Critical review of AMR risks arising as a consequence of using biocides and certain heavy metals in food animal production: Literature review

Existing reviews

As well as the specific studies that have been reviewed, the literature search identified a number of relevant reviews on this subject, or aspects of this subject, notably Wales & Davies (2015), these reviews are listed in Table 4**.**

Table 4: Key relevant reviews on the impact of biocides and heavy metals on AMR related to food animal production (in chronological order).

Title of review	**Reference**
Assessment of the antibiotic resistance effects of biocides	SCENIHR (2009)
Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture	Seiler & Berendonk (2012)
Antibiotic-resistant bacteria: A challenge for the food industry	Capita & Alonso-Calleja (2013)
Zinc and copper in animal feed - development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin	Yazdankhah et al. (2014)
Co-selection of resistance to antibiotics, biocides and heavy metals, and its relevance to foodborne pathogens	Wales & Davies (2015)
Aquaculture as yet another environmental gateway to the development and globalisation of antimicrobial resistance	Cabello et al. (2016)
Supplementation of pigs diet with zinc and copper as alternative to conventional antimicrobials	De?bski (2016)
Assistance in the update of the systematic literature review (SLR): “Influence of copper on antibiotic resistance of gut microbiota on pigs (including piglets)”	Van Noten et al. (2016)
EMA and EFSA Joint Scientific Opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety (RONAFA) *	EMA & EFSA (2017)
Antimicrobial resistance and its association with tolerance to heavy metals in agriculture production	Yu et al. (2017)
The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions	Watts et al. (2017)
Methicillin-resistant Staphylococcus aureus: livestock-associated, antimicrobial, and heavy metal resistance (particularly with reference to South Africa)	Dweba et al. (2018)
Environmental and public health related risk of veterinary zinc in pig production-using Denmark as an example	Jensen et al. (2018)
Resistance to metals used in agricultural production	Rensing et al. (2018)
Joint FAO/WHO Expert Meeting in collaboration with OIE on foodborne antimicrobial resistance: Role of the environment, crops and biocides	FAO/WHO (2019)
Antimicrobial resistance on farms, including the role of disinfectants in resistance selection	Davies & Wales (2019)
Selection and dissemination of antimicrobial resistance in Agri-food production	Cheng et al. (2019)
The role of emerging organic contaminants in the development of antimicrobial resistance	Alderton et al. (2021)
Scientific opinion on the role played by the environment in the emergence and spread of antimicrobial resistance (AMR) through the food chain **	EFSA BIOHAZ Panel (2021)

Factors influencing the transfer and abundance of antibiotic resistance genes in livestock environments in China	Wang et al. (2022b)
Interactions and associated resistance development mechanisms between microplastics, antibiotics and heavy metals in the aquaculture environment	Li et al. (2022b)
Evaluating the impact of heavy metals on antimicrobial resistance in the primary food production environment: A scoping review	Anedda et al. (2023)
* This EMA and EFSA opinion did not specifically consider biocides or heavy metals but did discuss their role as alternative antimicrobials and risks for co-selection. ** This EFSA opinion did not specifically consider biocides or heavy metals but did discuss co-selection and mitigation measures for reducing antimicrobial-resistant bacteria and genes in the food animal production environment. Many of the knowledge gaps identified are relevant to the subject of this report.	

The majority of these reviews are focussed on land-based food animal production. These reviews have repeatedly highlighted the lack of clear in-field evidence on the role of non-antibiotic drivers in co-selecting AMR in the environment. These reviews (for example, Davies & Wales, 2019) highlight that there is scarce evidence on the efficacy or selective effect of currently used farm and hatchery biocides on the survival or emergence of organisms with increased biocide tolerance or AMR. Investigations of bacterial strains recovered from the field appear to show some evidence of associations/correlations between biocide use and increased AMR although clear evidence for causal links is relatively scant. These studies were limited in terms of the organisms examined - most choose a specific bacterial spp., whether zoonotic or not, and ignored the rest of the microbial community.

The SCENIHR (2009) classified biocides according to their intrinsic potential for generating resistance/tolerance. Some biocides, due to the nature of their interactions with the bacteria, would be more prone to induce resistance/tolerance. SCENIHR considered that this group of high-risk biocides contains the QAC, biguanides (i.e., surface active agents), phenolics, and metallic salts. On the other hand, highly reactive biocides (for example, oxidising and alkylating agents) present a low risk of emergence of bacterial resistance, and that reduced susceptibility to these biocides results mainly from their inappropriate use. Several other biocides (isothiazolones, anilides, diamidines, inorganic acids and their esters or alcohols) have been classified as medium-risk in terms of emergence of bacterial resistance.

Few of these reviews have considered the impact of the use of biocides and/or heavy metals on AMR in aquaculture. Reviews by Seiler & Berendonk (2012), Cabello et al. (2016), Watts et al. (2017), EFSA BIOHAZ Panel (2021), and Li et al. (2022b) in relation to heavy metals mention heavy metals (and biocides, in the case of Cabello et al., 2016) as potential drivers for co-selection of AMR but they do not cite any specific studies that have addressed the use of heavy metals in feed or other compounds (biocides) used in aquaculture on co-selection of AMR. Nor has our literature review identified any compelling studies that have mapped the use of heavy metals (whether in feeds or in other uses) or biocides in aquaculture with co-selection of AMR.

Impact of biocides on AMR in food animal production

As highlighted by the SCENIHR (2009), Wales & Davies (2015), Cheng et al. (2019), Donaghy et al. (2019), and Giacometti et al. (2021) amongst others, and confirmed in our literature search, while there is much laboratory experimental evidence on the impact of biocides in selecting antibiotic resistance there are considerably less field data in relation to the food animal production context. As noted in a number of reviews, the efficacy of biocidal action in the field and ability to select AMR may be significantly reduced due to the presence of heavy organic soiling or dilution effects. In general, studies do not appear to have specifically quantified these effects on MICs in the context of AMR co-selection. A few studies (as discussed below) have observed that sustained exposure of livestock-associated bacteria to sub-inhibitory concentration of biocides may result in increased levels of AMR among these bacteria.

There has been a concern regarding the potential for poultry biocides used as antimicrobial processing aids during poultry processing operations to increase AMR (Rhoma et al., 2021). Biocides are used to treat poultry during processing in many countries, though not generally in Europe. Reviewing the evidence on peroxyacetic acid, chlorine, chlorite, and trisodium phosphate as a poultry carcass and meat decontaminant, the European Food Standards Authority Panel on Biological Hazards (EFSA BIOHAZ Panel) concluded that there was no evidence that their use would lead to acquired reduced susceptibility among contaminating bacteria, nor to acquired resistance to antibiotics (EFSA BIOHAZ Panel, 2008).

An investigative laboratory study that repeatedly exposed four strains of L. monocytogenes and S. Typhimurium from poultry to increasing sub-inhibitory concentrations of five decontaminants (trisodium phosphate (TSP), chlorine dioxide (CD), acidified sodium chlorite (ASC), citric acid (CA), and peroxyacetic acid (PA)) demonstrated an increase in resistance in the strains to various antibiotics of the 15 they were tested against (Alonso-Hernando et al., 2009). There was no strong pattern of association, although development of resistance to the aminoglycosides, streptomycin and neomycin was common. Strains exposed to ASC showed a higher number of increased resistances, 13 resistances to the 15 antibiotics against which they were tested than when exposed to TSP (7 resistances), CA (3 resistances), CD (5 resistances) or PA (7 resistances). A laboratory-based investigative study that dipped chicken legs in solutions of TSP, ASC, ascorbic acid (AA), CA, or water (control) showed that E. coli isolated from the samples had a higher level of resistance to ampicillin-sulbactam (treated with TSP), amoxicillin-clavulanic acid (treated with ASC, AA and CA), cefotaxime (treated with TSP), trimethoprim-sulphamethoxazole (treated with AA or CA), tetracycline (treated with CA), ciprofloxacin (treated with ASC, AA, or CA) and nitrofurantoin (treated with TSP) in comparison with controls (Capita et al., 2013).

In their review of evidence on this subject Rhouma et al. (2021) concluded that there is at present some evidence that sub-inhibitory concentrations of biocides may lead to selection, but that at present the published evidence is scare and derived from laboratory-based experiments. They suggested that due to the risk of co-selection that the poultry industry should consider non-chemical physical interventions, such as hot water and steam treatments. We would echo that and note that some poultry processors in the UK are currently employing such physical interventions.

