Research Paper

Development and validation of novel PCR primers for identification of plasmid-mediated colistin resistance (*mcr*) genes in various environmental settings

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ABSTRACT

Antibiotic resistance is considered one of the biggest threats to public health and has become a major concern for governments and international organizations. Combating this problem starts with improving global surveillance of antibiotic resistance genes (ARGs) and applying standardized protocols, both in a clinical and environmental context, in agreement with the One Health approach. Exceptional efforts should be directed to controlling ARGs conferring resistance to Critically Important Antimicrobials (CIA). In this study, a systematic literature review to synthesize data on the identification of *mcr* genes using a PCR technique was performed. Additionally, a novel set of PCR primers for *mcr-1 – mcr-9* genes detection was proposed. The developed primers were *in silico* and experimentally validated by comparison with *mcr*-specific PCR primers reported in the literature. This validation, besides being a proof-of-concept for primers' usefulness, provided insight into the distribution of *mcr* genes in municipal wastewater, clay and river sediments, glacier moraine, manure, seagulls and auks feces and daphnids from four countries. This analysis proved that commonly used primers may deliver false results, and some *mcr* genes may be overlooked in tested samples. Newly-developed PCR primers turned out to be relevant for the screening of *mcr* genes in various environments.

1. Introduction

Antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are considered some of the biggest threats to public health and have become a major concern for governments and international organizations (Ventola, 2015). In recent years, the detection of ARB and ARGs required ever-increasingly comprehensive methods of investigation. This way of thinking is a cornerstone of the One Health approach, a multidisciplinary concept combining the efforts of a wide range of specialists seeking to find solutions to preserve public health. In the case of antibiotic resistance (AR), the One Health approach underlines that microorganisms exist in a continuous biological space created by a dense network of multilateral relations between humans, animals and the environment (McEwen and Collignon, 2018). One Health policies to prevent the spread of AR call for the collection of knowledge and data concerning AR across distinct environmental compartments. Until now, multiple reports have pointed out the importance of reliable surveillance as a key strategy in recognizing and dealing with emerging AR (Schnall et al., 2019). It was also emphasized that for the efficiency of screening procedures and preventing AR dissemination, antibiotics should be categorized according to their clinical significance. This allows establishing a list of antibiotics critically important for medicine, i.e., drugs of the last resort, that need special attention and protection from AR development.

A currently recognized antibiotic of the last resort is colistin, a polymyxin (polymyxin E) first discovered in the 1940s. This natural antibiotic is synthesized by *Bacillus polymyxa* var. *colistinus* (Benedict and Langlykke, 1947). Initially, colistin was used for the treatment of problematic infections caused by Gram-negative bacteria. However, its usage was quickly restricted because of its high nephrotoxicity and neurotoxicity (Wolinsky and Hines, 1962). The situation changed in the late 1990s due to increasing AR globally. Nowadays, colistin is experiencing a true revival following the confirmation of its suitability in treating multi-drug resistant Gram-negative bacteria belonging to the *Gammaproteobacteria* class (in particular *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*) (Li et al., 2006). Initially, colistin worked very well as the majority of bacteria were susceptible, and the only known resistance mechanism, based on specific mutations in *pmrAB* and *phoPQ* regulatory systems, was encoded on the chromosome (Gunn et al., 1998; Lee et al., 2004). Colistin became popular in veterinary and animal production, where its usage in the European Union and China reached 12,000 tons in 2015 (Rhouma et al., 2016). A consequence of the uncontrolled usage of colistin was the development of novel resistance mechanisms. In 2016, the first evidence of the emergence of the plasmid-mediated polymyxin resistance mechanism (MCR-1, encoded by the *mcr-1* gene) was reported (Liu et al., 2016). The emergence of MCR-1 heralds the breach of colistin usage, which was restricted for use only in critical clinical situations. After that, WHO updated the Critically Important Antimicrobials list, which sets out a category of "critically important" antimicrobials with colistin on the top (WHO, 2019).

From a One Health perspective, colistin is a very interesting example of an antibiotic, which due to a long period of rejection in medicine, was mostly used in agriculture (Watkins et al., 2016), livestock and poultry production (Kempf et al., 2016). This created selection pressure in an environment suitable for the development of mobile colistin resistance

(MCR) mechanisms that are current clinical problems (Anon, 2018). Thus, most probably, MCR resistance has primarily an environmental origin, which exemplifies the role of non-nosocomial environments in the development of antibiotic resistance (McEwen and Collignon, 2018). Following the identification of *mcr-1* in 2016 (Liu et al., 2016), other *mcr* genes were found, including: (i) *mcr-2, mcr-3, mcr-4* and *mcr-5* in 2017 (Borowiak et al., 2017; Carattoli et al., 2017; Poirel et al., 2017; Yin et al., 2017), (ii) *mcr-6, mcr-7* and *mcr-8* in 2018 (AbuOun et al., 2017; Wang et al., 2018; Yang et al., 2018) and *mcr-9* in 2019 (Carroll et al., 2019).

Classification of *mcr* genes relies on the phylogeny of encoded MCR enzymes. This phylogenetic analysis revealed that *mcr* genes most probably have various ancestors. For example, it was proposed that *mcr-1* and *mcr-2* genes are closely related to *eptA* gene conferring intrinsic resistance to polymyxin E in *Neisseria* spp. (Xu et al., 2018), whereas, the *mcr-4* gene most probably originates from non-mobile, *NMCR-1* gene present in aquatic *Shewanella* spp. (Zhang et al., 2019). Various origin of *mcr* genes is also reflected by their reciprocal similarities, as separate clusters of *mcr* genes can be distinguished (Fig. 1). Moreover, a rapid evolution of *mcr* genes can be observed, which results in the development of the increasing number of variants of particular *mcr* genes (Sun et al., 2018).

