo can therefore not be ignored, given the noted links between environmental systems, wildlife, and humans. The global nature of the problem was noted by Hawkey and Jones (2009) in their review examining the 'changing epidemiology of resistance', which, they note, results in an increased 'morbidity, mortality, and cost of [treatment]'. The threat to human health resulting from antibiotics in environmental systems in particular was noted as recently as September 2013, when researchers at Johns Hopkins Bloomberg School of Public Health in the United States published findings based on evidence collected from nearly 500,000 patients. Their results indicated an association between an individual's proximity to livestock operations, and a notable increased risk of acquiring methicillin-resistant *Staphylococcus Aureus* (MRSA) –

which they conclude is a concern for public health (Casey *et al.*, 2013). The links may likely be either direct – between humans and livestock, and/or indirect via wildlife vectors. That such findings continue to be published suggest that it is a problem that is not likely to go away, and that action needs to be taken (e.g. implementation of effective policies regarding antibiotic use). In citing the 'excessive use of antibiotics [which can] negatively impact...human health,' Cabello *et al.* (2013) suggest the need for regulatory action, though given the complexity of the issue it is clear that an innovative approach might be necessary. The use of a One Health framework in addressing the problem has been suggested as useful approach given the variety of sectors involved (Wegener, 2012). The results here provide further evidence that a variety of stakeholders including zoologists, public health professionals, and individuals working in the agricultural industry may need to be involved under a One Health approach.

5.6 Conclusions & suggestions for future research

A diverse range of bacterial species was identified in wild Greater Flamingos at five sites in the Mediterranean Region, with *E. coli* and *Pseudomonas* isolates amongst the commonly identified species and genera. Many of the bacteria from Greater Flamingos demonstrated multidrug resistance. Variation in the resistances demonstrated might reflect the influence of land use between the sites. The existence of multidrug resistant bacterial species in the environment (i.e. the Greater Flamingo as a reservoir) presents a potential threat to human health, given that mechanisms of transfer exist whereby bacteria can be transferred between the environment and human populations. In particularl, potentially pathogenic bacteria species, including *E. coli* and *Pseuomonas* spp. represent a threat that must be addressed, through the development of appropriate legislation targeting antibiotic use.

To gain further insights on the mechanisms of transfer of and pathways of antibiotic resistance within environmental systems, it would be important for future research to investigate resistance in environmental samples at the same sites, and perhaps also consider analysis of antibiotics (and their derivatives) in these environmental samples. Although no direct links to land use could be made, this is an area worthy of further attention. Similarly, within bacterial samples, it would also be interesting to investigate the presence of antibiotic resistance genes. Investigation of resistance (both bacteria and genes) in adult flamingos would be an important component of future research efforts, given previously noted differences between juvenile and adult birds.

Suggestions for future research (summarised):

- Analysis of environmental samples from each of the sites, to evaluate the prevalence of resistant bacterial isolates as well as antibiotic residues, to determine the mechanisms by which resistance is either transferred to, or arises within sites
- Analysis of the prevalence of antibiotic resistance genes in the Greater Flamingo
- Evaluation of samples from adult flamingos (in addition to flamingo chicks) to investigate variation in antibiotic resistance of specific bacterial assemblages with age, given the suggestion that younger birds have more transient bacteria that later stabilise (Dongen *et al.*, 2013). As well, analysis of the microbial flora of adult Greater Flamingos is interesting to consider with reference to their movements between sites (i.e. metapopulation dynamics), and the potential transfer of resistant bacterial species.

The importance of embracing and using new technology and methodologies in future efforts will be critical. The use of functional metagenomics, for example, has been cited as a novel technique with which to investigate antibiotic resistance genes that has recently been used in

research on resistance in gulls (Martiny *et al.*, 2012). Together, these additional investigations would serve to provide a more comprehensive understanding of the mechanisms by which wild birds acquire antibiotic resistance, which is important in understanding the potential for transmission to human populations.

5.7 References

Aisenberg, G., Rolston, K.V., Safdar, A. 2004. Bacteremia caused by Achromobacter and Alcaligenes species in 46 patients with cancer (1989–2003). Article first published online: 23 SEP 2004. DOI: 10.1002/cncr.20604

Abulreesh, H.H., Goulder, R. and Scott, W.B. 2007. Wild birds and human pathogens in the context of ringing and migration. Ringing and Migration **23,** 193-20 DOI:10.1080/03078698.2007.9674363

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J. and Handelsman, J. 2010. Call of the wild: antibiotic resistance genes in natural environments. Nature Reviews Microbiology **8,** 251-259. doi:10.1038/nmicro2312

Amat, J. A., Rendón, M. A., Rendón-Martos, M., Garrido, A., & Ramírez, J. M. 2005. Ranging behaviour of greater flamingos during the breeding and post-breeding periods: linking connectivity to biological processes. Biological Conservation, **125**, 183-192.

Bala, M., Ray, K. & Gupta, S.M. 2005. Comparison of disc diffusion results with minimum inhibitory concentration (MIC) values for antimicrobial susceptibility testing of Neisseria gonorrhoeae. J Med Res **122**, 48-51

Balkiz, Ö., Özesmi, U., Pradel, R., Germain, C., Siki, M., Amat, J.A., Rendón-Martos, M., Baccetti, N. and Béchet, A. 2009. Range of the Greater Flamingo, *Phoenicopterus roseus,* metapopulation in the Mediterranean: new insights from Turkey. Journal of Ornithology **148,** 347-355.

Balkiz, O., Béchet, A., Rouan, L., Choquet, R., Germain, C., Amat, J.A., Rendón-Martos, M., Baccetti, N., Nissardi, S., Özesmi, U. and Pradel, R. 2010. Experience-dependent natal philopatry of breeding greater flamingos. Journal of Animal Ecology **79,** 1045-1056.

Banks, J.C., Cary, S.C. and Hogg, I.D. 2009. The phylogeography of Adelie penguin faecal flora. Environmental Microbiology **11,** 577-588.

Béchet., A., Germain, C., Sandoz, A., Hirons, G.J.M., Green, R.E., Walmsley, J.G. and Johnson, A.R. 2009. Assessment of the impacts of hydrological fluctuations and salt pans abandonment on Greater Flamingos in the Camargue, South of France. Biodiversity Conservation **18,** 1575-1588.

Benskin, C. M. H., Wilson, K., Jones, K. and Harley, I.R. 2009. Bacterial pathogens in wild birds: a review of the frequency and effects of infection. Biological Reviews **84,** 349-373.

Berger, S., Disko, R. and Gwinner, H. 2003. Bacteria in starling nests. Journal of Ornithology **144,** 317-322.

Blanco, G., Lemus, J.A., Grande, J., Gangoso, L., Donázar, J.A., Arroyo, B., Frías, O. and Hiraldo, F. 2007. Geographical variation in cloacal microflora and bacterial antibiotic resistance in a threatened avian scavenger in relation to diet and livestock farming practices. Environmental Microbiolgy **9.** 1738-1749.

Borghesi., F., Andreotti, A., Baccetti, N., Bianchi, N., Birke, M., Migani, F. and Dinelli, E. 2011. Flamingo feathers to monitor the presence of mercury at six Mediterranean sites. Chemistry and Ecology **27,** 137-151.