Studies by Randall et al. (2005, 2007) have observed that the use of certain types of biocides commonly used in UK farms can increase bacterial resistance/tolerance to both biocides and to antibiotics. A laboratory-based study (Randall et al., 2005) showed no co-selection effect with ciprofloxacin-resistant strains of E. coli to three commercial disinfectants (a tar oil phenol, which was a blend of high boiling point tar acids and organic acid, an oxidising compound, and a combination of formaldehyde, glutaraldehyde and QAC). There was a slight increase in cyclohexane tolerance among a minority of disinfectant-passaged strains (particularly those subjected to the phenolic biocide). A further laboratory based study (Randall et al., 2007) exposed eight S. Typhimurium isolates (including field isolates and laboratory mutants) to different farm biocides (a tar oil phenol; an oxidising compound; an aldehyde-based disinfectant; or QAC's). Results differed depending on the biocide and the Salmonella spp. strain tested. Exposure to an aldehyde-based disinfectant reduced susceptibility to the fluoroquinolone ciprofloxacin in some strains. An analysis of proteomes (the complete set of proteins made by an organism) revealed significantly increased expression of the AcrAB–TolC efflux system (responsible for resistance to antimicrobials) after exposure to a tar oil phenol disinfectant. The results showed that single exposure to biocides was insufficient to select for AMR strains. The authors concluded that biocides could be a selective pressure for the selection and/or maintenance of ciprofloxacin-resistant strains in the farm environment in the absence of ciprofloxacin itself.

Nhuyen et al. (2015) observed that the sustained exposure of E. coli and non-typhoidal Salmonella (isolated from farmed animals) to sub-inhibitory concentrations of a commonly used commercial disinfectant containing a mix of benzalkonium chloride (a QAC) and glutaraldehyde used on pork and poultry farms in Vietnam appeared to co-select AMR. Increases in MIC for the biocide were strongly correlated with reduced susceptibility shown by increases in MIC (or decreases in inhibition zone) for ampicillin, tetracycline, ciprofloxacin, and chloramphenicol, and to a lesser extent for gentamicin, trimethoprim/sulphamethoxazole. To investigate whether generic efflux pump expression was responsible for the observed changes, the study treated strains with a generic efflux pump inhibitor and measured the changes in AMR before and after treatment. Results suggested that mechanisms other than efflux pumps were responsible for co-selection.

Davies & Wales (2019) cited unpublished data from the Animal and Plant Health Agency (APHA) that there were concerns that the use of sub-inhibitory concentrations of QACs, as a consequence of cost and staff safety issues, was becoming a common practice in UK poultry hatcheries. They reported that there was evidence that certain quinolone-resistant, hatchery-resident Salmonella spp. strains appeared to have emerged from such situations and subsequently spread to broilers. This evidence does not appear to have been published elsewhere, or any similar studies undertaken.

Many essential oils (EOs), plant compounds, and extracts have been shown to act as antimicrobial agents and promoted as ‘natural’ alternative feed additives to antibiotics (De Souza, 2016; Stevanovi? et al., 2018; Álvarez-Martínez et al., 2021; Mariotti et al., 2022). While there is much evidence on the efficacy of EOs there are little data on their modes of action (Álvarez-Martínez et al., 2021) and potential to drive co-selection of resistance (De Souza, 2016). De Souza (2016) further concluded in their review on the effects of sub-inhibitory doses of EOs on AMR that EOs were not likely to impose a major hazard. There is some evidence that controlled exposure of bacteria to sub-inhibitory concentrations of EOs can alter and select for AMR (Al-Mnaser & Woodward, 2020), but field studies are lacking. There is evidence that different EOs may have different effects on resistance. A comparison of effects of low concentrations of cinnamon and oregano on resistance in Gram-negative bacilli observed that repeated use of cinnamon had no effect on AMR, but oregano could increase Proteus mirabilis resistance to ampicillin or decrease Serratia marcescens resistance to tetracycline (Becerril et al., 2012). Since EOs are composed of many chemical constituents, it is not surprising that different oils show synergistic or antagonistic effects. EOs have been advocated as alternatives to antibiotics in animal feeds, but studies do not appear to have considered whether their use may be co-selective. Thymus maroccanus (a species of thyme) EO has been shown in vivo to select for AMR in E. coli strains (Fadli et al., 2014). Resistance was associated with an overexpression of an efflux pump related to AcrAB-TolC (a RND-based tripartite efflux pump) in some variants. Sub-inhibitory concentrations of tea tree oil (Melaleuca alternifolia) have been associated with reduced susceptibility to antibiotics in E. coli, S. aureus, MRSA, and Salmonella spp. (McMahon et al., 2007b).

Menthol (an EO) has been suggested as an antibiotic alternative in cattle. A US study (Aperce et al., 2016), on feedlot cattle that had been fed menthol, reported no increased resistance in E. coli isolates to many antibiotics (amoxicillin, ampicillin, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfisoxazole, and sulphamethoxazole). They did observe an increased prevalence of tetracycline-resistant E. coli after 30 days of menthol supplementation (0.3 %) in feed. Similarly, another study by this research team (Murray et al., 2021), reported a trend (though not statistically significant) in increasing resistance with menthol (0.3 %) supplementation (also in combination with zinc [300 ppm]), particularly reduced sensitivity to tetracycline. No mechanistic explanations were pursued in these studies. It is not clear from these studies why a concentration of 0.3 % was used, and trials were not carried out to determine the effect of different concentrations of menthol in feed on co-selection. These studies note that menthol in feed has been shown to promote weight gain in poultry, and there are published studies on its use in fish feed. No other studies appear to have been undertaken to establish whether menthol in feed may select for AMR in other animal species.

It is usually assumed that conventional cleaning and disinfection procedures using biocides that are effective in eliminating non-antimicrobial-resistant bacteria will be equally effective against antimicrobial-resistant bacteria on farms (Davies & Wales, 2019). Davies & Wales (2019) cite some unpublished evidence that LA-MRSA may be more resistant than Salmonella spp., as well as evidence published by Kotb & Sayed (2015). A recent study by Montagnim et al. (2022) highlighted that MDR strains of E. coli may be more resistant to some commonly used farm disinfects than non-MDR strains. The efficacy of cleaning and disinfection regimes in reducing AMR on farms is discussed in reviews such as Davies & Wales (2019).

A strong association was found between frequent disinfection of pens and colonisation of nursery piglets with LA-MRSA in Canada. All LA-MRSA isolates carried at least one QAC BRG, with the most common genotype being qacG qacH smr (Slifierz et al., 2015b). Nursery herds testing positive for LA-MRSA reported more frequent use of disinfectants (as well as zinc therapy), as well as having a higher stocking density. This is one of the few animal food production in-field studies identified in our literature search that have addressed the impact of biocides on AMR. The study did not map the use of specific disinfectants to resistance.

Wieland et al. (2017) observed no association of increased didecyl dimethyl ammonium chloride (DDAC, a QAC) MICs with ESBL/AmpC isolates from poultry but they did observe significant positive correlations for MIC values of DDAC (?0.36–3.6 mg DDAC l-1) and four other antibiotics (chloramphenicol, florfenicol, piperacillin, sulphamethoxazole + trimethoprim) in E. coli, as well as for 13 antibiotics in enterococci, suggesting that residual QACs may select AMR enterococci.

A similar study showed an association in reduced susceptibility to sodium hypochlorite and AMR in Salmonella spp. from poultry in China (Xiao et al., 2022). Positive correlations between chlorine tolerance and clinical antibiotic resistance to ceftiofur, tetracycline, ciprofloxacin and florfenicol were observed. The most frequently detected chlorine BRG was qacE?1; thus cross-resistance was likely due to efflux pump over-expression. This gene is also associated with MGEs (such as class I integrons) and co-selection (Hu et al., 2018).

A study of E. coli from pigs, pig carcasses and pork in Thailand (Puangseree et al., 2021) showed a weak correlation between reduced susceptibility to biocides and AMR. Some cross-resistance between benzalkonium chloride (a QAC) and chloramphenicol, ciprofloxacin, sulphamethoxazole, and tetracycline; and chlorhexidine and ciprofloxacin, gentamicin, and streptomycin, was observed.

A study by Cufaoglu et al. (2022) investigated AMR and biocide susceptibility (as well as heavy metals, as will detailed in the next section) amongst E. coli isolates from chicken, cattle, and sheep in Turkey. A high prevalence of AMR were reported amongst isolates, along with a high prevalence of reduced susceptibility to N-alkyl-dimethyl-benzyl-ammonium [a QAC] (26%). In order to determine susceptibility to biocides (and heavy metals), field isolates were deemed tolerant, if the MIC of field isolates was above that of a control E. coli strain, ATCC 25922. This highlights an issue in the literature around breakpoints and how resistance is determined, in this study resistance/reduced susceptibility was reported if the MIC was one doubling dilution above the control and no replication indicated. The authors did report the presence of BRGs in a smaller proportion of isolates tested by PCR assay, with one MDR isolate subject to whole genome sequencing (WGS). Such genes were found in this isolate and were chromosomally located. Whilst HMRGs, BRGs and ARGs were found in the same isolates, there were limited data presented to suggest that the use of biocides specifically were driving AMR.