The recognition of critically important antibiotics raises the awareness for monitoring of resistance mechanisms, as well as screening their distribution in various environmental compartments. For this purpose, the public health institutions, including the European Center for Disease Prevention and Control (ECDC) and the Global Antimicrobial Resistance Surveillance System (GLASS) by WHO, prepared technical reports indicating genotypic surveillance as an efficient method for identification of ARGs (ECDC, 2020; Anon, 2017). These referred also to *mcr* genes. Molecular biology provides different methodologies for the detection of ARGs, however, PCR is regarded as one of the most useful
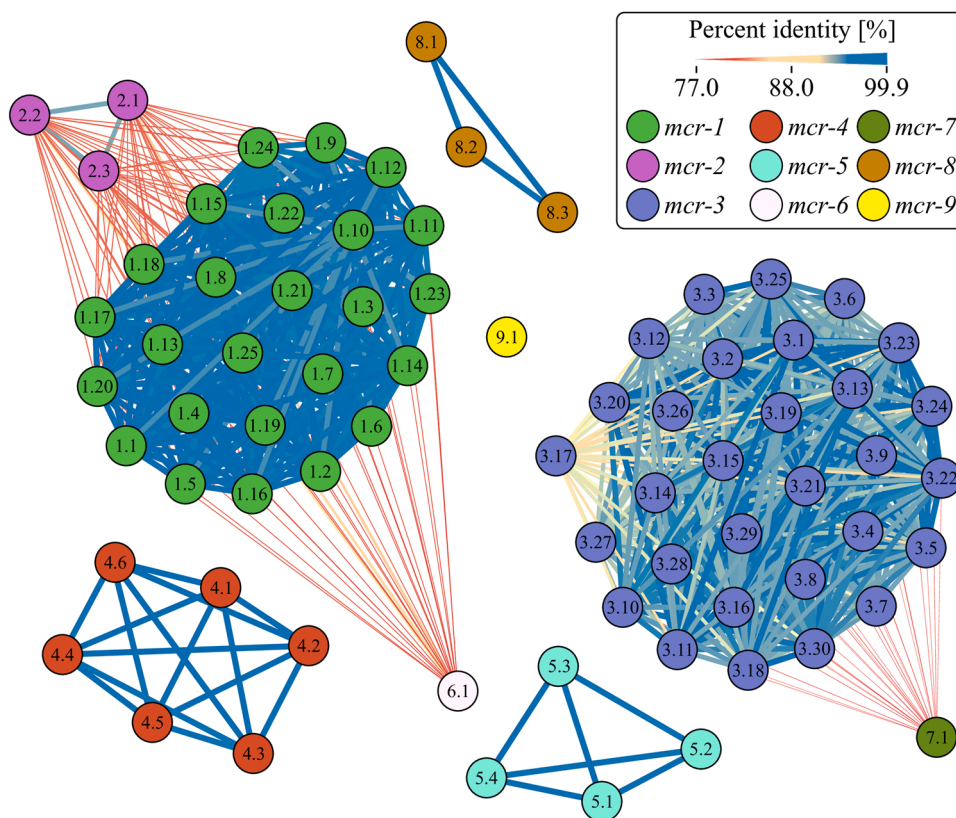

Fig. 1. Nucleotide-based similarity network of *mcr* gene variants. Each node represents a single variant of individual *mcr* gene denoted by different colors (in total 74 variants), while edges correspond to percent identity between nucleotide sequences of *mcr* variants. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

among them as it allows obtaining rapid and reliable results when applied properly. Notably, the scientific community provided a huge number of PCR primers for ARGs screening by PCR. Unfortunately, many of these PCR primers may generate unreliable (i.e., false-negative or false-positive) results (Gorecki et al., 2019).

In this study, we conducted a systematic literature review to synthesize data concerning the identification of *mcr* genes using a PCR technique. Retrieved PCR primers were then validated both, *in silico* and experimentally, which revealed the lack of a standard operating procedure (SOP) providing proper and reliable identification of *mcr* genes in various environmental settings. Moreover, it was proved that commonly used *mcr*-specific primers may generate false results and thus, their *in silico* pre-validation is critically important. We developed a system enabling such validation and building the rankings of PCR primers specific to a particular ARG. Finally, we proposed novel PCR primers for *mcr* genes identification and critically evaluated their usefulness by comparison with other literature-retrieved PCR primers. This, besides the applied value resulting from the development of novel diagnostic tools, provided us a comprehensive insight into the distribution of various *mcr* genes in municipal and hospital wastewaters, clay and river sediment, glacier moraine, manure, seagull and auks feces and daphnids from four European countries (Czech Republic, Norway, Poland and Portugal) differing in antibiotic consumption as well as legislation referring to antibiotic usage and prevention against AR development. Performed analysis showed that *mcr* genes are common in various environmental settings (including pristine Arctic regions) and that wild animals are important reservoirs of these genes.

2. Materials and methods

2.1. Systematic review search strategy

A critical, systematic review of the literature was conducted on 31st December 2019 to select pertinent scientific articles published in English and mentioning *mcr*-specific PCR primers. The Pubmed database was subjected to searches using the following terms: *mcr-1, mcr-2, mcr-3, mcr-4, mcr-5, mcr-6, mcr-7, mcr-8, mcr-9, mcr-1 PCR, mcr-2 PCR, mcr-3 PCR, mcr-4 PCR, mcr-5 PCR, mcr-6 PCR, mcr-7 PCR, mcr-8 PCR, mcr-9 PCR, mcr-1 primers, mcr-2 primers, mcr-3 primers, mcr-4 primers, mcr-5 primers, mcr-6 primers, mcr-7 primers, mcr-8 primers* and *mcr-9 primers*. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Liberati et al., 2009; Moher et al., 2009) guidelines were followed while searching, screening and selecting papers for this review. The following inclusion criteria were applied: (i) full-length articles published in English; (ii) articles published between 2016 and 2019; (iii) original articles using a PCR approach for the detection of *mcr* genes. Reviews and non-English articles were omitted. The following data were then extracted from the selected articles: *mcr* gene name, sequence of primers and predicted PCR product size.

2.2. Sampling and DNA extraction

2.2.1. Wastewater treatment plant (WWTP) – Poland

A sample of primary sludge was collected from the municipal WWTP located in Lodz (Poland) in October 2016. The WWTP has a capacity of about 195 m^3 of wastewater per day. The sample (5 litres in total) was collected into sterile bottles and transferred directly to the laboratory. In the laboratory, sludge samples were distributed into sterile 50-ml Falcon tubes and centrifuged for 10 min at 5,900 g. After removing the supernatant, sludge samples were stored at - 20 ℃ for further analysis. Total DNA was extracted in triplicate from 500 mg of raw material using a Fast DNA Spin Kit for Feces (MP Biomedicals, Illkirch, France) according to the manufacturer's instructions.