Boucheker, A., Samraoui, B., Prodon, R., Amat, J.A., Rendón-Martos, M., Baccetti, N., I Esquerre, F.V. Nissardi, S., Baliz, Ö., Germain, C., Boulkhssai, M. and Béchet, A. 2011. Connectivity between the Algerian population of Greater Flamingo *Phoenicopterus roseus* and thos of the Mediterranean basin. Ostrich: Journal of African Ornithology **82,** 167-174.

Boyce, W.M., Sandrock, C., Kreuder-Johnson, C., Kelly, T. and Cardona, C. 2009. Avian influenza viruses in wild birds: A moving target. Comparative Immunology, Microbiology and Infectious Diseases **32**, 275-286.

Brittingham, M.C., Temple, S.A. and Duncan, R.M. 1988. A survey of the prevalence of selected bacteria in wild birds. Journal of Wildlife Disease **24,** 299-307.

Brooke, J.S. 2012. Stenotrophomonas maltophilia: an Emerging Global Opportunistic Pathogen **25,** doi: 10.1128/CMR.00019-11 Clin. Microbiol. Rev. January 2012 vol. 25 no. 1 2-41

Busse, H.-J. and Stolz, A. 2006. The Prokaryotes Achromobacter, Alcaligenes and Related Genera pp 675-700

Casey, J. A., Cosgrove, S. E., Stewart, W. F., Pollak, J., & Schwartz, B. S. 2013. A population-based study of the epidemiology and clinical features of methicillin-resistant Staphylococcus aureus infection in Pennsylvania, 2001–2010. Epidemiology and Infection **141**, 1166-1179.

Clinical and Laboratory Standards Institute (CLSI), 2013. Performance standards for antimicrobial susceptibility testing: 23rd informational supplement (M100-S23). CLSI, Wayne, PA.

Coenye, T., Vancanneyt, M., Falsen, E., Swings, J. and Vandamme, P. 2003. *Achromobacter insolitus* sp. nov. and *Achromobacter spanius* sp. nov., from human clinical samples. International Journal of Systematic and Evolutionary Microbiology **53,** 1819-1824. doi: 10.1099/

Cole, D., Drum, D. J., Stallknecht, D. E., White, D. G., Lee, M. D., Ayers, S., ... & Maurer, J. J. 2005. Free-living Canada Geese Antimicrobial Resistance. Emerging Infectious Diseases **11**.

Costanzo, S.D., Murby, J. and Bates, J. 2005. Ecosystem response to antibiotics entering the aquatic environment. Marine Pollution Bulletin **51,** 218-223.

da Costa, P.M., Loureiro, L. and Matos, A.J.F. 2013. Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans, animals, and the environment. International Journal of Environmental Research and Public Health **10,** 278-294.

Dobbin, G., Hariharan, H., Daoust, P.-Y., Hariharan, S., Heaney, S., Coles, M., Price, L. and Muckle, C.A. 2005. Bacterial flora of free-living Double-crested cormorant (*Phalacrocorax auritus*) chicks on Prince Edward Island, Canada, with reference to bacteria and antibiotic resistance. Comparative Immunology, Microbiology and Infectious Disease **28,** 71-82.

Dolejska, M., Cizek, A. & Literak, I. 2007. *High prevalence of antimicrobial-resistant genes and integrons in* Escherichia coli *isolates from black-headed gulls in the Czech Republic.* Journal of Applied Microbiology **103,** 11-19.

Drancourt, M., Bollet, C., Carlioz, A., Martelin, R., Gayral, J.P., and Raoult, R. 2000.. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. Journal of Clinical Microbiology **38**, 3623–3630.

Dyar, O.J., Hoa, N.Q., Trung, N.V., Phuc, H.D., Larsson, M., Chuc, N.T.K. and Lundborg, C.S. 2012. High prevalence of antibiotic resistance in commensal *Escherichia coli* among children in rural Vietnam. BMC Infectious Disease **12**:92. DOI: 10.1186/1471-2334-12-92.

Ernoul, L., Mesléard, F., Gaubert, P. and Béchet, A. 2013. Limits to agri-environmental schemes uptake to mitigate human-wildlife conflict: lessons learned from Flamingos in the

Camargue, southern France. International Journal of Agricultural Sustainability. DOI: 10.1080/14735903.2013.798897.

EUCAST. 2013. European Committee on Antimicrobial Susceptibility Testing, Breakpoint tables for interpretation of MICs and zone diameters. Available online at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_3.1.pdf

Falcucci, A., Maiorano, L., & Boitani, L. 2007. Changes in land-use/land-cover patterns in Italy and their implications for biodiversity conservation. Landscape Ecology **22**, 617-631.

Ferraro, M.J. 2001. Should we reevaluate antibiotic breakpoints? CID **Supplement 3,** S227-S229.

Geraci, J., Béchet, A., Cézilly, F., Ficheuz, S., Baccetti, N., Samraoui, B. and Wattier, R. 2012. Greater flamingo colonies around the Mediterranean form a single interbreeding population and share a common history. Journal of Avian Biology **43,** 341-354.

Good, R.C. 1985. Opportunistic Pathogens in the Genus Mycobacterium. Annual Review of Microbiology **39**, 347-369.

Guenther, S., Grobbel, M., Lubke-Becker, A., Goedecke, A., Friedrich, N.D., Wieler, L.H., Ewers, C. 2009. Antimicrobial resistance profiles of E. coli from common European wild bird species, Veterinary Microbiology (2008), doi:10.1016/j.vetmic.2009.12.016

Hombach, M., Mouttet, B. and Bloemberg, G.V. 2013. Consequences of revised CLSI and EUCAST guidelines for antibiotic susceptibility patterns of ESBL- and AmpC β-lactamase-producing clinical Enterobacteriaceae isolates. Journal of Antimicrobial Chemotherapy **68,** 2092-2098

Hubalek, Z. 2004. An annotated checklist of pathogenic microorganisms associated with migratory birds. Journal of Wildlife Diseases **40,** 638-659.

Hupton, G., Portocarrero, S., Newman, M. And Westneat. D.F. 2003. Bacteria in the reproductive tracts of red-winged blackbirds. The Condor **105,** 453-464.

Iverson, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R. and Joosten, H. 2007. The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter* genomospecies. BMC Evolutionary Biology **7:**64. doi:10.1186/1471-2148-7-64

Jordain, E., Gauthier-Clerc, M., Bicout, D. and Sabatier, P. 2007. Bird migration routes and risk for pathogen dispersion into Western Mediterranean Wetlands. Emerging Infectious Diseases **13,** 365-372.

Jorgensen, J.H. and Ferraro, M.J. 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clinical Infectious Diseases **49,** 1749-1755. doi: 10.1086/647952

Kahlmeter, G., Brown, D.F.J., Goldstein, F.W., MacGowan, A.P., Mouton, J.W., Österlund, A., Rodloff, A., Steinbakk, M., Urbaskova, P. and Vatopoulos, A. 2003. European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. Journal of Antimicrobial Chemotherapy **52,** 145-148.

Klomp, J.E., Murphy, M.T., Smith, S.B., McKay, J.E., Ferrera, I. and Reysenbach, A.-L. 2008. Cloacal microbial communities of female spotted towhees *Pipilo maculates*: microgeographic variation and individual sources of variability. Journal of Avian Biology **39,** 530-538.