Other studies on biocides (as discussed below) have not observed any evidence of cross-resistance or co-selection between antibiotics and biocides.

A Brazilian comparison of biocide (sodium hypochlorite and benzalkonium chloride [a QAC]) use on AMR of S. Heidelberg isolated from poultry flocks in 2006 with those isolated in 2016 showed no increase in resistance/tolerance over this time period (to either biocides or antibiotics [with the exception of tetracycline resistance which showed an increase]), suggesting no signs co-selection from biocide use (Bassani et al., 2021).

A survey of field strains of S. enterica from pigs (132 strains) and poultry (125 strains) in Thailand commonly exhibited the multiple antibiotic resistance (MAR) phenotype (42%), but this was not associated with reduced susceptibility to benzalkonium chloride (a QAC) or chlorhexidine among the 257 strains (Chuanchuen et al., 2008).

A study of Salmonella isolates from two commercial US turkey processing plants found that all Salmonella isolates were chlorhexidine tolerant, but no cross-resistance between chlorhexidine and five antibiotics (gentamicin, kanamycin, sulphamethoxazole, streptomycin, and tetracycline) was found in the 130 Salmonella spp. serovars compared in the laboratory (Beier et al., 2011). A series of later studies (Beier et al., 2013; Beier et al., 2019 and Beier et al., 2021) by the same US research group compared biocide tolerance and AMR in strains of E. coli O157:H7, Campylobacter coli, and C. jejuni isolated from cattle, pigs, and poultry, respectively. In all cases no correlation between biocide tolerance and AMR was observed. The beef study (Beier et al., 2013) compared biocide tolerance and AMR of 244 strains of E. coli O157:H7 isolated from cattle carcasses, faeces, and hide. It failed to find any correlation between biocide tolerance and AMR. Although only a low prevalence of AMR isolates was observed (14%), mainly to chloramphenicol, streptomycin, and tetracycline. Fifteen different biocides were tested, including QAC based sanitisers, and organic acids. Tolerance to certain biocides (chlorhexidine or benzalkonium chloride) and antibiotics was observed in some strains. The same group compared the tolerance/resistance of 111 C. coli isolated from pigs and pork meat to 22 biocides and 9 antibiotics, finding that 84 to 96% of strains were resistant to chlorhexidine, and 75% were resistant to tetracycline (Beier et al., 2019). In the poultry study (Beier et al., 2021) the resistance of 96 strains of C. jejuni isolated from the litter of chicken houses to 9 antibiotics and 22 biocides (mainly a range of QAC based sanitisers, but also included formaldehyde) was studied. This study found 13.5%, 12.5%, and 22% of the strains demonstrated reduced susceptibility to ciprofloxacin, nalidixic acid, and tetracycline, respectively. In total, 32% of strains were resistant to chlorhexidine, but not to other biocides; and no cross-resistance was observed between the antibiotics and the 22 biocides (disinfectants). An unrelated laboratory study observed no evidence that tolerance to biocides (benzalkonium chloride (a QAC), chlorhexidine, cetylpyridinium chloride (CPC, a QAC), trisodium phosphate, and sodium dodecyl sulphate) was connected to AMR (resistance to erythromycin and/or ciprofloxacin) in 27 C. coli and 15 C. jejuni isolates from food, animal, human and environmental water sources (Mavri et al., 2012).

A study of four French poultry slaughterhouses observed no increased resistance or cross-resistance in Campylobacter isolates after cleaning and disinfection (Peyrat et al., 2008). Similarly, a study of over 500 isolates from Danish pig slaughterhouses showed there was little evidence of an association between biocide use and AMR in Salmonella spp. (Gantzhorn et al., 2014). There was no increased tolerance in the isolates to the biocides tested (a QAC and an acid/hydrogen peroxide mix) in comparison to a reference strain of S. Typhimurium and there was no evidence that cleaning and disinfection co-selected for antimicrobial-resistant bacteria. Likewise, a study by Bridier et al. (2019) of cleaning and disinfection procedures over three months in a French pig slaughterhouse observed no evidence of reduced susceptibility to biocides of Salmonella spp. isolates or co-selection of AMR in such isolates.

A Belgium survey of disinfectant use and resistance in E. coli in both poultry and pig production observed no indications for the co-selection of AMR through the use of commonly-used biocides (i.e., glutaraldehyde, benzalkonium chloride, formaldehyde, and a formulation of peracetic acid and hydrogen peroxide) in these environments (Maertens et al., 2019). In a further study by this research team (Maertens et al., 2020) the susceptibility of field E. coli isolates from a broiler and pig pilot farm to 14 antibiotics and the 4 disinfectants was monitored over a one-year period. No change in biocide tolerance to these disinfectants was observed and no association was found between biocide use and AMR.

As well as their use for cleaning and disinfecting biocides are used in footbaths and to clean udders of animals used for milk production (SCENIHR, 2009; Wales & Davies, 2015; Donaghy et al., 2019; VKM, 2016). Few studies on the impact of such practices on co-selection for AMR were found in our literature search.

A number of studies have identified a concern that inappropriate application of teat dipping biocides applied to dairy cattle could co-select for AMR. Few studies have demonstrated whether this may occur. A study by Abd El-Aziz et al. (2021) of Streptococcus uberis from bovine clinical mastitis in dairy farms with diverse hygienic interventions in Egypt showed that qac resistance genes were positively correlated with ARGs/AMR phenotypes in the isolates studied. No details were provided on the type of antiseptic used, and no clear evidence of a link between disinfectant use and AMR was demonstrated. A German study (El Behiry et al., 2012) observed no cross-resistance in S. aureus from cows with subclinical mastitis that showed reduced susceptibility to commercial teat dips (nonoxinol-9 iodine complex and chlorhexidine). This suggested that reduced susceptibility to these biocides may be distinct from AMR. An Italian study observed that coagulase-negative staphylococci from milk had a low prevalence of qac genes encoding for disinfectant efflux pumps and there was no evidence of cross-selection (Turchia et al., 2020).

A study by Roedel et al. (2021) on E. coli from broiler meat farms did not find a link between phenotypic biocide tolerance to commonly-used biocides and AMR on those farms. The study determined both the MIC and MBC and determined reduced susceptibility based on the MIC95 or MBC95 (the antibiotic concentration that would inhibit the growth of 95% of the tested bacterial isolates) therefore, arguably providing more robust results. When WGS was undertaken for a small number of isolates' ARGs (aadA1 and sul1) were found closely linked with qacE?1 on a class 1 integron and which included a mercury-resistance gene operon. There was an association between blaCMY-2 and sugE(p) (conferring resistance to QACs) found on the same contig (a consensus region of DNA from overlapping DNA sequences from WGS mapping a region of DNA) and which was associated with genes suggestive of localisation on a plasmid.

Biocides and heavy metals (copper and zinc, as discussed in the relevant section of this report) are routinely used in antimicrobial footbaths in commercial dairy farming to prevent lameness caused by bacterial infections (Bell et al., 2014; Yu et al., 2017). A number of different biocides may be used, with Bell et al. (2014) reporting formalin to be commonly used in the UK. Few studies have investigated their impact on AMR co-selection. A study of disinfecting footbaths used in six Norwegian dairy farms found that Serratia marcescens may survive and multiply in these baths but there were no indications of cross-resistance between biocides and AMR in surviving isolates (Langsrud et al., 2003). The contents of these footbaths are usually disposed into slurry tanks, which is likely to lead to soil contamination and a potential driver for co-selection of AMR (Williams et al., 2019).

The effects of biocides and their impact on AMR in the aquacultural environment (whether marine or fresh) appears to be poorly studied. Our literature search identified very few published studies. According to Burridge *et al.* (2010) compounds used are water soluble and of low toxicity depending on the quantities used. They report that Virkon ®, iodine + detergents, chloramine-T, hypochlorite chlorine dioxide, benzalkonium chloride, Superquats ®, glutaraldehyde, formalin 40%, calcium oxide, calcium hydroxide, sodium carbonate, Creolina, synthetic phenols, halophenols and ethanol are used in Chile in salmon aquaculture. We have not found data on which compounds are used in the UK. Defra currently list the products which have demonstrated effectiveness again aquatic disease through testing in the UK done via The Centre for

Environment, Fisheries and Aquatic Science (Cefas). These include many of the products above and interestingly state that testing is done at 4+1 degrees celsius so under industry appropriate conditions.