2.2.2. Daphnids – Poland

Daphnia spp. were collected in July 2020 from the Ksiazeca Pond,

which is located near the city center of Warsaw, Poland. Ksiazeca is a small (area about 0.24 ha), shallow (maximum depth ca. 60 cm, average ca. 35 cm), fishless, artificial water reservoir with a concrete bottom located in a city park. Every year, the pond is dried and partially cleaned just prior to the onset of winter and is filled again with fresh water at the beginning of spring. Each year, daphnid re-colonization from the remaining resting egg bank takes place. Ducks appear intermittently at the pond and bring a steady supply of new *Daphnia* genotypes to the population gene pool. The selected pond was one of several reservoirs in Warsaw under permanent scientific study and appears to be a good representative of urban reservoirs in the city (Mikulski et al., 2017). Zooplankton samples were collected from the Ksiazeca pond using a plankton net (150 μm mesh size). Subsamples were harvested from the three sites reflecting the diversity of the habitat: the edge, the deep middle and the intermediate zone. Samples were directly transported to the laboratory for further processing. In the laboratory, zooplankton samples were pooled and examined. Only *Daphnia* spp. individuals were selected for further microbiome analysis. Total DNA was extracted in triplicate from 250 mg of *Daphnia* per sample using a Fast DNA Spin Kit for Feces (MP Biomedicals) according to the manufacturer's instructions.

2.2.3. Clay sediments – Poland

Sediment samples were collected on July 2020 from the clay water reservoir (clay pit) located in Szczesliwice park in Warsaw, Poland. The reservoir occupies an area of about 5.95 ha, and the maximum depth is 10 m. It is a habitat for numerous species of birds, amphibians, reptiles, fish and invertebrates.

Sediment samples were collected using a small shovel and placed in sterile containers. Samples were harvested from the four sites representing the diversity of Szczesliwice pond: natural shore, concrete shore, macrophyte area and wooded shore. Samples were directly transported to the laboratory for further processing. Before DNA extraction, all samples were pooled. Total DNA was extracted in triplicate from 500 mg of sediments per sample using a Fast DNA Spin Kit for Feces (MP Biomedicals) according to the manufacturer's instructions.

2.2.4. Seagull feces and river sediments – Portugal

Seagull feces and river sediment samples were collected from the Local Nature Reserve Douro Estuary located in Vila Nova de Gaia (Portugal) in August 2020. The Local Nature Reserve Douro Estuary occupies an area of 62 ha and is composed of an estuary area from the Douro river. It is a project developed and maintained by the Biological Park of Gaia (Portugal) with the main objective of protecting birds and the landscape. Migratory and resident species of seagulls can be observed, such as *Larus fuscus graellsii, Larus cachinnans, Larus marinus, Larus canus, Larus delawarensis, Chroicocephalus ridibundus* and *Ichthyaetus melanocephalus*. Seagull feces were collected close to the ebb tide since this is the area with the largest group of seagulls. Individual feces samples were carefully collected into a sterile flask to avoid the collection of river sediment and transported in refrigerated containers for further processing in the laboratory. Two composite samples of seagull feces were obtained by pooling 0.5 g of each individual fecal sample from 5 and 10 individuals. Total DNA was extracted from 0.24 g of seagull feces composite sample and from composite sediment samples obtained by pooling 8 g of 8 individual river sediment samples. Extractions were made in triplicate with the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2.5. Hospital effluents – Czech Republic

A sample of hospital effluent was collected on August 2020 from hospital WWTP situated in Prague, Czech Republic. The WWTP has the capacity of about 80 m^3 of infectious wastewater per day. Total DNA extraction was performed according to the protocol for the DNeasy PowerSoil kit (Qiagen). The sample (1 litre in total) was collected into

sterile bottles and transferred directly to the laboratory. In the laboratory, four aliquots of 20 ml were filtered through a 0.45-µm membrane filter. Each filter was placed into a sterile 5-ml tube into which the contents of the PowerBead tube was placed. C1 solution was added, and the tubes with filters were placed on a Vortex-Genie 2 (MO BIO Laboratories, Carlsbad, CA, USA). Next steps of DNA extraction were performed according the manufacturer's instructions.

2.2.6. Manure – Czech Republic

Approximately 100 ml of pig manure was collected in August 2020 from a pig farm (16,200 specimens) situated in Karlovy Vary district (Czech Republic). Sample was transported directly to the laboratory for further processing. Four aliquots (0.251–0.280 g) were used to extract DNA using the DNeasy PowerSoil kit (Qiagen) according to the manufacturer's instructions.

2.2.7. Little auks feces and glacier moraine – Norway (Arctic)

Little auks feces (N 7700'296" E 01530'526") and soil from the glacier moraine ("N 7700'434" E 01535'451") samples were collected in June 2018 from sites in ice-free areas, in the vicinity of the Polish Polar Station Hornsund in Spitsbergen, Svalbard, Norway. Four aliquots of 250 g soil (0–10 cm depth) or feces were collected from approximately 1 m^2 area at each sampling site using sterile tools. Frozen (- 20 ℃) samples were transported to laboratory, where all aliquots from each sampling sites were pooled and homogenized. Total DNA was extracted in triplicate from 500 mg of a homogenized sample using a Fast DNA Spin Kit for Feces (MP Biomedicals) according to the manufacturer's instructions.

2.3. Polymerase chain reaction (PCR)

The occurrence of the *mcr* genes was investigated using PCR assays. All PCR reactions were performed in triplicate using 1.0 µl of extracted DNA (which is equal to approximately 5–20 ng of DNA, depending on the sample), 0.1 pmol of each primer (Table 1, Supplementary Materials - Table S1) with a DreamTaq PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The DNA concentration was determined using the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The PCR programs differed depending on the recommended annealing temperature for each primer pair (Supplementary Materials - Table S1). PCR products were resolved by electrophoresis in 1.5% agarose gels. All PCR products of the desired size were purified and sequenced using a specific primer with an ABI-PRISM 377 capillary DNA analyzer (Applied Biosystem, Foster City, CA, USA) at the DNA Sequencing and Oligonucleotide Synthesis Laboratory, IBB Polish Academy of Science (Warsaw, Poland). Obtained DNA sequences were subjected to similarity searches using