Kohl, K.D. 2012. Diversity and function of the avian gut microbiota Journal of Comparative Physiology B **182,** 591-602. doi: 10.1007/s00360-012-0645-z.

Kuper, K.M., Boles, D.M., Mohr, J.F. and Wanger, A. 2012. Antimicrobial susceptibility testing: a primer for clinicians. Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy **29**, DOI: 10.1592/phco.29.11.1326

Lambiase, A., Rossano, F., Del Pezzo, M., Raia, V., Sepe, A., de Gregorio, F., and Catania, M.R. 2009. Sphingobacterium respiratory tract infection in patients with cystic fibrosis BMC Research Notes, 2:262 doi:10.1186/1756-0500-2-262

Levy, S.B. 1997. Antibiotic resistance: an ecological imbalance. Antibiotic Resistance: Origins, Evolution, Selection, and Spread. CIBA Foundation Symposium. John Wiley and Sons, West Sussex, England. 250pp.
Literak, I., Vanko, R., Dolejská, M.,. Čížek, A. and Karpíšková, R. 2007. Antibiotic resistant *Escherichia coli* and *Salmonella* in Russian rooks (*Corvus frugilegus*) wintering in the Czech Republic. Letters in Applied Microbiology **45,** 616-621.

Literak, I., Dolejska, M., Janoszowska, D., Hrusakova, J., Meissner, W., Rzyska, H., Bzoma, S. and Cizek, A. 2010. Antibiotic-resistant *Escherichia coli* bacteria, including strains with genes encoding the Extended-Spectrum Beta-Lactamase and QnrS, in waterbirds on the Baltic sea coast of Poland. Applied Environmental Microbiology **76,** 8126-8134.

Livermore, D.M. 2002. Multiple mechanisms of antibiotic resistance in *Pseudomonas aeruginosa: our worse nightmare?* Clinical Infectious Diseases **34,** 634-640.

Lombardo, M.P., Thorpe, P.A., Cichewicz, R., Henshaw, M., Millard, C., Steen, C. and Zeller, T.K. 1996. Communities of cloacal bacteria in tree swallow families. The Condor **98,** 167-172.

Lombardo, M.P., Thorpe, P.A. and Power, H.W. 1999. The beneficial sexually transmitted microbe hypothesis of avian copulation. Behavioural Ecology **10, 333-337.**

Lu, J., Domingo, J.W.S. and Hill, S. 2008. Phylogenetic diversity and molecular detection of bacteria in gull feces. Applied and Environmental Microbiology **74,** 3969-3976.

Lucas, F.S. and Feeb, P. 2005. Environmental factors shape cloacal bacterial assemblages in great tit *Parus major* and blue tit *P. C caeruleus* nestlings. Journal of Avian Biology **36,** 510-516. DOI: 10.1111/j.0908-8857.2005.03479.x

Macrae, A. 2000. The use of 16S rDNA methods in soil microbial ecology. Brazilian Journal of Microbiology **31,** 77-82.

Maldonado, L.A., Stach, J.E.M., Pathom-aree, W., Ward, A.C., Bull, A.T. and Goodfellow, M. 2005. Diversity of cultivable actinobacteria in geographically widespread marine sediments. Antonie van Leeuwenhoek **87,** 11-18.

Martinez, J. L. 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environmental Pollution **157**, 2893-2902.

Martiny, A.C., Martiny, J.B.H., Weihe, C., Field, A. and Ellis, J.C. 2011. Functional metagenomics reveals previously unrecognized diversity of antibiotic resistance genes in gulls. Frontiers in Ecology and Evolution **2,** doi:10.3389/fmicb.2011.00238

Mathevet, R., Tourenq, C. and Mesléard, F. 2002. Agricultural policies, land-use and waterbird conservation: the case study of a major Mediterranean wetland, the Camargue. European Journal of Geography, Environment, Nature, Paysage, document 221. DOI: 10.4000/cybergeo.3755.

Matsuyama, H., Katoh, H., Ohkushi, T., Satoh, A., Kawahara, K., and Yumoto, I. 2008. Sphingobacterium kitahiroshimense sp. nov., isolated from soil. International Journal of Systematic and Evolutionary Microbiology **58,** 1576-1579.

Matyar, F., Akkan, T., Uçak, Y. and Eraslan, B. 2010. *Aeromonas* and *Pseudomonas*: antibiotic and heavy metal resistance species from Iskenderun Bay, Turkey (northeast Mediterranean Sea). Environmental Monitoring and Assessment **167,** 309-320.

Mills, T.K., Lombardo, M.P. and Thorpe, P.A. 1999. Microbial colonization of the cloacae of nestling tree swallows. The Auk **116,** 947-956.

Mulet, M., Lalucat, J., García-Valdés, E. 2010. DNA sequence-based analysis of the Pseudomonas species DOI: 10.1111/j.1462-2920.2010.02181.x

Nager, R.G., Johnson, A.R., Boy, V. Rendon-Martos, M., Calderon, J. and Cézilly, F. 1996. Temporal and spatial variation in dispersal in the greater flamingo (*Phoenicopterus ruber roseus*). Oecologia **107,** 204-211.

O'Brien, T.F. 2002. Antimicrobial resistance: how use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. Clinical Infectious Diseases **34,** S78-S84. doi: 10.1086/340244

Oliveira, M., Santos, V., Fernandes, A., Nunes, S.F., Bernado, F. And Vilela, C.L. 2010. Pitfalls of antimicrobial susceptibility testing of enterococci isolated from farming broilers by the disk diffusion method. In: Mendez-Vilas, A. (ed) Current Research, Technology and Education Topics in Applied Microbiolgy and Microbial Biotechnology, vol 2. Formatex: pp. 415-418.

Patel, J.B., Leonard, D.G.B., Pan, X., Musser, J.M., Berman, R.E. and Nachamkin, I. 2000. Sequence-based identification of *Mycobacterium* species using the MicroSeq 500 rDNA bacterial identification system. Journal of Clinical Microbiology **38,** 246-251.

Pereira, A., Santos, A., Tacão, M., Alves, A., Henriques, I. and Correia, A. 2013. Genetic diversity and antimicrobial resistance of Escherichia coli from Tagues estuary (Portugal). Science of the Total Environment **461-462,** 65-71.

Piddock, L.J.V. 1990. Techniques used for the determination of antimicrobial resistance and sensitivity in bacteria. Journal of Applied Bacteriology **68**, 307-318. DOI: 10.1111/j.1365-2672.1990.tb02880.x

Porter Jr, R.E. 1998. Bacterial enteritides of poultry. Poultry Science **77,** 1159-1165.

Radhouani, H., Igrejas, G., Pinto, L., Gonçalves, A., Coelho, C., Rodrigues, J., & Poeta, P. 2011. Molecular characterization of antibiotic resistance in enterococci recovered from seagulls (Larus cachinnans) representing an environmental health problem. Journal of Environmental Monitoring, **13**, 2227-2233.

Radhouani, H., Poeta, P., Gonçalves, A., Pacheco, R., Sargo, R., & Igrejas, G. 2012. Wild birds as biological indicators of environmental pollution: antimicrobial resistance patterns of Escherichia coli and enterococci isolated from common buzzards (Buteo buteo). Journal of Medical Microbiology **61**, 837-843.