A study by Romero *et al.* (2017) has been widely cited in the literature as providing evidence of the co-selection of biocides (and heavy metals) on AMR in seafoods. This study observed multiple /tolerances resistances (to biocides, heavy metals, and antibiotics) in 76% of isolates from a wide range of seafoods. ARGs detected included *sul1* (43.33% of tested isolates), *sul2* (6.66%), *bla*$_{\text{TEM}}$ (16.66%), *bla*$_{\text{CTX?M}}$ (16.66%), *bla*$_{\text{PSE}}$ (10.00%), *bla*$_{\text{IMP}}$ (3.33%), *bla*$_{\text{NDM?1}}$ (3.33%), *floR* (16.66%), *aadA1* (20.0%), and *aac(6?)-Ib* (16.66%) and is of concern given that *bla*$_{\text{IMP}}$ and *bla*$_{\text{NDM?1}}$ encode resistance to carbapenems (CIAs). The only BRG detected was q*acE ?1* (10.00%). These results suggest that exposure to biocides may co-select for AMR. The fish and seafood sampled was purchased at supermarkets and fish markets in the region of Jaen, Spain. While many samples were sea-caught fish; sea bass, salmon, and prawn samples were farmed and showed patterns of tolerance/resistance to biocides and antibiotics. No direct comparison with any pattern of use of biocides during the husbandry of these seafoods was made.

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Overall, the literature on investigations of bacterial isolates recovered from the field appear to show some evidence of associations/correlations between biocide use and increased resistance to antibiotics. Particularly there is some evidence that QACs, such as benzalkonium chloride, that are widely used in food animal production for disinfection of farm environments and equipment, and chlorhexidine, a biguanide used as an antiseptic and disinfectant for example as a dairy teat disinfectant, may co-select AMR, although there appears to be little clear evidence in the literature for causal links in the field. These biocides have also been identified as risks in other reviews, such as SCENIHR (2009). There is clearly a need to establish whether current cleaning and disinfection regimes in use in food animal production in the UK represent a real hazard with respect to the selection of AMR.

Impact of heavy metals on AMR in food animal production

As highlighted by Wales & Davies (2015), Cheng et al. (2019), Donaghy et al. (2019), and Giacometti et al. (2021), while there is some laboratory experimental evidence on the impact of heavy metals on the selection or development/dissemination of AMR, there are considerably fewer field data (though considerably more than on the impact of biocides). In common with the evidence on the impact of biocides, while there are some data showing an association/correlation in resistance, there is little clear evidence for causal links. A summary of in-field studies that have addressed the impact of heavy metals on AMR is shown in Table 5. Evidence is mainly on the supplementation of pig feed with zinc or copper, or the therapeutic use of zinc oxide in pig production. There are little data on the impact of heavy metals on AMR in other forms of food animal production, particularly aquaculture. These studies do not directly address whether an increase in AMR or ARGs as an impact of heavy metal use may in turn lead to an increased risk to public health, but only to the dissemination of AMR and ARGs in the environment.

There is concern that therapeutic use of zinc oxide in pig production (which is used to prevent postweaning diarrhoea) and in feed (as a growth promotor) at high concentrations (as high as 3,000 ppm rather than 50 to 150 ppm required for nutritional use) may co-select for LA-MRSA. This is due to the co-location of the zinc/copper resistance gene (czrC) and methicillin resistance gene (mecA) within the staphylococcal cassette chromosome mec (SCCmec) element (Aarestrup et al., 2010; Cavaco et al., 2010; Argudín et al., 2016; Hau et al., 2017; Poole et al., 2017; Jensen et al., 2018). SCCmec is a MGE that carries the mecA gene [or its homologue mecC] (encoding resistance to methicillin and all ?-lactam drugs) and other functional genes, and can transfer to other Staphylococcus spp.

An association between reduced zinc susceptibility and the development of LA-MRSA CC398 in Danish pigs was shown to be a consequence of the frequent presence of czrC in SCCmec (type V) in both pig and human isolates (Aarestrup et al., 2010; Cavaco et al., 2010). Alen et al. (2018) reported an increase in the percentage of zinc tolerant LA-MRSA CC298 isolated from patients of a German university hospital located in a pig farming-dense area between 2000 and 2014 which they associated with the use of zinc in pig feed. Prior to 2009, about half of the LA-MRSA CC398 isolates were zinc tolerant, whereas by 2014 all tested LA-MRSA CC298 isolates were found to be zinc tolerant. Zinc tolerance was found to correlate with the presence of the czrC gene in all cases. A small-scale study from USA confirmed a strong association of S. aureus CC398 from pigs and czrC-positive (Hau et al., 2017). The same study suggests that for certain other lineages (pig-associated LA-MRSA ST5) the contribution of zinc to the emergence of LA-MRSA may be negligible. This may be due to the variations in SCCmec cassettes among LA-MRSA lineages and not all types will harbour SCCmec type V, with ST5 isolates found to carry either SCCmec type III or IV, or untypeable cassettes (Hau et al., 2017). Argudi?n et al. (2016) demonstrated that the czrC gene was almost exclusively found (98%) in the presence of SCCmec V in both CC398 and non-CC398 LA-MRSA isolates (CC1 and CC97 LA-MRSA). No evidence of zinc contributing to the prevalence of CC398 and CC5 LA-MRSA strains in pig farms in Korea was observed by Eom et al. (2019).

Other genes potentially conferring metal tolerance, including copB (copper), have been found to be present in LA-MRSA and associated with SCCmec and integrons (Argudín et al., 2016). The plasmid pAFS11 obtained from CC398 isolates has been shown to harbour 5 different ARGs and

2 HMRG operons [including copA, encoding copper tolerance] (Feßler et al., 2017). LA-MRSA strains have been described harbouring plasmids carrying tolerance genes for copper (copA and mco HMRGs) and for multiple antibiotics including macrolides, lincosamides, streptogramin B, tetracyclines, aminoglycosides, and trimethoprim (erm(T), tet(L), aadD, and dfrK ARGs) (Gomez-Sanz et al., 2013).

Other studies have shown LA-MRSA in weaner pigs are influenced by exposure to therapeutic doses of in-feed zinc (?2,000 ppm) when compared to the recommended dietary concentration (100 ppm). Slifierz et al. (2015a) reported was a significant association between the prevalence of LA-MRSA-positive pigs (followed from birth to weaning) and zinc concentration (3,000 vs 100 ppm) at four and five weeks of age. In both groups LA-MRSA-positive animals were similarly infrequent by seven weeks of age in the randomised controlled trial. A further report by the same group found a strong association between the concentration of zinc in the nursery ration and colonisation of nursery piglets with LA-MRSA (Slifierz et al., 2015b). Samples from 390 pigs from 26 farms were compared. Nursery herds testing positive for LA-MRSA reported more frequent use of zinc therapy (?2,000 ppm in-feed), as well as having a higher stocking density. In this study czrC (a HMRG encoding resistance to zinc and copper) was detected in about two-thirds of isolates in association with a lower susceptibility to zinc compared with czrC-negative isolates.

An in vitro study by Peng et al (2020), investigated the growth of two ESBL-producing E. coli strains carrying blaCTX-M-1, with the gene either on a plasmid or chromosomally- encoded, in pig faecal material containing an increasing concentration of zinc (0-8 mM). Interestingly, expression of the gene increased with increasing zinc concentration. The authors suggest that zinc may be inducing the promoter activity of an insertion element ISEcp1 normally found upstream of the blaCTX-M-1 gene and increased zinc efflux and thus higher level zinc tolerance. Furthermore, at higher zinc concentrations there was a higher proportion of CTX-M-1 resistant E. coli relative to the total flora, but only for the strain where the gene was plasmid encoded. These results suggest that exposure to therapeutic zinc concentrations may give a selective growth advantage to bacteria carrying such plasmid-encoded genes and thereby induce their expression. Such selection does therefore not have to be linked to co-carriage of specific HMRGs and ARGs.

Increasing concern over the therapeutic use of zinc in animal production potentially leading to an increased prevalence of LA-MRSA contributed to a phase-out of these products in the EU (Rensing et al., 2018). Therapeutic use of zinc was banned from June 2022 within the EU and whilst the UK was included with legislation passed before the UK left the EU, product still in date and remaining within the supply chain are permitted for use as part of the UK phase-out. Within the EU 27, zinc is now only permitted at concentrations up to 150 ppm for nutritional use, compared to concentrations of 2,500 ppm used previously for therapeutic use (Veterinary Medicines Directorate, 2022).