BLAST (Altschul et al., 1990) against the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020) to verify product specificity. All *mcr* sequences in the .abi format are available under the following address: http://ddlemb.com/bioinformatic-tools-and-databases/. The pUC57 vectors carrying *de novo* synthesized *mcr* genes [*mcr-1* (CARD ARO no. 3003689), *mcr-2* (CARD ARO no. 3004110); *mcr-3* (CARD ARO no. 3004139); *mcr-4* (CARD ARO no. 3004325); *mcr-5* (CARD ARO no. 3004332); *mcr-6* (CARD ARO no. 3004501); *mcr-7* (CARD ARO no. 3004517); *mcr-8* (CARD ARO no. 3004516); *mcr-9* (CARD ARO no. 3004684)] were used as the positive controls for the validation of the PCR primers. In the case of negative results in all tested samples, spiking with four amounts (1.0, 0.1, 0.01 and 0.001 ng) of specific standards (i.e. selected *mcr* gene cloned into the pUC57 vector), generated by *de novo* gene synthesis, was performed. This was conducted to test the possibility of a potential inhibition of the PCR reaction.

2.4. Quantitative PCR (qPCR)

The quantitative PCR assays targeting *mcr-1* and *mcr-3* were performed using primers developed in this study - MCR1_DEMB_F/ MCR1_DEMB_R and MCR3_DEMB_F/MCR3_DEMB_R, respectively (Table 1). The primers final concentration in each reaction was 250 nM. The following conditions were applied for the reactions: (i) for *mcr-1* – 95 ℃ for 15 min (1 cycle), 95 ℃ for 15 s, 60 ℃ for 30 s and 72 ℃ for 20 s (40 cycles); (ii) for *mcr-3* – 95 ℃ for 15 min (1 cycle), 95 ℃ for 15 s, 60 ℃ for 30 s and 72 ℃ for 20 s (45 cycles). Quantifications were made based on the Standard Curve method using a StepOnePlusTM Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The genes *mcr-1* and *mcr-3* were quantified using the SYBR® Select Master Mix (Thermo Fisher Scientific, Austin, USA). As qPCR standards, *de novo* synthesized *mcr-1* (CARD ARO no. 3003689) or *mcr-3* (CARD ARO no. 3004139) genes cloned into the pUC57 vector were used. For the optimization of the reaction conditions and validation of primers the following DNA matrixes were used: (i) analysed in this study, DNA originating from WWTP (Poland) and seagull feces (Portugal); (ii) DNA from hospital effluents (Portugal) and the river water (Portugal) (Rocha and Manaia, 2020).

2.5. PCR primer design

Primers were designed with an in-house-developed pipeline – the ConPrimise v1.0 (https://gitlab.com/gorecki413/ConPrimise) – written in Python v3.6 (https://www.python.org/) and UniPriVal (Gorecki et al., 2019). The ConPrimise v1.0 script designs the maximum possible number of primers using a consensus sequence calculated from all

Table 1
Primer pairs designed in this study.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product length (bp)	Annealing temperature (℃)
mcr-1	MCR1_DEMB_F: GTCGGTATGCTCGTTGGCTTAG	MCR1_DEMB_R: CATAGGCATTGCTGTGCGTCTG	271	54.7
mcr-2	MCR2_DEMB_F: ATGGCGGTCTATCCTGTATCG	MCR2_DEMB_R: CACAAGCAACAGCACAAGG	396	49.1
mcr-3	MCR3_DEMB_F: CCGCTACACCGATTTCGTGATTG	MCR3_DEMB_R: TCACCATGATCGGAGACGTAGAG	99	57.1
mcr-4	MCR4_DEMB_F: CTGCTGACTGGGCTATTACC	MCR4_DEMB_R: GTCTTGAGCATTAGCACGG	534	49.1
mcr-5	MCR5_DEMB_F: GAGAATGCTGCCCTACTTG	MCR5_DEMB_R: GTTTGTCGTTCATAGCCGCTC	412	49.1
mcr-6	MCR6_DEMB_F: ACTGACCAAGCCGAGTCTAAG	MCR6_DEMB_R: GCATCACGGGATTGACATAGC	259	52.4
mcr-7	MCR7_DEMB_F: GCGACCTCCTACCTGAATG	MCR7_DEMB_R: CCCTTTGGCGACGACTTTG	345	51.3
mcr-8	MCR8_DEMB_F: TTGTCGTCGTGGGCGAAAC	MCR8_DEMB_R: CTGTCGCAAGTTGGGCTAAAG	514	51.3
mcr-9	MCR9_DEMB_F: CGGCGAACTACGCTTACAG	MCR9_DEMB_R: CGCACAGTTTCGGGGTTATCAC	465	51.3

known variants of the particular gene after multiple sequence alignments using ClustalW (Chenna et al., 2003). All reference sequences of *mcr* genes used for designing of novel primers are available under the following address http://ddlemb.com/uploads/all_mcr_nucl_fasta.fna. In the next step, all primers were filtered out, searching for those which pass previously set thresholds. In this study, we implemented the following thresholds: (i) primer lengths between 18 and 22 bp, (ii) melting temperatures between 50 and 58 ℃, (iii) mononucleotide tracks shorter than 3, (iv) GC content between 45% and 55%, (v) product length between 90 and 600 bp, (vi) self-dimerization of primers – not acceptable. Additionally, primers were checked for hairpin structure formation and cross-dimerization using commonly available tools (https://www.bioinformatics.org/sms2/pcr_primer_stats.html). Designed sets of primers were additionally validated using the UniPriVal software (Gorecki et al., 2019). Only those which exhibited maximal basal statistics (specificity, efficacy, taxonomic efficacy) were then ranked by the updated model success metric to choose the best possible primers for further application. Designed primers are presented in Table 1.