Radimersky, T., Frolkova, P., Janoszowska, D., Dolejska, M., Svec, P., Roubalova, E., Cikova, P. & Literak, I. 2010. *Antibiotic resistance in faecal bacteria (Escherichia coli, Enterococcus spp.) in feral pigeons.* Journal of Applied Microbiology **109,** 1687-1695.

Rendon, M.A., Green, A.J., Aguilera, E. and Almaraz, P. 2008. Status, distribution and long-term changes in the waterbird community wintering in Doñana, south–west Spain Biological Conservation

Rex, J.H. and Pfaller, M.A. 2002. Has Antifungal Susceptibility Testing Come of Age? Clinical Infectious Diseases **35**, 982-989. doi: 10.1086/342384

Rollin, P.E., Rollin, D., Baylet, R. and Walmsley, J.G. 1983. Bacterial and fungal digestive flora of herring gulls (*Larus argentatus*) in the Camargue. Avian Pathology **12,** 147-150.

Sacchi, C.T., Whitney, A.M., Mayer, L.W., Morey, R., Steigerwalt, A., Boras, A., Weyant, R.S. and Popovic, T. 2002. Sequencing of 16S rRNA gene: a rapid tool for identification of *Bacillus anthracis.* Emgerging Infectiuos Diseases **8,** 1117-1123. doi: 10.3201/eid0810.020391

Santos, S.S., Pardal, S., Proença, D.N., Lopes, R.J., Ramos, J.A., Mendes, L. and Morais, P.V. 2012. Diversity of cloacal microbial community in migratory shorebirds that use the Tagus estuary as stopover habitat and their potential to harbour and disperse pathogenic microogranisms. FEMS Microbiology Ecology **82,** 63-74.

Sato, M., Ahmed, A.M., Noda, A., Watanabe, H., Fukumoto, Y. and Shimamoto, T. 2009. Isolation and molecular characterisation of multidrug-resistant Gram-negative bacteria from imported flamingos in Japan. Acta Veterinaria Scandinavica **51.**

Schwartz, T., Kohnen, W., Jansen, B., & Obst, U. 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiology Ecology **43**, 325-335.

Seimenis, A.M. 2008. Zoonotic diseases in the Mediterranean region: a brief introduction. Veterinaria Italiana **44,** 573-576.

Shawkey, M.D., Mills, K.L., Dale, C. and Hill, G.E. 2005. Microbial Diversity of Wild Bird Feathers Revealed through Culture-Based and Culture-Independent Techniques. Microbial Ecology **50,** 40-47.

Singleton, D.R. and Harper, R.G. 1998. Bacteria in old house wren nests. Journal of Field Ornithology **69,** 71-74.

Sjölund, M., Bonnedahl, J., Hernandez, J., Bengtsson, S., Cederbrant, G., Pinhassi, J., ... & Olsen, B. 2008. Dissemination of multidrug-resistant bacteria into the Arctic. Emerging Infectious Diseases **14**, 70.

Soler, J.J., Martín-Vivaldi, M., Peralta-Sánchez, J.M., Ruiz-Rodríguez, M. 2010. Antibiotic-Producing Bacteria as a Possible Defence of Birds against Pathogenic Microorganisms. The Open Ornithology Journal **3**, 93-100 93 1874-4532/10 2010 Bentham Open

Spiers, A., Buckling, A. and Rainey, P.B. 2000. The causes of *Pseudomonas* diversity. Microbiology **146,** 2345-2350.

Spilker, T., Vandamme, P. and Lipuma, J.J. 2012. A multilocus sequence typing scheme implies population structure and reveals putative novel Achromobacter species. Journal of Clinical Microbiology **50,** 3010-3015.

Stern, N.J., Meinersmann, R.J., Cox, N.A., Bailey, J.S. and Blankenship, L.C. 1990. Influence of host lineage on cecal colonization by *Campylobacter jejuni* in chickens. Avian Diseases **34,** 602-606.

Sunqvist, M. 2010. Antibiotic resistance and population dynamics of *Escherichia cola* in relation to a large scale antibiotic consumption intervention. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 512. 78 pp. Uppsala.

Tourenq, C., Aulagnier, S., Durieux, L., Lek, S., Mesléard, F., Johnson, A. and Martin, J.-L. 2001. Identifying rice fields at risk from damage by the greater flamingo. Journal of Applied Ecology **38,** 170-179.

Tsubokura, M., Matsumoto, A., Otsuki, K., Animas, S. B., & Sanekata, T. 1995. Drug resistance and conjugative R plasmids in Escherichia coli strains isolated from migratory waterfowl. Journal of Wildlife Diseases **31**, 352-357.

Turnidge, T. and Paterson, D.L. 2007. Setting and revising antibacterial susceptibility breakpoints. Clinical Microbiology Reviews **20,** 391-408.

van der Bij, A.K., van Dijk, K., Thijsen, S.F.T., Notermans, D.W., de Greeff, S. and van de Sande-Bruinsma, N. 2012. Clinical breakpoint changes and their impact on surveillance of antimicrobial resistance in *Escherichia coli* causing bacteraemia. Clinical Microbiology and Infection 18, 466-472. DOI: 10.1111/j.1469-0691.2012.03996.x

van Dongen, W.F.D., White, J., Brandl, H.B., Moodley, Y., Merkling, T., Leclaire, S., Blanchard, P., Danchin, E., Hatch, S.A. and Wagner, R.H. 2013. Age-related differences in the cloacal microbiota of a wild bird species. BMC Ecology **13**:11, doi:10.1186/1472-6785-13-11.

Vittecoq, M., Thomas, F., Jourdain, E., Moutou, F., Renaud, F. and Gautheir-Clerc, M. (in press). Risks of emerging infectious diseases: evolving threats in a changing area, the Mediterranean Basin. Transboundary and Emerging Diseases. DOI: 10.1111/tbed.12012

Walker, S. E., Sander, J. E., Cline, J. L., & Helton, J. S. 2002. Characterization of Pseudomonas aeruginosa isolates associated with mortality in broiler chicks. Avian Diseases **46**, 1045-1050.

White, J., Mirleau, P., Danchin, E., Mulard, H., Hatch, S.A., Heeb, P. and Wagner, R.H. 2010. Sexually transmitted bacteria affect female cloacal assemblages in a wild bird. Ecology Letters **13,** 1515-1524.

Wiegand, I., Hilpert, K. and Hancock, R.E.W. 2008. Agar and broth dilution methods to determine the minimum inhibitory concentration of antimicrobial substances. Nature Protocols **3,** 163-170.

Woo, P.C., Lau, S.K., Teng, J.L., Tse, H. and Yuen, K.Y. 2008. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clinical Microbiology Infections **14**, 908-934. doi: 10.1111/j.1469-0691.2008.02070.x.

6 Nearly 70 years on from one man's 'note of warning' on antibiotic resistance: where are we now, and what is the way forward?

6.1 Chapter overview

The value of this thesis lies in its multi-faceted approach, involving both desk- and field-based methodologies, which has allowed for the exploration of a variety of aspects of antibiotic resistance in wild birds. In order to fully realize the benefits of this approach, this chapter will bring together the findings of each of the separate investigations. Examination of where the respective methodologies have yielded similar findings, as well as consideration of apparent differences, is important in identifying the overall implications of this research. The first section provides a synthesis of the notable research findings as presented in the previous chapters. In the section that follows, the implications for human health of these findings are reviewed with reference to the wider body of research on antibiotic resistance. Methodological limitations of this thesis are then discussed, in providing suggestions for future research. This chapter – and indeed, this thesis in its entirety – then concludes with a look to the future, in addressing the question: what is the way forward?