Two related studies by Agga et al. (2014, 2015) on the effects of copper supplementation (125 ppm vs 16.5 ppm) on AMR in weaned pigs used data from the same trial but analysed different parameters. The first study (Agga et al., 2014) found that copper MIC was not affected by copper supplementation or by pcoD gene carriage (a plasmid-borne copper HMRG). The second study (Agga et al., 2015) reported that copper supplementation was associated with a significant increase in tetP genes (which impart resistance to tetracyclines) among faecal E. coli but did not show a link with the pcoD gene (Agga et al., 2015). These studies observed that copper supplementation was associated with lowered blaCMY-2 gene copies from faecal E. coli. According to their results tetA and blaCMY-2 were positively associated with each other and negatively associated with both pcoD and tetB genes. They suggested that this points to the potential opportunity to select for a less harmful tetracycline resistance profile in E. coli by replacing in feed antibiotics by copper. A point highlighted in Van Noten et al.’s (2016) systematic review of this data. In their review of these data Wales & Davies (2015) concluded that it is possible that baseline levels of antibiotic and copper resistance/tolerance were sufficiently high in this study population that the copper supplementation was insufficient to select for reduced copper susceptibility or associated ARGs. Van Noten et al. (2016) judged the trials to be of intermediate methodological quality because of uncertainty concerning the independence of the samples (the same piglet could have been sampled at different weeks). We would agree that the data are not particularly compelling.

A Bavarian study observed that high concentrations of zinc and copper in pig manure (indicative of high concentrations in feed) may promote the spread of AMR of gut microbiota in pigs (Holzel et al. 2012). In the survey of manure samples from 305 pig farms the study found that supramedian concentrations of copper (388.5 ppm) and zinc (1,199.2 ppm) showed significant associations with the phenotypic antibiotic resistance of E. coli isolates in the manure. Bacterial resistance against ampicillin, augmentin (amoxicillin plus clavulanate), and piperacillin was significantly higher in E. coli from pig manure containing copper. While resistance rates against piperacillin and doxycycline in E. coli from pig manure were associated with zinc.

A possible effect of zinc feed supplementation on the mobility of ARGs in E. coli was observed by Bednorz et al. (2013), who reported an increased diversity of genotypes and plasmid profiles and increased MDR among weaning pigs supplemented with high concentrations of zinc (>2,000 ppm). The study found that 18.6% of the E. coli clones from the high zinc group were multi-resistant but not a single clone from the control group (50 to 70 ppm). An independent second study (Ciesinski et al., 2018) used the same feeding setup, but changed the experimental setup to focus on a complex analysis of resistance phenotypes rather than clonal diversity. They also observed that high dietary zinc feeding increased the proportion of MDR E. coli from weaned piglets, corroborating the finding of the previous study. The impact of zinc was observed in all three habitats tested (faeces, digesta, and mucosa). The authors suggested several possible mechanisms for their observations. One was co-selection, as some isolates had both zinc tolerance and antibiotic resistance. Another was enhanced exchange of MGEs under the influence of zinc. Differences in the plasmid profiles of clones of the zinc and control group were observed in the initial study (Bednorz et al., 2013).

In contrast, a further study by this group (Ghazisaeedi et al., 2020) argued against a co-selection mechanism of zinc and AMR suggesting that an explanation for an increase in MDR isolates from piglets with high zinc dietary feeding could be that antimicrobial-resistant bacteria are more tolerant to stresses such as zinc or copper exposure. In this further study, the group screened the phenotypic zinc/copper tolerance of 210 isolates (including antibiotic resistant, MDR, and non-resistant E. coli) selected from two, independent zinc-feeding animal trials. Importantly, no significant association was observed between AMR and phenotypic zinc/copper tolerance of the same isolates.

Medardus *et al.* (2014) also observed an effect of zinc as a feed supplement. They reported that among 349 *Salmonella* spp. isolates from nine pig units in the USA studied over a two-year period, an elevated zinc MIC was associated with the occurrence of the *czcD*-encoded zinc efflux pump but not with the concentration of faecal zinc. By contrast faecal copper concentration was associated with an elevated copper MIC, but not with the occurrence of a copper efflux gene *pcoA*. The same study reported that specific serovars were associated both with copper and zinc susceptibility and with patterns of antibiotic resistance; such resistances were not independently associated with copper or zinc susceptibility once serovar was taken into account. Concentrations of zinc and copper in the feed were between 79-7,384 ppm and 3-1,384 ppm, respectively.

In a study of faecal E. coli among 180 weaner pigs in the US, in-feed copper supplementation at a growth-promoting concentration (125 ppm) was associated with reduced susceptibility to chlortetracycline and oxytetracycline in E. coli (Shelton et al., 2009). No significant effects were observed for high concentrations (3,000 ppm) of added zinc.

Studies have reported conflicting evidence on an association between the supplementation of

copper in feed of different animals and resistance in faecal enterococci. The development of tolerance to copper in enterococci is associated with the presence of tcrB, a copper HMRG, which is often located on a conjugative plasmid that may carry ARGs, thus contributing to co-selection (Yazdankhah et al., 2014). In a study of Enterococcus faecium isolated from pigs on Danish farms, tcrB was more frequently detected from the more intensively copper-supplemented livestock (Hasman & Aarestrup, 2002). Copper tolerance was strongly correlated with macrolide and glycopeptide resistance in isolates from pigs, and tcrB genes shown to be located on the same conjugative plasmid as ARGs erm(B) and vanA (associated with both vancomycin and teicoplanin resistance). In a further study by this group, weaner and grower pigs were given a heterogeneous inoculum of tcrB-positive and -negative E. faecium and reported that exposure at a commercial in-feed concentration of copper (175 ppm vs 6 ppm) was associated with a higher detection frequency of tcrB and of the linked erm(B) and vanA genes (Hasman et al., 2006). They also identified the tcr genes in the enterococcal species E. mundtii, E. casseliflavus, and E. gallinarum.
In contrast, two US studies by Amachawadi et al. (2010, 2015a) found no relationship between feeding weaned piglets with feed with elevated copper concentrations (125 ppm) compared to the control diet (16.5 ppm) and an increased prevalence of copper-resistant enterococci. Though one study by this team (Amachawadi et al., 2011), similar to the other trials, did show that elevated copper in feed could increase the prevalence of tcrB-positive enterococci. These studies did demonstrate a positive correlation between the presence of the tcrB gene and tolerance to copper and the possibility of transferring this gene to enterococci from the same and from different species (Amachawadi et al., 2010, 2011), but did not specifically examine AMR in these enterococci. A study by Ragland et al. (2006) reported no increase in vancomycin-resistant enterococci isolates from piglets (17 to 20 days old) receiving an increased copper or zinc supplementation (192.4 and 2,712.68 ppm, respectively) compared to the control group (11.2 and 120 ppm, respectively).

In a further study by Amachawadi et al. (2015b) copper fed to USA feedlot cattle at a growth promotion concentration (100 ppm) was observed to be associated with modest, but significantly increased frequencies (4.5% vs 2.0% in controls) of detection of tcrB-positive and macrolide-resistant erm(B)-positive E. faecium, whilst resistances to other screened antibiotics, including vancomycin, were unaffected (Amachawadi et al., 2015b), although an earlier study reported that feeding elevated copper (up to 100 ppm) and /or zinc (up to 300 ppm) to feedlot cattle had marginal effects on AMR of faecal E. coli (resistance to clindamycin, erythromycin, penicillin, tiamulin, tilmicosin, and tylosin) and enterococci [classified as susceptible or intermediate to chloramphenicol, ciprofloxacin, gentamicin, linezolid, penicillin, streptomycin, and vancomycin] (Jacob et al., 2010). In E. coli and Enterococcus spp., only minimal differences in MICs of copper, zinc, and antibiotics were noticed. The tcrB gene was not detected in faeces or in enterococcal isolates. The proportions of erm(B) and tet(M) were unaffected by copper or zinc supplementation although this was a relatively small-scale trial involving only 20 animals, with only 5 animals per treatment.

As previously mentioned, a study by Cufaoglu et al. (2022) investigated AMR and heavy metal tolerance (as well as biocide tolerance) amongst E. coli from chicken, cattle, and sheep in Turkey.

A high prevalence of AMR (with 99% and 78% resistant to erythromycin and/or fosfomycin, respectively) were reported amongst isolates, along with a high prevalence of reduced susceptibility to zinc (62 %). Whilst HMRGs, BRGs, and ARGs were found in the same isolates, there were limited data presented to suggest that the use of heavy metals and/or biocides specifically were driving AMR.

A further study that undertook WGS of E. coli from veal calves found a higher proportion of AMR isolates with BRGs, sugE (80%), sugE1 (27%), and with 50% of isolates carrying the qacE?1 gene (Haley et al., 2022). Furthermore, ARGs mph(A), dfrA17, aadA5, and blaCTX-M-15 were positively associated with silver (sil) and copper (pco) HMRGs. But a negative association was observed between HMRGs and some frequently identified ARGs (sul2, aph(3”)-Ib, and aph(6)-Id). The authors speculated that since copper is found in milk replacer and calf starter diets it may also be co-selecting for AMR, with some association between MGEs and HMRGs, BRGs, and ARGs.