2.6. In silico primer validation

All primers used in this study were subjected to *in silico* validation using the UniPriVal software. For the preparation of rankings of tested primers, a model success metric (MSM), described previously, was used (Gorecki et al., 2019). MSM is a value obtained by the normalization of basic statistics with an accuracy parameter. Accuracy was calculated based on a modified confusion matrix. It may be also used for describing particular primers as it is directly proportional to the number of correct *in silico* PCR products (i.e., true positive) and inversely proportional to the number of incorrectly omitted *in silico* PCR products (i.e., false-negative) (Gorecki et al., 2019). In this study, for more reliable validation of primers, a novel parameter – precision parameter ($PP_{i,j}$) – was developed. It indicates the contribution of a specific product in a pool of *in silico* amplified PCR products of sizes not exceeding 5000 bp. This parameter is crucial for the selection of primers, which under laboratory conditions will generate the smallest possible number of unspecific products of different lengths, causing background noise or multiple melting curves in case of real-time PCR. The precision parameter is calculated as the ratio of the number of specific *in silico* PCR products for a particular primer pair i amplifying ARG j using the NT database as a matrix ($CP_{i,j}NT$) and the number of all *in silico* products observed for this particular primer pair using the NT database as a matrix ($P_{i,j}NT$) (i).

$$PP_{i,j} = \frac{CP_{i,j}NT}{P_{i,j}NT} \qquad (i)$$

Previously described parameters, i.e. specificity (showing if primer pair is specific to the target gene), efficacy (showing if primer pair cover all gene variants), taxonomic efficacy (showing if primer pair cover all putative bacterial hosts) as well as accuracy and precision parameters were used for the normalization of basal statistics for the calculation of an updated model success metric (MSM), which is used to rank primer pairs (Supplementary Materials - Table S2). All parameters are expressed in values from 0.0 to 1.0.

2.7. Construction of the similarity network of mcr gene variants

A similarity network was constructed based on an undirected weighted topology using numeric values corresponding to the percent identity between nucleotide sequences of all *mcr* gene variants (http://ddlemb.com/uploads/all_mcr_nucl_fasta.fna) obtained after alignment of sequences using BLAST tool (Altschul et al., 1990). Variants are represented as nodes and clustered in the network according to their weight (value of percent identity). Visualization of the network has been prepared using the Gephi software (Bastian et al., 2009) by a combination of Reingold–Fruchterman and ForceAtlas 2 layouts (Jacomy et al., 2014).

3. Results

3.1. Literature-based identification and in silico validation of PCR primers for mcr genes

A PRISMA approach was employed for a literature systematic review in this study (Liberati et al., 2009; Moher et al., 2009). Overall 1056 publications (after the removal of duplicates) were found. Non-English papers (4), reviews (46), articles with no access (36) and errata (11) were excluded from the analysis. In the next step, 412 original articles not applying PCR for the identification of *mcr* genes were also excluded. This resulted in 547 papers being included in the quantitative systematic synthesis (Fig. 2). Examination of these scientific works revealed that within 100 of them, authors designed primers *de novo*. The highest number of articles contained primers designed for *mcr-1*, which resulted in retrieving 84 primer pairs for that gene. For other *mcr* genes, there were: (i) 21 primer pairs for *mcr-2*, (ii) 18 for *mcr-3*, (iii) 12 for *mcr-4*, (iv) 12 for *mcr-5*, (v) 2 for *mcr-6*, (vi) 3 for *mcr-7*, (vii) 4 for *mcr-8* and (viii) 2 for *mcr-9* (Supplementary Materials - Table S2). The low number of primers for genes *mcr-6* to *mcr-9* probably results from a recent release date of the reference sequences for those variants, which falls between 2018 and 2019.

Interestingly, results of the performed systematic review showed, additionally, that over a 4-year period (2016–2019), only a few of the designed primers specific to *mcr* genes were frequently used. It was revealed that primers for *mcr* identification that were designed in 60 articles were probably used only by their developers, as they have never been cited by other authors, while 16 other articles introducing new primer sets were cited only once (Supplementary Materials - Fig. S1). Notably, amongst publications presenting novel *mcr* primers, eight were cited more than 10 times, including six studies presenting primer pairs for a particular *mcr* gene (Borowiak et al., 2017; Carattoli et al., 2017; Chabou et al., 2016; Liu et al., 2016; Xavier et al., 2016; Yin et al., 2017) and two introducing sets of primer pairs for more than one *mcr* gene (Rebelo et al., 2018; Wang et al., 2018) (Supplementary Materials - Fig. S1).

It was shown that PCR screening with properly designed primers is one of the most common and efficient approaches allowing reliable epidemiological risk assessment in clinical strains and surveillance of antibiotic resistance genes in various experimental settings (Abdelgader et al., 2018; Chung et al., 2017; Jin et al., 2018; Zurfluh et al., 2017). However, a crucial step toward obtaining the least biased results is proper *in silico* validation of primers. In 2019, we proposed a system for *in silico* validation of PCR primers based on 3 parameters (specificity, efficacy and taxonomic efficacy) using a heuristic algorithm for alignments of primer pairs with global databases and metagenomic data (Gorecki et al., 2019). In this work, we supplemented the validation system by adding two additional parameters – accuracy and precision, which increased the strength of the model success metric for the building of the primers' rankings. Then 166 *mcr*-specific primer pairs, found by a systematic literature review, were subjected to *in silico* validation.

The validation step revealed that 22 (13%) primer pairs exhibited MSM values equal to zero, which indicated their lack of usability in the screening of *mcr* genes. As many as 55 primer pairs showed maximal values of MSM. Obtained ranking of primers revealed that commonly used ones were placed on far positions, which suggested that they were less suitable for *mcr* gene screening than the primers proposed by other scientists, but not so commonly used (Supplementary Materials – Table S2).

The methodology proposed by us is very sensitive, and at the same moment very strict, as calculations for *in silico* validation of ARG-specific primers are made using the National Center for Biotechnology
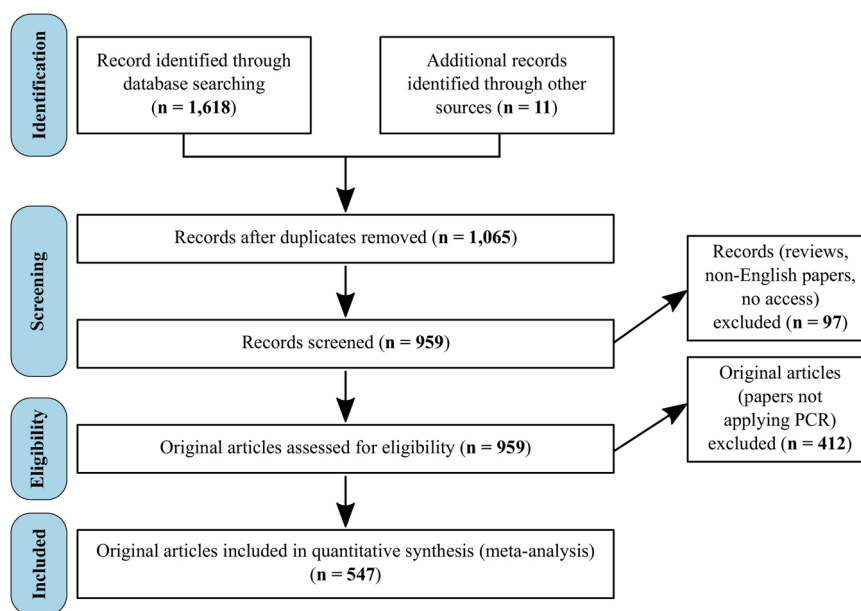
Fig. 2. PRISMA-adapted flow diagram of included and excluded publications during the preparation of the systematic literature review in this study. The flow diagram was adapted from the PRISMA website (http://www.prisma-statement.org/) (Moher et al., 2009) and further modified.