6.2 Synthesis of research chapters

This dissertation began with the story of 'mould juice' – the serendipitous discovery of penicillin by Alexander Fleming in 1928. Less than twenty years later, just as the 'era of antibiotics' was gaining momentum, Fleming was interviewed for an article that appeared in the New York Times in June 1945. In the prescient piece, he suggested that, "microbes [can be] educated to resist penicillin" (Fleming, cited in Edqvist and Pedersen, 2001). Six months later, he delivered a lecture in accepting his Nobel Prize. At the end of his address, Fleming again cited the potential for the development of resistance to penicillin, the use of which was increasing in a clinical context (Rosenblatt-Farrell, 2009; Spellberg, 2011).

> "But I would like to sound one note of warning. There may be a danger in underdosage. It is not difficult to make microbes resistant to penicillin...The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant."

That the very man who first discovered antibiotics issued a warning about their potential failure so soon after their use had commenced was notable. However, due to the coincidence of several factors – including a pharmaceutical industry that eagerly seized the opportunity for maximizing profits, and the subsequent expansion of the use of antibiotics to include

applications in a variety of agricultural contexts – his warning was not taken on board (Hall, 1994; Kunin, 2008; Edqvist and Pedersen, 2001). As we learned in Chapter Two, in the decades that followed the discovery of penicillin and its subsequent introduction to the consumer market, the use of antibiotics increased dramatically as new ones were developed. As Fleming predicted in his lecture, it was not long before antibiotics were readily available on the consumer market, and as had occurred with penicillin, resistances to almost all of the emerging antibiotics were identified within years of their introduction (Davies and Davies, 2010; Wellington *et al.*, 2013). In 1969, the 'Swann Committee' was formed in the U.K. to investigate reported links between the use of antibiotics in veterinary agriculture and a reported spread of antibiotic resistance; they concluded that a significant problem existed (Wise, 2007). Within several years, scientific research began to provide case-study evidence of antibiotic resistant bacteria outside of clinical environments, in a diverse range of environmental systems across the globe (Allen *et al.*, 2010).

Since then, a large body of research has demonstrated the prevalence of antibiotic resistant bacteria in both the biotic and abiotic components of environmental systems. In doing so, this research has suggested pathways and mechanisms of transfer by which antibiotic resistant bacteria, resistance genes, and antibiotics (and residues) themselves can persist in a given environment (Finley *et al.*, 2013). Much of this research has investigated the extent to which wildlife species serve as reservoirs of resistance, and study after study has provided evidence indicating that resistance is widespread (Allen, 2010). However, much remains to be determined with regard to the factors that affect the prevalence of, and patterns in, resistance at regional and global scales. Nearly seventy years after Fleming voiced his concerns, this thesis has used a variety of research methodologies to address several of the remaining questions, with specific attention to wild birds as reservoirs of antibiotic resistant bacteria and including a focused evaluation of the Greater Flamingo.

6.2.1 Antibiotic resistance in wild birds

The research framework used here – which consisted of desk-based research along with fieldwork and laboratory-based analyses – has provided insights into the prevalence of antibiotic resistance in wild birds at a variety of spatial scales. The meta-analysis provided an overall picture of the patterns of resistance in wild birds on a global scale, which could be explored with field-based research on a regional scale through use of the Greater Flamingo. Indeed, the data provided by fieldwork and laboratory analysis of bacterial samples from wild Greater Flamingos have contributed new, comprehensive, and compelling evidence of the prevalence of antibiotic resistant bacteria at five of its breeding sites across the Mediterranean region. Together, the three chapters provide the opportunity for consideration of variables

affecting antibiotic resistance in environmental systems, including taxonomic, geographic, and landscape (i.e. ecosystem) factors.

In the third chapter, a meta-analysis approach was used to evaluate antibiotic resistance in wild birds at a global scale. Despite the recognition of wild birds as important vectors of infectious disease – and numerous research efforts that have provided case-study evidence of wild birds as reservoirs of antibiotic resistant bacteria – a comprehensive understanding of the prevalence, patterns, and factors affecting antibiotic resistant bacteria in wild birds has been lacking. In this chapter, these shortcomings were addressed by combining the results of previous research efforts to evaluate antibiotic resistance in wild birds with reference to a variety of factors. The results of this meta-analysis provided evidence of widespread resistance in a variety of bird species across the globe, which inhabit terrestrial, freshwater, and/or marine systems. In comparing the overall prevalence of resistance between the ten different antibiotics used in the meta-analysis, ampicillin demonstrated a significantly higher prevalence of resistance than five of the nine other antibiotics assessed. Interestingly, this corresponded with the results of the preliminary analysis of antibiotic resistance in the Greater Flamingo (presented in Chapter Four), in which ampicillin was found to be the antibiotic to which the highest proportion of samples demonstrated resistance. Together, these findings suggest that ampicillin resistance is particularly widespread, and that understanding the factors that might be driving this are important to investigate in more detail. Trimethoprim-sulfamethoxazole was the antibiotic with the lowest overall proportion of resistance demonstrated to it in the meta-analysis (it was significantly lower than ampicillin and tetracycline). This confirms the finding of the preliminary analysis of resistance in the Greater Flamingo, in which trimethoprim-sulfamethoxazole was one of the antibiotics to which the lowest proportion of samples demonstrated resistance. Assessing the factors that might be underlying the variation demonstrated to different antibiotics can provide interesting insights into overall processes that affect resistance in environmental systems.

6.2.2 Factors affecting the prevalence of antibiotic resistance in environmental systems

One of the more interesting findings of the meta-analysis was that the Falconiformes were an order with a significantly higher prevalence of resistance than other orders of birds investigated. Relating the results of the investigation of resistance in the Greater Flamingo to this taxonomic subgroup analysis of the meta-analysis was complicated by the fact that, along with the five other species of flamingo, the Greater Flamingo comprises its own order, the Phoenicopteriformes, which was not represented in the meta-analysis, because no research on flamingos was amongst the included studies (IUCN, 2013). Thus, direct comparisons could not be made with reference to taxonomic factors, though it is interesting to consider the extent to which food chain dynamics might affect variation between orders (given the suggestion that

the Falconiformes demonstrated a significantly higher prevalence of resistance because the order is comprised of predators and/or scavengers, as discussed in the meta-analysis chapter). Only recently has research begun to investigate the fate of antibiotics in aquatic and terrestrial systems with reference to its effect within the dynamics of food chains, so its specific impacts with reference to taxonomy remain to be determined (Boxall and Ericson, 2012). Some of the most recent evidence has indeed suggested that one effect of antibiotics in environmental systems might be an increased occurrence of antibiotic resistance corresponding with the increasing levels of the food chain, so this is clearly an area for which further research is required (Boonsaner and Hawker, 2013).