A Chinese study (Yang et al., 2020a) examining E. coli and Salmonella spp. isolates from broiler farms and broiler meat found no association between ARGs and HMRG or BRGs in E. coli, but there was a positive association between HMRGs (including pcoR and zntA) and BRGs (sugE(c), emrE, mdfA, ydgE/ydgF, qacF, sugE(p) and qacE?1). In Salmonella spp. isolates ARGs (including ß-lactam resistance genes (blaCTX, blaTEM and blaSHV), tetracycline resistance genes (tetA, tetB, and tetC) and sulfonamide resistance genes (sul1, sul2, and sul3)) were associated with HMRGs (including pcoR, pcoA, and pcoC), with some HMRGs (including pcoR and pcoA) associated with qacE?1. No details of the Salmonella spp. were provided by the authors.

Whilst not evidence of co-selection, a novel genomic island (clusters of genes within a bacterial genome that appear to have been acquired by HGT) likely to be due to insertion of a plasmid was found in monophasic S. Typhimurium isolates from the UK and Italy during 2005-2012. The genomic island included a number of ARGs genes, but also gene clusters associated with tolerance to arsenic, cadmium, zinc and copper. These isolates formed a single clade (isolates composed of a common ancestor) distinct from recent monophasic epidemic clones previously described from North America and Spain. Furthermore, isolates within this clade had a significantly higher MIC for copper sulphate than those outside the clade and without the genomic island. The authors concluded that heavy metal supplements in feed within the gastrointestinal tract of pigs may have contributed to the success of this clade (Petrovska et al., 2016). This is also supported by work in the USA by Medardus et al. (2014) who also found a strong association between AMR and heavy metal tolerance among serotypes of Salmonella spp. of public health importance.

In two opinions, the EFSA FEEDAP Panel concluded that co-selection in the gut bacteria for tolerance to zinc and copper could not be excluded (EFSA FEEDAP Panel, 2014, 2016). While the opinion on zinc did not consider its impact on AMR in any detail, the opinion on copper in feed did consider its impact on AMR in detail, which was supported by a systematic literature review by Van Noten et al. (2016). While both of these opinions made recommendations (that were later actioned into regulations) for lower permitted concentrations of zinc and copper in animal feeds (as quoted in this report in a previous section) these concentrations were primarily based on dietary requirements rather than on any impact on co-selection risk.

Table 5: In-field studies that have addressed the impact of heavy metals on antibiotic resistance (AMR).

Form of animal production	Context	Heavy metal	Species and strains	Impact on resistance	Stated antimicrobial resistance profiles (antimicrobial or class)	Antimicrobial Resistance Gene (ARGs) present	Heavy metal Resistance Genes (HMRGs) present	Country	Reference
Feedlot cattle	Feed supplementation	Copper, zinc	E.Coli, Enterococcus sp	No association with increased resistance	NS	erm(B), tet(M)	NS	USA	Jacob et al, 2010
Weaning pigs	Therapy	Zinc	LA-MRSA	Association with increased resistance	Methicillin	mecA	czrC	Canada	Slifierz et al. 2015a
Weaning pigs	Therapy	Zinc	LA-MRSA	Association with increased resistance	Methicillin	mecA	czrC	Canada	Slifierz et al. 2015b
Weaned pigs	Feed supplementation	Copper	E.Coli	Association with change in resistance	NS	tetA, tetB, blaCMY-2	pcoD	USA	Agga et al., 2014, 2015
Weaning pigs	Feed supplementation	Copper, zinc	E.Coli	Copper associated with increased resistance, but not zinc	Chlortetracycline, Neomycin, Oxytetracycline, Tiamulin	NS	NS	USA	Shelton et al., 2009
Weaning pigs	Feed supplementation	Zinc	E.Coli	Association with increased resistance	NS	blaTEM, tet(A), tet(B), sul1, sul2	NS	Germany	Bednorz et al., 2013
Pigs	Therapy	Zinc	LA-MRSA	Association between zinc resistance gene and methicillin resistance.	Erythromycin, Penicillin, Tetracycline	mecA	czrC	Denmark	Aarestrup et al. 2010; Cavco et al. 2010
Pigs	Feed supplementation	Copper	E.faecium	Association with increased resistance	Macrolides, Glycopeptide	erm(B), vanA	tcrB	Denmark	Hasman & Aarestrup, 2002
Cattle	Feed supplementation	Copper	E. faecium, E. faecalis.	No association with increased resistance	NS	NS	tcrB	USA	Amachawadi et al., 2015a
Cattle	Feed supplementation	Copper	E. faecium	Association with increased resistance	Macrolides	erm(B)	tcrB	USA	Amachawadi et al., 2015b
Weaning pigs	Feed supplementation	Zinc	E.Coli	Association with increased resistance	NS	NS	NS	Germany	Ciesinski et al., 2018
Weaning pigs	Feed supplementation	Zinc	E.Coli	Association with increased resistance, but no evidence of co-selection	?-lactamases (Ampicillin or cefotaxime), Tetracyclines (Tetracycline), Aminoglycosides (Streptomycin) and Potentiated Suphonamides (sulphamethoxazole/trimethoprim)	NS	NS	Germany	Ghazisaee di et al., 2020

As well as in feed, heavy metals may also be used as antimicrobial agents against multiple types of bacteria. Heavy metals, such as zinc and silver, are used for the treatment of burned skin surfaces, open wounds, and specific eye infections and have also been incorporated in medical devices (McDonnell & Russell, 1999; Maillard & Hartemann, 2012). AMR isolates of E. coli that also showed reduced susceptibility to silver (and copper) have been isolated from UK pig abattoirs, suggesting that co-selection is possible (Yang et al., 2020b). Few studies appear to have addressed whether their use as antimicrobial agents could be drivers for co-selection. Maillard & Hartemann (2012) called for a better understanding and control of silver usage to prevent its possible contribution to the spread of AMR. There is still clearly a need to evaluate the potential risk of the use of silver contributing to antibiotic resistance.

One of the few published studies to have considered co-selection (Elbehiry et al., 2019), demonstrated no cross-resistance between silver or gold tolerance (used in the form of nanoparticles) in adapted strains (previously subjected to sub-inhibitory treatments) of S. aureus associated with mastitis and isolated from dairy cattle and AMR. Gold nanoparticle treatments were observed to cause less development of resistance than silver treatments.

Copper and zinc are routinely used in the UK in antimicrobial footbaths in commercial dairy farming to prevent lameness caused by bacterial infections (Bell et al., 2014; Yu et al., 2017; Williams et al., 2019). Though there appears to be no evidence on their impact on co-selection. It is likely that their disposal into slurry tanks will lead to soil contamination and thus may be a driver for co-selection of AMR. This has been recognised (Williams et al., 2019) but does not appear to have been studied. Williams et al. (2019) estimated that nearly 400 million litres of cattle footbath waste is likely to be disposed annually into slurry tanks in the UK. They demonstrated that layered double hydroxides are effective in removing copper and zinc from a commercially available cattle footbath solution.

Few studies on the impact of heavy metals used in aquaculture on AMR were identified in our literature search. While there are studies on the impact of heavy metals in the environment on AMR in fish and seafood, these studies appear to relate mainly to the impact of pollution on wild caught species rather than on the impact of heavy metal use in aquaculture.

For example, a study of E. coli from pond sediment from fish farms in Nigeria showed co-occurrence of metal (copper and zinc) tolerance and antibiotic resistance (to ? -lactams including 3rd-generation cephalosporins, the fluoroquinolones, potentiated sulphonamides, tetracyclines, aminoglycosides and phenicols) and a significant correlation between concentrations of these metals and AMR (Ajewole, 2021). There was an absence of detailed information from farms on the use of biocides or feed containing these metals to correlate use with development of AMR.

Studies have shown a correlation between heavy metal tolerance and AMR in bacteria associated with aquacultural environments. But no direct causative link between the use of heavy metals in feed or as an antifoulant have been made. Aeromonads and Pseudomonads from Australian rainbow trout and sediments displayed resistance to ?-lactams, trimethoprim and florfenicol and reduced susceptibility to heavy metals [including zinc and copper] (Akinbowale et al., 2007). No link was made to any use of these heavy metals in aquaculture beyond speculation regarding the use of copper to control algae and parasites. Chenia & Jacobs (2017) observed a correlation between erythromycin resistance and copper tolerance in bacteria isolated from a tilapia aquaculture system, but while the authors suggested that copper use in feed and as an antifoulant could be responsible, this was not investigated. Neither of these studies investigated the presence of specific ARGs.