Information (NCBI Resource Coordinators, 2017) database, which is a global resource. This enables testing primers for all possible applications, including heterogeneous environmental samples such as soil, sludge or waters and not only (somehow homogenous) clinical settings. Such validation will identify whether a particular pair of primers covers all variants of the analysed ARG. If not, this will ultimately lower the position of primers in the overall ranking. However, as shown during analyses of *mcr* genes, not all clinically valid ARG variants are equally distributed, and some of them are rare (mostly absent) in some environments. This raises the question as to whether their omission is negligible? This is unlikely since, for a proper surveillance of ARGs, we should eliminate the programmed methodology bias at the very beginning to limit the number of false-positive and false-negative results. We assume that the situation as described above may be relevant in the case of *mcr-1*-specific primers designed by Liu et al. (Liu et al., 2016). These primers were mostly used for the screening of clinical or animal samples (260 citations), where they probably cover the majority of *mcr-1* variants. However, they were also used for environmental sample screening (29 publications over the 2016–2019 period), which might result in obtaining false-negative results, as those primers do not cover all variants of the *mcr-1* gene that proved to be present in various settings. Our analysis revealed that primers designed by Liu et al. (Liu et al., 2016) had maximal specificity and taxonomic efficacy values but dropped in ranking due to their efficacy value, which equals 0.88. This indicated that some of the clinically relevant *mcr-1* variants may be simply overlooked during the screening (Supplementary Materials - Table S2).

3.2. Genotypic surveillance of mcr genes in various environmental samples – a proof-of-concept for novel mcr-specific primers

In respect to the problems identified above, we made an attempt to create a standardized model for surveillance of *mcr* genes in various environments by developing a novel set of PCR primer pairs for identification of *mcr-1 – mcr-9* genes and evaluating their utility in comparison to other *mcr*-specific primers, i.e. 23 primer pairs selected from the literature, including: 4 primer pairs for *mcr-1*, 4 for *mcr-2*, 4 for *mcr-3*, 4 for *mcr-4*, 4 for *mcr-5*, 1 for *mcr-6*, 1 for *mcr-7*, 1 for *mcr-8*, designed previously and commonly used (as judged from our analysis) (Supplementary Materials - Fig. S1). Novel primers were designed based on the consensus sequence of all variants of a particular *mcr* gene, and they

were presented in Table 1. The procedure for designing novel primers was supplemented with an additional step of initial *in silico* validation, which allowed selecting those that amplify all variants of a particular *mcr* gene present in global public databases of bacterial nucleotide sequences, i.e. the NCBI nucleotide collection (NCBI Resource Coordinators, 2017).

Experimental validation of designed primers was performed using nine environmental DNAs isolated from samples originating from four countries (i.e., the Czech Republic, Norway, Poland and Portugal). PCR screening (combined with subsequent sequencing of PCR products) performed with these primers, enabled identification of all *mcr* genes except for *mcr-7*. Most prevalent were: *mcr-3* (identified in six samples, i. e. daphnids, WWTP, hospital effluents, manure, glacier moraine and little auks feces from the Czech Republic, Norway and Poland) and *mcr-2* (in five samples, i.e. daphnids, seagull feces, river sediments, hospital effluents and little auks feces from all four countries). In case of *mcr-1*, *mcr-5* and *mcr-6*, they were identified in four samples, while *mcr-4*, *mcr-8* and *mcr-9* were detected in two samples (Fig. 3).

After demonstrating the utility of newly designed primers for the identification of *mcr* genes, their efficiency in specific ARGs screening was evaluated by comparison to other commonly used primers, retrieved from the literature. It was shown that, in the case of *mcr-1*, *mcr-5*, *mcr-6* and *mcr-8*, primers newly designed in this study enabled the identification of *mcr* genes in more samples (Fig. 3). Genes that were identified exclusively by primers designed in this study were: (i) *mcr-1* in daphnids and WWTP from Poland, (ii) *mcr-5* in seagull feces and river sediments, (iii) *mcr-6* in clay sediments, seagull feces and river sediments, and (iv) *mcr-8* in daphnids and river sediments. The *mcr-7* gene was not detected in analysed samples (Fig. 3). An interesting situation was observed for the *mcr-3* gene, which was detected in all analysed samples but only when complementing results obtained for two different primer pairs, i.e. designed in this study and by Lescat et al. (Lescat et al., 2018) (Fig. 3).