An interesting, if unexpected, finding of the meta-analysis was that there was very little significant variation between the prevalence of resistance within the different environmental systems (i.e. terrestrial, freshwater, and marine). This might suggest that in general, birds inhabiting all systems are equally likely to serve as reservoirs of antibiotic resistant bacteria, given the extent to which terrestrial and aquatic systems are linked (Schneider *et al.*, 2002). The relative influence of different landscape factors, such as land cover, may thus be negligible. Landscape factors were investigated empirically by taking bacterial samples from wild Greater Flamingos at multiple sites that were within a variety of landscape matrices, while keeping other factors constant (e.g. the bird species, methodologies, etc.). To achieve a better understanding of the impact of landscape on the prevalence of resistance, the results of the preliminary screening of bacterial samples from the Greater Flamingo were analysed via GIS analysis of land cover data provided by the EU CORINE programme. The results of this suggested that for only four antibiotics (ampicillin, erythromycin, kanamycin, and tetracycline), did land cover variables at certain scales (assessed with reference to 1km, 5km, and 10km buffers around each of the sites) predict antibiotic resistance. The lack of many significant results of land cover – and their inconsistency – provides further evidence that it is likely that environmental systems as reservoirs of antibiotic resistant bacteria are largely homogenous given the numerous pathways which link them. Similarly, that very little significant variation between continents was found in the meta-analysis of antibiotic resistance in wild birds suggests that pathways have led to the spread of resistant bacteria. Certain areas have been described as potential 'hotspots' for the development of resistance – and it is possible that when it first arises, resistance varies significantly with reference to geographic factors. A recent study led by the EU PHARMAS project has provided some insights into spatial variation of the risks of pharmaceutical pollution in aquatic environments, which included consideration of eleven antibiotics (Oldenkamp *et al.*, 2013). The output of the study is a map that indicates which regions have the highest level of risk; in most cases, these correspond to areas with high populations and/or pharmaceutical prescription rates. Interestingly, within Europe, the Mediterranean was described as being at a relatively high risk (Oldenkamp *et al.*, 2013). The high prevalence of antibiotic resistance found in analysis of the Greater Flamingo makes

sense in light of this recent evidence. Taken together, the results presented in this thesis as derived from the multiple approaches demonstrate the extent to which wild birds serve as reservoirs of antibiotic resistant bacteria, and illustrate the need for consideration of the implications for human health. Both within the meta-analysis, and in considering the results of the Greater Flamingo analyses, it is clear that the potential implications for human health are wide-ranging. This is addressed, with reference to relevant literature, in the following section.

6.3 Implications for human health

Research on antibiotic resistant bacteria in wildlife is critical in evaluating the extent to which environmental systems may serve as reservoirs of resistance. Given noted pathways between wildlife and humans, knowledge on the extent to which environmental reservoirs might exist will help project future threats to health (McCallum *et al.*, 2004).

The research conducted for this thesis has provided evidence of threat to human health on both regional and global scales. Specific bacterial genera and species were identified through a desk-based meta-analysis approach, which indicated that wild birds serve as reservoirs for a range of resistant bacterial species worldwide. Empirical evidence was provided by fieldwork-based approach, in which bacterial samples were acquired from wild Greater Flamingos in the Mediterranean region. Analysis of these samples indicated widespread resistance to eight antibiotics, while DNA identification of resistant isolates provided details on the specific bacteria that were found to be resistant. Together, these approaches have provided a list of bacteria that have demonstrated resistance to a variety of antibiotics, many of which are potentially pathogenic in a human health context. DNA identification of resistant isolates from the Greater Flamingo in the fifth chapter indicated that *E. coli*, *Enterococci*, and *Pseudomonas* were amongst the isolates that frequently demonstrated resistance. The meta-analysis provided evidence that suggested that this problem of antibiotic resistant bacteria is not limited to the Greater Flamingo, nor within the Mediterranean region, given that similar bacteria genera and species were found to demonstrate resistance in multiple species of wild birds. Taken together, these findings provide robust evidence of bacteria that, if transferred to human populations, would be cause for concern. Further insights into the potential threat to human health were provided with the results of MIC determination, which involved a more detailed analysis of antibiotic resistance than that achieved in the preliminary screening. The results of this analysis provided evidence for a range of bacterial genera and species that resistance was at levels that were equivalent to or greater than the clinical breakpoints. Although breakpoints were not considered in the statistical analysis component of the meta-analysis (studies used various criteria in defining a baseline or cut-off for 'resistance'), the discussions of the chapters were reviewed, which indicated that the authors of the included studies concluded that there was a threat to human health.

The threat to human health cannot be ignored. In the past decade, overwhelming evidence linking human activity to the prevalence of antibiotic resistant bacteria and resistance genes has emerged (Guenther *et al.*, 2011). In mid-September 2013, the United States Centers for Disease Control and Prevention (CDC) issued a report on antibiotic resistance in which, citing an estimated 23,000 deaths per annum (in the United States), they warned of "potentially catastrophic consequences" of antibiotic resistance. This call to action echoed a similar statement earlier this year by the U.K.'s Chief Medical Officer Dame Sally Davies, in which she described antibiotic resistance as a catastrophic threat. Cooper and Shlaes (2011) suggest that the lack of new antibiotic development will result in the death of 'millions' per annum as a result of drug resistant infections.

6.4 What is the way forward? A global perspective

The urgency of the problem became apparent in 2009 as indicated in a technical report, produced by a joint working group consisting of the European Centre for Disease Prevention and Control (ECDC) and the European Medicines Agency (EMEA) (ECDC, 2009). The report aimed to highlight the disparity between the growing rate of antibiotic resistance in Europe and the decline in drug research and development – a gap that they asserted "threaten[s] to take us back to the pre-antibiotic era". A similar sentiment was expressed by the aforementioned Dame Sally Davies, who has asserted that, "If we don't get this right we will find ourselves in a health system not dissimilar to the early 19th century" (cited in Torjesen, 2013). It should be noted that this is not the first time that a call to action has been sounded. In a widely cited *Science* paper that appeared in 1992, Dr. Harold Neu, an infectious disease expert who specialized in antibiotics and antibiotic resistance, warned that both the scientific community and society had become complacent towards the threat of antibiotic resistance (Neu, 1992). Yet, more than two decades later, the problem persists; the theme of World Health Day in 2011 was antibiotic resistance, for which the slogan 'No Action Today, No Cure Tomorrow' was used (WHO, 2011). It is clear that action needs to be taken, but what is appropriate?

A seemingly obvious step would be the development of new antibiotics; as described in Chapter Two, the lack of new antibiotics has exacerbated (if not driven) the proliferation of antibiotic resistance. Lewis (2012) suggested that we return to the 'golden era' of antibiotic drug discovery and make an effort to renew research approaches that were at one time promising, but were for one reason or another abandoned. Wright and Sutherland (2007) also highlighted the potential that exists in renewing investigation of antibiotics that were previously discovered, but were not ultimately brought to market. However, there are several challenges with this approach, and the development of new antibiotics is only one of several options currently considered (Spellberg *et al.* 2013). As outlined in the second chapter, there are a