A study of the dissemination of resistance genes in duck/fish polyculture ponds, a typical farming model in some parts of China, showed significant correlations between concentrations of copper and zinc and numerous ARGs (Zhou et al., 2019). Concentrations of copper were significantly and positively correlated with the relative abundance of sul3, tetT, tetW, qnrB, qnrS, fexB, sul1, sul2, tetM, and qnrA genes. With zinc concentrations significantly correlated to relative abundance of sul2, sul3, tetM, tetA, tetT, tetW, qnrA, qnrB, qnrS, aac(6?)-Ib, qepA, blaSHV, cmlA, floR, fexA, cfr, and fexB genes. Again, while the authors suggested that differences in metal levels could be related to different feed formulations no levels of metals were measured in the feeds used.

A study by Romero et al. (2017) has been widely cited in the literature as providing evidence of the co-selection of heavy metals on AMR in seafoods. This study observed multiple tolerances (to biocides, heavy metals, and antibiotics) in 76% of isolates from a wide range of seafoods. ARGs detected included sul1 (43.33% of tested isolates), sul2 (6.66%), blaTEM (16.66%), blaCTX?M (16.66%), blaPSE (10.00%), blaIMP (3.33%), blaNDM?1 (3.33%), floR (16.66%), aadA1 (20.00%), and aac(6?)-Ib (16.66%). The copper HMRGs pcoA/copA, and pcoR were detected in 36.66% and 6.66% of selected isolates, respectively. These results suggest that exposure to heavy metals may co-select for AMR, although the fish and seafood sampled was purchased at a wide range of retail supermarkets and fish markets in the region of Jaen, Spain. While many samples were sea-caught fish; sea bass, salmon, and prawn samples were farmed and showed patterns of tolerance/resistance to heavy metals, and antibiotics. No direct comparison with any pattern of use of heavy metals during the husbandry of these seafoods could be made.

Overall, the literature on investigations of bacterial strains recovered from the field show evidence of associations/correlations between heavy metal use and increased resistance/tolerance to antimicrobial agents. Particularly there is evidence that high concentrations of copper or zinc may co-select AMR. This has led to a reduction in permitted concentrations in recent years. There is a need to establish whether current use (in feed and other uses) in food animal production in the UK represent a real hazard with respect to the selection of AMR.

Persistence of biocides and/or heavy metals in the environment

The environmental persistence of biocides depends on the nature, action, and use of the biocide. While non-oxidising biocides (such as QACs) are likely to persist in the environment (Wales & Davies, 2015; Mulder et al., 2018; Davies & Wales, 2019), oxidising agents, (such as ozone, hydrogen peroxide, chlorine dioxide, sodium hypochlorite, peracetic acid and iodophors) by their nature are unstable and prone to degradation and rapidly breakdown during use. While several reviews (Capita & Alonso-Calleja, 2013; Wales & Davies, 2015; Davies & Wales, 2019) express concern regarding the persistence of biocides, such as QACs, in the environment they cite no specific studies that appear to have studied this or provide evidence exactly of how long biocides, such as QACs, may persist in the environment. Hegstad et al. (2010) cite several studies on the fate of a QAC in the environment, one of which reported a biodegradation of 36% in 28 days, but there does not appear to be any studies that have looked at the fate and persistence of on-farm biocides in-field. A comprehensive review of predicted and measured concentrations and fate of QACs in soils and their implications on AMR development was undertaken by Mulder et al. (2018). They predicted that concentrations of QACs in manure-amended soils could reach 3.5 ppm after 1 year, assuming zero biodegradation, but highlighted the lack of data on this and whether QACs could accumulate in soil over time.

While many biocides breakdown during use, heavy metals do not biodegrade, are very persistent and will accumulate in the environment. Heavy Metals, along with antimicrobial-resistant bacteria, ARGs, and HMRGs, may be introduced into soil and water through sewage systems, direct excretion, land application of biosolids or animal manures as fertilisers, and irrigation with wastewater or treated effluents (Yazdankhah et al., 2018). In England and Wales, food animal production has been estimated to be a major source of environmental contamination by zinc and copper (Nicholson et al., 1999, 2003, 2006). Livestock manure was found to be responsible for an estimated 37%–40% of total zinc and copper inputs. Denmark has maintained a national monitoring program of heavy metals in the environment for the last 28 years to better understand the effects of these practices on the environment. The values and analyses published in 2016 indicate that the use of pig slurry has led to a significant increase in the measured concentrations of copper and zinc in soil (Rensing et al., 2018). The persistence of heavy metals in agricultural soil may lead to leaching into natural water thus impacting on irrigation and aquaculture.

A recent EFSA report highlighted that further research is required to quantify the concentrations of potentially co-selective residues of biocides and heavy metals in manures, agricultural, and aquaculture environments to facilitate risk assessment of the role that they may play in co-selection for AMR (EFSA BIOHAZ Panel, 2021).

Dissemination of AMR from animal manures to agricultural soils

Land application of animal manure is a common agricultural practice potentially leading to dispersal and propagation of ARGs in environmental settings. The dissemination of antimicrobial-resistant bacteria and ARGs from animal manure and slurry to agricultural soils has been addressed in numerous studies and reviews. It was not the purpose of this study to review this evidence, only the specific evidence on the impact of biocides and/or certain heavy metals used in animal production on AMR in this context. There is clear evidence that agricultural soils are a vast reservoir of antimicrobial-resistant bacteria and ARGs, and that the application of animal manure and/or slurry contributes to this reservoir. Overall, environmental factors can have a high impact on selective pressures, distribution, and diversity of AMR in agricultural soil. Namely, soil characteristics, such as silt, clay, organic matter, and pH, have been shown to correlate with the relative abundance of antimicrobial-resistant bacteria and ARGs (Wang et al., 2022a). This has been reviewed by Zalewska et al. (2021) and Wang et al. (2022a). Different manure sources may influence the fate of resistome in agri-ecosystems with studies demonstrating that the application of pig and poultry manures leading to a greater abundance of ARGs than cattle manure (Zhang et al., 2017b; Duan et al., 2019).

There have been numerous studies on how animal manure may be treated or processed to reduce the transmission of antimicrobial-resistant bacteria and ARGs into the environment. These methods have been reviewed by Liu et al. (2021a), Ezugworie et al. (2021), EFSA BIOHAZ Panel (2021), Wang et al. (2022a, b), amongst others. Commonly used methods include aerobic composting, anaerobic digestion, and aerobic digestion. Other alternative methods include the use of biochar, nano-materials, and phage (though, as previously discussed, phage have also been implicated in the transfer of resistance genes within the soil microbiota, albeit experimentally). Ezugworie et al. (2021) concluded that no single composting protocol completely eliminated ARGs and that a combination of protocols could yield better results. Available data indicate that none of these methods are effective at eliminating antimicrobial-resistant bacteria and ARGs. It is clear that additional research is needed to determine optimum methods in a UK context for reducing/eliminating antimicrobial-resistant bacteria and ARGs from stored manure prior to use in the environment. A recent EFSA report also highlighted that such measures may increase storage and equipment resources requirements and may reduce the fertiliser value (EFSA BIOHAZ Panel, 2021), although the report did not cite specific evidence in relation to this conjecture.

There is some evidence that a delay between the application of manure and plant life cycle (germination, growth, or harvest) of crops may reduce contamination and internalisation with antimicrobial-resistant bacteria and ARGs (EFSA BIOHAZ Panel, 2021). According to a recent EFSA BIOHAZ Panel report (EFSA BIOHAZ Panel, 2021) further research is required to define what a suitable delay may be.

Impact of biocides on AMR in animal manures and agricultural soils

No evidence has been found in the literature on the impact of biocides used in food animal production on antimicrobial-resistant bacteria or genes detected in manure and manure enriched soils. A review on the occurrence of biocides in 2016 by Wohde et al. (2016) cited only three studies at the time on the occurrence of biocides in manure. The studies cited were mainly on methods of detection and contained no evidence on what biocides may persist in animal manure. Wohde et al. (2016) at the time highlighted that the occurrence of biocides in manure had been neglected, which would appear to still be the case.