Among results obtained for all *mcr* genes, these for *mcr-1* and *mcr-3* are especially important in terms of public health. These genes are of special clinical relevance as they proved to be hosted mostly by bacterial strains isolated from hospital patients, while other *mcr* genes are also identified in environmental strains (Elbediwi et al., 2019). Importantly, *mcr-1*-specific PCR primers designed in this study revealed to be most effective in the detection of these genes compared with other
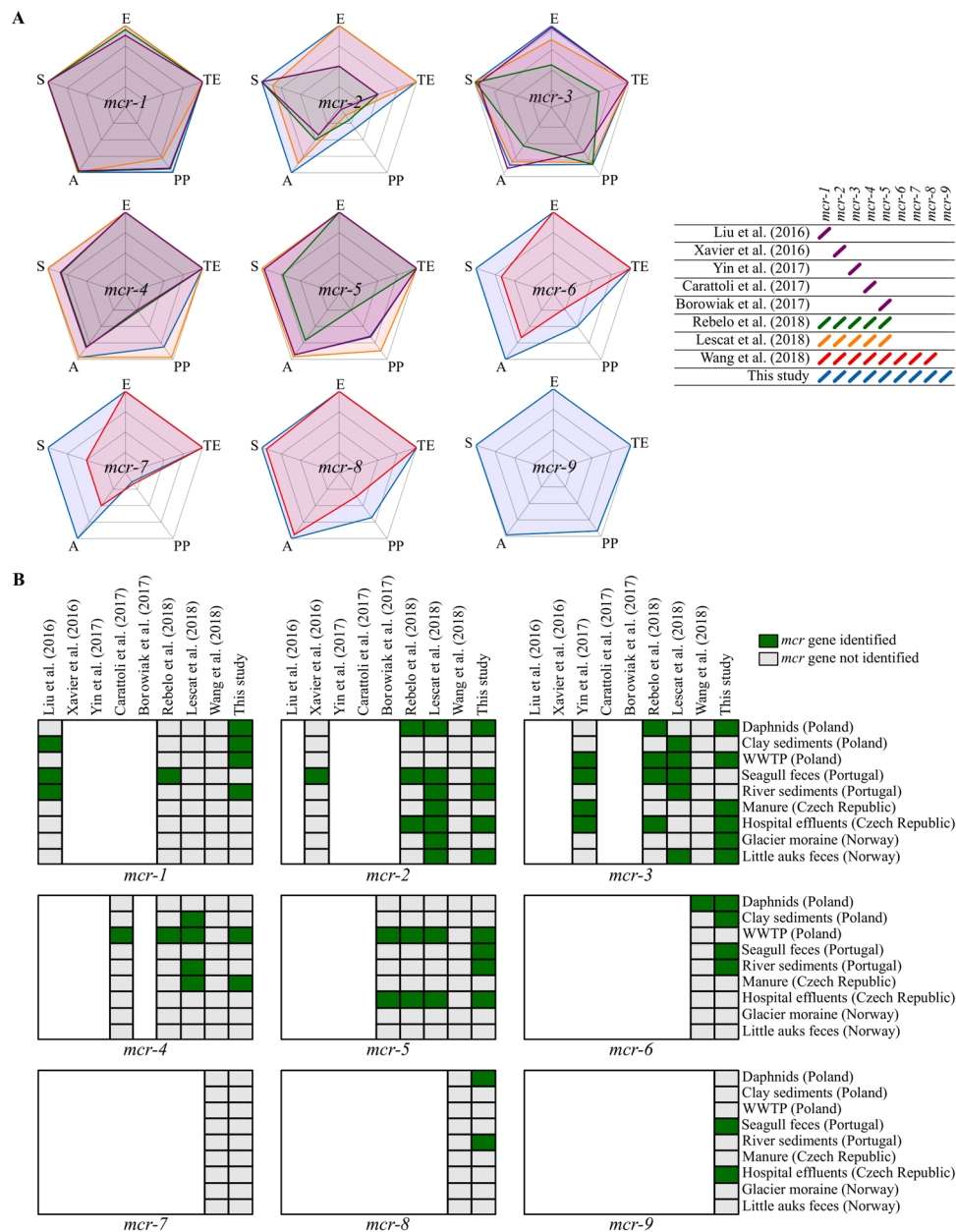
Fig. 3. *In silico* and experimental validation of *mcr*-specific PCR primers. (A) Distribution of values calculated after *in silico* validation of *mcr*-specific PCR primer pairs. Values range from 0 in the middle of the spider chart to 1 at the border. A – accuracy; E – efficacy; PP – precision; S – specificity; TE – taxonomic efficacy. (B) Distribution of the *mcr* genes in analysed environments tested with the use of various PCR primers.

literature-retrieved primers, whose application resulted in the omission of some genes. Thus, these primers seem to be highly useful for understanding the real epidemiological risk. Taking into account the importance of tracking *mcr-1* and *mcr-3* genes in various environmental settings, we developed a quantitative PCR (qPCR) protocols for testing the abundance of these genes using primers designed in this study. We validated the protocols using samples originated from various environments, i.e. (i) primary sludge from WWTP (Poland) and seagull feces (Portugal), analysed in this study; (ii) hospital effluents (Portugal) and the river water (Portugal), analysed previously (Rocha and Manaia, 2020). We detected: (i) for *mcr-1* – 9.35×10^2 copies per 1 ml of hospital effluents, 3.89×10^1 copies per 1 ml of river water and 6.26×10^3 copies per 1 ml of primary sludge from WWTP; (ii) for *mcr-3* – 3.17×10^4 copies per 1 ml of hospital effluents, 5.08×10^3 copies per 1 ml of river water and 3.17×10^5 copies per 1 ml of primary sludge from WWTP. We did not detect *mcr-1* and *mcr-3* in seagull feces

(Supplementary Materials - Fig. S2 and Fig. S3).

4. Discussion

Information concerning the first MCR-1-positive bacterial strains was published together with the first set of primers suitable for the identification of the *mcr-1* gene in *Escherichia coli* and *Klebsiella pneumoniae* isolated from animals and humans (Liu et al., 2016). Shortly after this, it was observed that the *mcr-1* variants spread globally and were identified also in other strains, including *Enterobacter cloacae* (Wong et al., 2016), *Salmonella* spp. (Doumith et al., 2016) and *Aeromonas* spp. (Gogry et al., 2019), which were isolated not only from humans and animals but also from various environmental settings, like soils. Subsequent, complex scientific investigations led to the recognition of multiple *mcr* genes and variants. As of 31st December 2019, nine different *mcr* genes have been described with an overall 74 variants (Supplementary Materials -

Table S3).

The global spread of *mcr* genes requires sufficient methods for rapid and reliable detection of colistin resistance. The WHO's Global Antimicrobial Resistance Surveillance System (GLASS) indicated genotypic surveillance as a basis for tracing the spread of colistin-resistant strains. However, the report also emphasized the importance of the PCR-based, molecular methods used for *mcr* genes detection (Anon, 2017). Primers are the key components for these molecular methodologies of plasmid-mediated colistin resistance detection, thus, they should be carefully selected (Osei Sekyere, 2019).