variety of reasons for which pharmaceutical companies have largely halted the research and development of novel antibiotics. Incentive schemes have been suggested as a mechanism that could promote antibiotic discovery (Towse and Sharma, 2011). Interestingly, the lack of investment in developing new antibiotics is a problem acknowledged by the drugs companies themselves: in calling for increased financial incentives for antibiotic research and development, GlaxoSmithKline (2012) cited a lengthy, expensive and highly regulated process that ultimately yields insufficient profits. It might be that an entirely new business model needs to be introduced (So *et al.*, 2011) In 2009, Theuretzbacher proposed that antimicrobial research and development inevitably occurs in waves, and at the time asserted that (after a period during which very little new research and development was conducted), a new wave of research was beginning. Three years on from this, however, the same author presented a review in which the current status of research and development was described as 'dry' (Theuretzbacher, 2012). The need for the development of legislation to address antimicrobial resistance – and inspire pharmaceutical companies to act – is apparent. In the United States, proposed acts include the Strategies to Address Antimicrobial Resistance (STAAR) and the Generating Antibiotic Incentives Now (GAIN) Acts. In 2011, the Infectious Diseases Society of America (ISDA) offered a list of recommended actions for the United States Congress. At present, however, many of the proposed or recommended legislative actions have not yet been enacted, and previous regulatory reform has not always been successful in getting pharmaceutical companies to act (Cooper and Shlaes, 2011; Shlaes *et al.*, 2013). Many legislative actions (such as these) are developed on a national basis. This will likely be ineffective at thwarting the global problem of antibiotic resistance, given evidence (from both the present research and the wider body of literature on the topic) that resistance readily spreads without regard to international borders.

The necessity of a global strategy has been referenced by individuals from a variety of sectors, including doctors, economists, and infectious disease experts (Cars, 2008; Palmer and Call, 2013; Spellberg *et al.*, 2013). Elinor Ostrom, a Nobel Laureate in Economic Sciences (2009) stated, "The issue is comparable to that of climate change in the sense that both phenomena involve non-renewable global resources, both are caused by human activity and are intrinsically linked to our behavior. The problem can only be addressed through international cooperation". Suggestions include the creation of collaborative multinational task forces, such as the Trans Atlantic Task Force on Antimicrobial Resistance. The World Health Organization has established the Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR), and routinely convenes groups of experts with the aim of minimizing health impacts and preventing health impacts in the future. Within a multinational approach, a multi-sector approach is also likely going to be key, and involve not only scientists and policy makers, but also researchers from a variety of disciplines (Palmer and Call, 2013; Spellberg *et al.*, 2013). Along these lines, the importance of understanding the underlying historical and

societal dimensions of this and other emerging infectious diseases is recognized (Morens *et al.*, 2004; Weiss and McMichael, 2004). The impact that antibiotics had on society throughout the middle of the 20th century served as a driving force behind the proliferation of their use and the resulting problem of antibiotic resistance as it exists today, and it is likely going to be a societal response that will ultimately provide a solution (Alanis, 2005; Davies and Davies, 2010).

6.5 Concluding remarks: #pinkisthenewblack

As first introduced at the start of this thesis, the impact of human activity on environmental systems has, throughout history, been wide ranging in both its nature and its impacts. Antibiotic resistance provides an interesting present-day example of how human activity serves to impact environmental systems, which can – via various pathways, systems, and networks – feedback and have unintended consequences for humans. The use of antibiotics in a variety of contexts over the past several decades has resulted in the emergence and spread of antibiotic resistant bacteria, antibiotic resistance genes, and antibiotic residues in environmental systems – which can adversely affect human health. The effects of these so-called 'reservoirs' in environmental systems are well documented in the scientific literature, which includes a large body of research on wildlife reservoirs of resistance. Despite this relatively large amount of research that has provided evidence of the breadth of the problem, and numerous assertions by experts from a variety of fields that the situation is critical, a solution is still lacking. It is therefore likely that an entirely new approach is required; that a paradigm shift is necessary (Cars *et al.*, 2008).

In the long term, the approach to antibiotic resistance needs to change. One of the problems noted in synthesizing the results of this thesis with the wider body of research on antibiotic resistance in wild birds, wildlife, and more generally, in environmental and urban systems, is that much of the research is effectively conducted (and presented) in silos – that wildlife biologists, clinicians, economists, and legislators often work in isolation from one another. Breaking down these silos by developing truly collaborative, multidisciplinary research efforts, may address some of the problems resulting from the typically separate research efforts. The One Health initiative offers a very useful framework within which antibiotic resistance can be approached (Palmer and Call, 2013) Along these lines, at present, there exist very few mechanisms by which research findings can be disseminated across different disciplines. In the same way that the meta-analysis conducted here served to bring together the results of multiple investigations to answer research questions about antibiotic resistance on a global scale, the development of a website may help achieve similar objectives on an even larger scale. A website at which researchers (from a variety of disciplines) could share their research

findings in relation to antibiotic resistance may provide a way of integrating and mapping emerging data, and could be developed with reference to input from the various stakeholders who would be involved under a One Health approach.

While these changes may, in the long term, prove to be a worthwhile approach, a short-term solution to raise public awareness is also required, and a new framework that capitalizes on society's potential to incite change is worthy of consideration. As a growing awareness of the 'miracle drug' phenomenon impacted society in the mid-20th century, the widespread occurrence of antibiotic resistance now needs to similarly affect society. Indeed, Carlet *et al.* (2011) suggested that it is society's failure to protect antibiotics as a resource that has led to the proliferation of resistance. Although the general public has a certain level of understanding of drug resistance as a result of news media coverage of MRSA and 'the superbugs', in addition to both small-scale educational campaigns within local communities and large-scale attempts at raising awareness (e.g. European Antibiotic Awareness Day, launched in 2008), antibiotics continue to be misused by patients and doctors alike and it is likely that the full scope of the problem (i.e. the prevalence of resistance in environmental systems) is less well understood (Trepka *et al.*, 2001; Levy, 2002; Earnshaw, 2009). Whereas in the mid-20th century, knowledge and awareness of antibiotics spread throughout that day's available media (consider, for example, the postbox advertisement for penicillin that was featured in Chapter 2), today we have the advantage of social media, which can make any number of items (from serious news articles to silly videos) 'go viral' and reach millions of people within hours across multiple platforms. As a charismatic species (for which underlying research is relatively inexpensive and easy to coordinate), there is potential for the flamingo to bring attention to the problem if it were to be used as a 'poster child' to raise awareness amongst society that the antibiotic resistance is not limited to hospitals, and that it is a problem with serious implications for human health. As the findings of this thesis have shown, the presence of antibiotic resistance in environmental systems is widespread, and has serious implications for human health. As a result, a novel approach to raising awareness on a large scale is critical, and the insights provided by this thesis may provide a mechanism by which this problem can be addressed.

6.6 References

Alanis, A.J. 2005. Resistance to antibiotics: are we in the post-antibiotic era? Infectious Diseases: Revisiting Past Problems and Addressing Future Challenges **36,** 697-705.

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J. and Handelsman, J. 2010. Call of the wild: antibiotic resistance genes in natural environments. Nature Reviews Microbiology **8,** 251-259. doi:10.1038/nrmicro2312.

Boonsaner, M. and Hawker, D.W. 2013. Evaluation of food chain transfer of antibiotic oxytetracycline and human risk assessment. Chemosphere **(in press).**

Boxall, A.B.A. and Ericson, J.F. 2012. Environmental fate of human pharmaceuticals. Human Pharmaceuticals in the Environment **4,** 63-83.

Carlet, J., Collignon, P., Goldmann, D., Goossens, H., Gyssens, I. C., Harbarth, S. *et al.* and Voss, A. 2011. Society's failure to protect a precious resource: antibiotics. The Lancet **378**, 369-371.