Impact of heavy metals on AMR in animal waste and agricultural soils

Due to negligible absorption in the gut, high concentrations of zinc and copper in feed results in high concentrations of these metals in animal waste. In the United Kingdom, Nicholson et al. (1999) analysed manure samples from commercial farms in England and Wales, finding zinc concentrations in 12 samples ranging from < 5 to 2500 ppm with typical concentrations of approximately 500 ppm. Data collected in China (Table 6) report that concentrations of copper and zinc are higher in pig manure than other animal manures (Wang et al., 2013). This is likely to be the case in the UK, but detailed data are lacking. It is also likely that reductions in the concentrations of heavy metals permitted in food animal production in the UK (as previously discussed in this report) may have reduced the concentration of these metals in animal waste, but again data are lacking. The environment close to aquaculture production sites have also been reported to contain elevated concentrations of copper and zinc due to the added minerals in fish feed (Burridge et al., 2010). We have found no clear evidence in the literature linking copper and zinc concentration in fish feed used in aquaculture to levels at production sites, whether in the water or sediment. Heavy metal concentrations in water, sediment, soil, and manure reported in the literature were compiled by Seiler & Berendonk in 2012. They introduced the notion that concentrations of heavy metals may need to accumulate to critical concentration before they can trigger co-selection of AMR. As also highlighted by the FAO and WHO (FAO/WHO, 2019) and Arya et al. (2021), there are little data on what these threshold values should be in order to inform suitable standards for metal concentrations in food animal production. Arya et al. (2021) developed a general model to provide a general mechanistic framework to predict minimal co-selective concentrations for metals, based on knowledge of their toxicity at different concentrations. They predicted MSCs of 5.5, 1.6, and 0.152 mg/L for copper, zinc, and silver, respectively. Comparing these thresholds with metal concentrations from slurry and slurry-amended soil from a UK dairy farm that used copper and zinc as additives for feed and in an antimicrobial footbath (at current permitted concentrations) they predicted that the slurry (which contained 22.31 and 32.16 mg/L of copper and zinc, respectively) would be co-selective, but not the slurry-amended soil (which contained only 0.068 and 0.16 mg/L of copper and zinc, respectively).

Table 6: Heavy metal contents (mean (standard deviation) ppm) reported in animal feed and manures (adapted from Wang et al., 2013).

Animal type	Copper content in feed	Zinc content in feed	Copper content in manure	Zinc content in manure
Pig	82.0 (87.7)	149.2 (280.1)	288.6 (382.3)	599.1 (1194.4)
Sow	42.9 (66.9)	82.2 (48.5)	136.4 (193.3)	483.5 (522.7)
Cattle (dairy)	19.8 (13.1)	98.6 (78.2)	56.1 (51.7)	212.6 (103.3)
Poultry	30.4 (41.4)	102.6 (46.1)	141.7 (265.1)	432.3 (287.1)
Poultry (broiler)	49.0 (57.0)	111.2 (55.0)	144.3 (208.5)	351.6 (198.2)

Studies (examples being Ji et al., 2012; Zhou et al., 2016; Peng et al., 2017; Guo et al., 2018; Duan et al., 2019; Wu et al., 2020; Dong et al. 2021; Liu et al., 2021a; Peng et al., 2021; Xue et al., 2021; Mazhar et al., 2021; Liu et al., 2022) have shown a positive correlation between the presence of heavy metals in animal manure and in agricultural soils enriched with animal manure containing heavy metals. The specific source of these heavy metals was not identified. While feed and/or medicines are often cited as probable sources of these metals, studies fail to provide clear evidence of a correlation between concentrations of these metals existing in feed and/or medicines and corresponding levels in manure and manure-enriched soils. Thus, there is no firm evidence of a causative relation in this matter. Microbial communities may be shaped by exposure to different agents and furthermore heavy metals within natural environments have been shown to significantly impact on the structure of microbial communities (Li et al, 2020). There is also some evidence that presence of heavy metals may have a positive effect on the HGT potential of ARGs in soil (Peng et al., 2017; Tongyi et al., 2020; Yuan et al., 2020; Liu et al., 2021b; Li et al., 2022c). Though how heavy metals enhance the mechanism of genetic transfer is not clear, although there is some evidence that livestock-associated bacteria may carry more MGEs than bacteria from other sources, such as clinical environments (Yuan et al., 2020) and manure has been shown to be rich in MGEs carrying HMRGs co-occurring with ARGs (Liu et al., 2022), indicating the importance of MGEs mediating in co-selection. Positive correlations between the presence of heavy metals and ARGs/MGEs in manures and enriched soils have been observed (Peng et al., 2021; Tu et al., 2023). A positive correlation between concentrations of copper and zinc and the integron-integrase gene, intI1, (linked to genes conferring resistance/tolerance to antibiotics, biocides, and heavy metals) has been observed in soil continuously amended with manure for 30 years (Peng et al., 2017). Although this particular study observed that heavy metals, and the source of manure influenced ARGs in the soil, it did not directly trace the source of these heavy metals to use in feed, although feed was suggested as a likely source. A recent study by Li et al. (2022a) suggests that while low levels of copper and zinc in pig manure may alter resistance and MGE compositions they may not be the primary drive for ARG transmission. The type of soil, which as discussed may impact on AMR, is often not stated in the literature (Wang et al., 2022a). It should also be noted that these studies have been carried out in China, where different production regimes may be practiced than in the UK (including the more widespread use of antibiotics) and there appears to be little evidence regarding the impact of European food animal production practices on ARGs in soil. The studies also lack comparison with control farms with no use of metals and/or antibiotics which would allow strong conclusions regarding the use of heavy metals.

Since heavy metals are non-biodegradable, they may be a continuous pressure on co-selection of AMR during the composting of animal manure and waste bedding (Li et al., 2015; Wang et al., 2019; Liu et al., 2021a; Ezugworie et al., 2021). Limits on heavy metals concentrations in compost have been issued by different countries, limits for copper and zinc are shown in Table 7.

Table 7: Copper and zinc limits (ppm) in compost permitted by different countries (adapted from Wang et al., 2019).

Heavy metal	Austria	Belgium	Switzerland	Germany	Italy	Spain	Canada
Copper (ppm)	400	100	150	100	300	1750	100
Zinc (ppm)	1000	1000	500	400	500	4000	500

Some studies have investigated the impact of biochar in mitigating the impact of the presence of heavy metals in animal manure on AMR during composting or digestion, or in manure-enriched soil (Liu et al., 2021a, Wang et al., 2022a; Ejileugha, 2022). Biochar has been reported to reduce the bioavailability of heavy metals, prevent HGT, and eliminate ARGs carried by MGEs (Ejileugha, 2022; Wang et al., 2022a). More novel mitigation treatments for removing heavy metals include electro remediation, which passes an electric current through liquid manure and metal ions are precipitated on an electrode (Hejna et al., 2018). At present the technology is unproven at the farm-scale and is unlikely to be cost-effective.

AMR transfer from soil to crops and foods of plant origin

The dissemination of antimicrobial-resistant bacteria and genes from manure to agricultural soils to crops and foods of plant origin has been addressed in numerous studies and reviews (Hölzel et al. 2018; FAO/WHO, 2019; Zalewska et al. 2021; EFSA BIOHAZ Panel, 2021; Wang et al. (2022a), amongst others). A number of reviews (including recent reviews by FAO/WHO, 2019 and EFSA BIOHAZ Panel, 2021) have cited evidence that antimicrobial-resistant bacteria and ARGs from manure-amended soils can potentially disseminate from soil microbiota to plant microbiota, thus may be an important route for AMR transmission in foods of plant origin. Since fruits and vegetables are frequently eaten raw or with minimal processing they can potentially serve as a source of dietary exposure to antimicrobial-resistant bacteria and ARGs of animal-origin.

It was not the purpose of this study to review this evidence, only the evidence on the impact of biocides and/or certain heavy metals used in animal production on AMR. The FAO/WHO (2019) report also suggested that heavy metals (for example, copper and zinc in feed) should be considered in terms of probability of selection.

While studies, such as Buta et al. (2021), have observed an association between the presence of heavy metals and ARGs in animal manure which migrated with the manure to agricultural soils enriched with animal manure and these to crops, a causal link to the use of heavy metals in animal production is not identified. Longitudinal studies on the impact of biocides and/or heavy metals during animal production on the transmission of AMR to crops and foods of plant origin are lacking.

Overall, there appears to be little compelling evidence in the literature on this subject and a better understanding is needed on how ARGs may transfer from the soil to plants and the risk of ARGs to humans from consumption of plants containing antimicrobial-resistant bacteria and/or ARGs, particularly those intended to be consumed raw.

AMR transfer from soil to animals and foods of animal origin

While faeces, fertilisers of animal origin (for example, manure and slurry), and bedding have been identified as potential transmission routes to the dissemination of antimicrobial-resistant bacteria and genes in animals and foods of animal origin there is little information on their importance (EFSA BIOHAZ Panel, 2021). Longitudinal studies on the impact of biocides and/or heavy metals during animal production on the transmission of AMR to animals and foods of animal origin are lacking. While there is evidence of co-carriage of BRGs/HMRGs and ARGs in retail meats (for example Zou et al. 2014 and Yang et al., 2020a) which is of concern, studies showing a clear relationship between the use of biocides and/or heavy metals on the farm contributing to AMR in retail meats is lacking.