In this study 166 *mcr*-specific primer pairs, found by a systematic literature review of over 1000 articles, were identified and subjected to complex *in silico* validation. This analysis revealed that only 33% of primers reached maximal scores of parameters in the validation test. Interestingly, obtained ranking of primers revealed that some commonly used ones were less suitable for *mcr* gene screening comparing with the primers proposed by other scientists. The most surprising results were obtained for primers designed by Wang et al. (Wang et al., 2018) which turned out to be either unspecific (primers for *mcr-1* to *mcr-5*) or exhibited MSM values between 0.07 and 0.33 (primers for *mcr-6* to *mcr-8*), which, in respect to the accurate surveillance of *mcr* genes in all possible environmental settings, is rather insufficient. Another surprising observation was made for *mcr-2*-specific primers (named MCR2-IF and MCR2-IR) designed by Xavier et al. (Xavier et al., 2016), which exhibited low efficacy and taxonomic efficacy parameters and were ranked on 16th position out of 22 (Supplementary Materials - Table S2). It is worth mentioning that these primers are recommended by the National Food Institute (Denmark) for *mcr-2* gene screening (Anon, 2016). Our finding suggests that in the case of both sets of primers, false-negative results could be obtained.

Over the last 5 years, there was an increasing need for screening of colistin resistance genes, which resulted in the development of primers for all known *mcr* genes. However, our analysis clearly demonstrated that many commonly used primers were, in fact, not the best possible ones, as they could not cover all known variants of the particular *mcr* gene collected in the CARD database and thus generate false-negative results. A possible solution to hinder further misuse of *mcr*-targeting primers is the implementation of fast, cheap, reliable and standardized validation methods, which allows selecting relevant primers for sufficient screening of ARGs from a global perspective. Here, novel *in silico* tools for the pre-testing of ARG-specific primers could be successfully applied, e.g. these presented by Gorecki et al. (Gorecki et al., 2019) and in this study.

In this work we also designed novel primers specific to *mcr-1 – mcr-9* genes. We added an additional step to the production process, i.e. initial *in silico* validation, which allowed selecting primers that amplify all variants of a particular *mcr* gene present in NCBI database. As we are aware that novel primers are as good as recent reference database has been used for their creation, and in a constantly changing environment *mcr* genes evolve, which may reduce the usefulness of primers in time, we tried to develop and present the universal approach allowing for regular and automated *mcr*-specific primer pair designing and validation, that can be easily repeated when reference databases are updated.

Experimental validation of designed primers was performed using nine environmental DNAs isolated from samples originating from four countries (i.e., the Czech Republic, Norway, Poland and Portugal). Moreover, their efficiency in *mcr* genes screening was evaluated by comparing to other commonly used primers, retrieved from the literature. PCR screening combined with subsequent sequencing of obtained amplicons revealed various types of *mcr* genes in all analysed environmental samples. However, developed primers indeed have some limitations, since they failed with the following samples (*mcr-1* in seagull feces, *mcr-2* in manure from the Czech Republic and glacier moraine from Norway; *mcr-3* in clay sediments from Poland plus seagull feces and river sediments from Portugal; *mcr-4* in clay sediments from Poland and river sediments from Portugal). It is worth mentioning that successful

PCR is not only depended on a proper primer design. It was previously described that composition of the original sample can have important implications during amplification (Miłobedzka et al., 2021). Main problem which can occur is related to difficulties during DNA purification. Impurities or high salt concentration may interfere with the template DNA or inhibit DNA polymerase which strongly affects the results of PCR (Wilson, 1997). Moreover, in case of environmental samples (which complexity is unknown) we cannot predict and precisely estimate the contribution of unspecific amplification which may result in retention of primers. Such retention may result in obtaining false-negative results or problematic replicability of results. Another implication arising from the nature of the sample is related to low abundance of the tested gene. For some genes in a given sample it is impossible to perform their quantification (since the copy number of the gene is below the limit of quantification [LOQ]) or detection (since the gene occurs in a quantity below the limit of detection [LOD]). Such situation was, for example, observed for ARGs of clinical relevance is soils (Fortunato et al., 2018).

Testing the occurrence of ARGs in various environments is important, but also the abundance of these genes should be analysed. For such studies the qPCR technique is used (Miłobedzka et al., 2021). Here, we developed a qPCR protocols for testing the abundance of the *mcr-1* and *mcr-3* genes, since they are of special clinical relevance being hosted mostly by bacterial strains isolated from hospital patients. For the qPCR assays we used primers designed in this study and we proved their usefulness in environmental screening of *mcr-1* and *mcr-3* genes.

5. Conclusions

PCR screening is one of the most common approaches for molecular genotyping in the surveillance of antibiotic resistance genes as emerging pollutants in various environments. However, for obtaining reliable results, special attention should be paid to the selection of proper PCR primer pairs to minimize the number of false-negative results. As the antimicrobial resistance phenomenon is a global multi-dimensional problem, which was recently recognized by One Health, standardized validation methods that allow for the design and selection of relevant primers are needed for the sufficient screening of ARGs in various environments. This study indicated that the lack of such standardized validation models for selecting ARGs and especially *mcr*-specific primers may result in the use of primers that are specific only to a particular variant of the gene, which significantly narrows their application and excludes them as tools for environmental screening. We proposed an *in silico* validation system for primers. Based on this system, we designed novel primers for *mcr-1* to *mcr-9* genes conferring resistance to colistin, i. e. one of the drugs of the last resort. These newly developed primers were experimentally validated proving to be relevant for screening of *mcr* genes in various environmental settings. Moreover, the presented system provides a solid foundation for further enhancement of the selection of the best primers for *mcr* genes, as well as other ARGs.

CRediT authorship contribution statement

Adrian Gorecki: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft preparation, Writing – review & editing, Visualization. **Marcin Musialowski:** Investigation. **Mikolaj Wolacewicz:** Investigation. **Przemyslaw Decewicz:** Software. **Catarina Ferreira:** Investigation, Writing – review & editing. **Dana Vejmelkova:** Investigation, Writing – Review & Editing. **Malgorzata Grzesiuk:** Investigation, Resources, Writing – review & editing. **Celia M. Manaia:** Resources, Writing – review & editing. **Jan Bartacek:** Resources, Writing – review & editing, Funding acquisition. **Lukasz Dziewit:** Conceptualization, Methodology, Validation, Formal analysis, Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.127936.

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