Cars, O., Högberg, L. D., Murray, M., Nordberg, O., Sivaraman, S., Lundborg, C. S., ... & Tomson, G. (2008). Meeting the challenge of antibiotic resistance. *Bmj*, *337*.

Centers for Disease Control and Prevention (CDC). 2013. Antibiotic resistance threats in the United States, 2013. Available online at: http://www.cdc.gov/drugresistance/threat-report-2013/. Accessed 29 September 2013.

Cooper, M. A. and Shlaes, D. 2011. Fix the antibiotics pipeline. Nature **472**, 32-32.

Davies, J. and Davies, D. 2010. Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews **74,** 417-433.

Earnshaw, S., Monnet, D.L., Duncan, B., O'Toole, J., Ekdahl, K., Goossens, H., European Antibiotic Awareness Day Technical Advisory Committee, European Antibiotic Awareness Day Collaborative Group. 2009. European Antibiotic Awareness Day 2008 – the first Europe-wide public information campaign on prudent antibiotic use: methods and survey of activities in participating countries. Euro Surveillance: Bulletin Europeen sur les Maladies Transmissibles = European Communicable Disease Bulletin **14,** 19280.

Edqvist, L. E., & Pedersen, K. B. 2001. 9. Antimicrobials as growth promoters: resistance to common sense. *Late lessons from early warnings: the precautionary principle 1896–2000*, 93.Fleming, A. Penicillin. Nobel Lecture. 11 December 1945.

European Centre for Disease Control (ECDC). 2009. The bacterial challenge: time to react. Technical Report. Available online at: http://www.ecdc.europa.eu/en/publications/Publications/0909_TER_The_Bacterial_Challenge_Time_to_React.pdf.

Finley, R. L., Collignon, P., Larsson, D. J., McEwen, S. A., Li, X. Z., Gaze, W. H., *et al.* and Topp, E. 2013. The scourge of antibiotic resistance: the important role of the environment. Clinical Infectious Diseases. doi: 10.1093/cid/cit355

GlaxoSmithKline. 2012. Bacteria Battle: How we do R&D. Available online at: http://www.gsk.com/explore-gsk/how-we-do-r-and-d/bacteria-battle.html. Accessed 29 September 2013.

Guenther, S., Ewers, C. and Wieler, L. H. 2011. Extended-spectrum beta-lactamases producing E. coli in wildlife, yet another form of environmental pollution? Frontiers in microbiology, **2**.

Hall, G.C. 1994. The evolution of antibiotic resistance: a modern catastrophe. Bios **65,** 133-138.

IUCN. 2013. Phoenicopterus roseus. In: IUCN Red List of Threatened Species. Version 2013.1 Available online at: www.iucnredlist.org. Accessed 28 September 2013.

Kunin, C.M. 2008. Why did it take the Infectious Diseases Society of America so long to address the problem of antibiotic resistance? Clinical Infectious Diseases **46,** 1791-1792.
Levy, S.B. 2002. Factors impacting on the problem of antibiotic resistance. Journal of Antimicrobial Chemotherapy **49,** 25-30. doi: 10.1093/jac/49.1.25

Lewis, K. 2012. Antibiotics: Recover the lost art of drug discovery. Nature **485**, 439-440.

McCallum, H. I., Kuris, A., Harvell, C. D., Lafferty, K. D., Smith, G. W. and Porter, J. 2004. Does terrestrial epidemiology apply to marine systems? Trends in Ecology & Evolution **19**, 585-591.

Morens, D.M., Folkers, G.K. and Fauci, A.S. 2004. The challenge of emerging and re-emerging infectious diseases. Nature **430,** 242-249.

Neu, H.C. 1992. The crisis in antibiotic resistance. Science **21,** 1064-1073.

Oldenkamp, R., Huijbregts, M. A., Hollander, A., Versporten, A., Goossens, H., & Ragas, A. M. 2013. Spatially explicit prioritization of human antibiotics and antineoplastics in Europe. Environment International **51**, 13-26.

Palmer, G.H. and Call, D.R. 2013. Antimicrobial resistance: a global public health challenge requiring a global one health strategy. Commentary, Institute of Medicine, Washington D.C. http://www.iom.edu/Global/ Perspectives/2013/AntimicrobialResistance. Accessed 8 August 2013.

Rosenblatt-Farrell, N. 2009. The landscape of antibiotic resistance. Environmental Health Perspectives **117**, A244-A250.

Shlaes, D. M., Sahm, D., Opiela, C. and Spellberg, B. 2013. The FDA Reboot of Antibiotic Development. Antimicrobial agents and chemotherapy **57**, 4605-4607.

Schneider, R. L., Mills, E. L. and Josephson, D. C. 2002. Aquatic-terrestrial linkages and implications for landscape management. Integrating landscape ecology into natural resource management. Cambridge University Press, Cambridge, 241-262.

So, A. D., Gupta, N., Brahmachari, S. K., Chopra, I., Munos, B., Nathan, C. *et al.* and Weigelt, J. 2011. Towards new business models for R&D for novel antibiotics. Drug Resistance Updates **14**, 88-94.

Spellberg, B. 2011. The antibiotic crisis: can we reverse 65 years of failed stewardship? Archives of Internal Medicine **27,** 1080-1081.

Spellberg, B., Bartlett, J. G. and Gilbert, D. N. 2013. The future of antibiotics and resistance. New England Journal of Medicine **368**, 299-302.

Theuretzbacher, U. 2009. Future antibiotics scenarios: is the tide starting to turn? International journal of antimicrobial agents **34**, 15-20.

Theuretzbacher, U. 2012. Accelerating resistance, inadequate antibacterial drug pipelines and international responses. International journal of antimicrobial agents **39**, 295-299.

Torjesen, I. 2013. Antimicrobial resistance presents an "apocalyptic" threat similar to that of climate change, CMO warns. BMJ **346,** doi: http://dx.doi.org/10.1136.bmj.f1597

Towse, A. and Sharma, P. 2011. Incentives for R&D for New Antimicrobial Drugs. International Journal of the Economics of Business **18**, 331-350.

Trepka, M.J., Belongia, E.A., Chyou, P.-H., Davis, J.P. and Schwartz, B. 2001. The effect of a community intervention trial on parental knowledge and awareness of antibiotic resistance and appropriate antibiotic use in children. Pediatrics **107,** doi: 10.1542/peds.107.1.e6

Weiss, R.A. and McMichael, A.J. 2004. Social and environmental risk factors in the emergence of infectious disease. Nature Medicine **10,** S70-S76/

Wellington, E.M., Boxall, A.B., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., Johnson-Rollings, A.S., Jones, D.L., Lee, N.M., Otten, W., Thomas, C.M. and Williams, A.P. 2013. The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. Lancet Infectious Disease **13,** 155-165.

Wise, R. 2007. An overview of the Specialist Advisory Committee on Antimicrobial Resistance (SACAR). Journal of Antimicrobial Chemotherapy **60,** i5-i7.

Wright, G. D. and Sutherland, A. D. 2007. New strategies for combating multidrug-resistant bacteria. Trends in Molecular Medicine **13**, 260-267.

World Health Organisation. 2011. World Health Day – 7 April 2011. www.who.int/world-health-day/2011/en. Accessed 20 September 2013.