Journal of Biosafety and Biosecurity 6 (2024) 16–26

Contents lists available at ScienceDirect

Journal of Biosafety and Biosecurity

journal homepage: www.keaipublishing.com/en/journals/journal-of-biosafety-and-biosecurity/

Research Article

Effectiveness of chemical inactivation of infectious liquid biological waste: A randomized sample study of research laboratories in Switzerland

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ARTICLE INFO

Article history:
Received 18 December 2023
Received in revised form 5 February 2024
Accepted 18 February 2024

Keywords:
Chemical inactivation
Infectious liquid biological waste
Lentivirus (HIV-1)
Adenovirus

ABSTRACT

Laboratory wastewater has been suggested as an important escape route for microorganisms from research environments. Likely reasons for the unintentional release of laboratory organisms are shortcomings in the handling of infectious liquid biological waste (LBW) and inadequate inactivation procedures. We developed an analytical approach to investigate the use of chemical inactivation (CI) procedures in Swiss research laboratories by on-site random sampling of presumably inactivated infectious LBW and testing it for the presence of infectious lentiviruses (HIV-1) and adenoviruses (AdV). In addition, standard operating procedures (SOPs) for CI were collected and evaluated, and laboratory-staff knowledge of CI processes was assessed using a questionnaire. Although we found several deficiencies in the technical knowledge and training of laboratory staff on the CI of LBW, as documented by 27 returned questionnaires, no infectious viruses were detected in the eight LBW samples collected. Whilst we acknowledge that the number of LBW samples and SOPs is small, we conclude that CI of LBW containing infectious lentiviruses and adenoviruses does not result in the systematic release of considerable amounts of infectious viruses into the environment from research laboratories in Switzerland.

1. Introduction

Wichmann et al.[1] reported that between 2012 and 2014 almost half the wastewater samples collected during a longitudinal study at a Swiss research site contained viable genetically modified *Escherichia coli* (*E. coli*). This finding suggests that laboratory wastewater is an important escape route for microorganisms from research environments and could be an additional source of antibiotic resistance genes in wastewater systems, potentially worsening the issue of multidrug-resistant bacteria.[2,3] One study detected an Enterobacteriaceae plasmid sequence in non-Enterobacteriaceae bacteria, indicating interspecies transfer of plasmids in laboratory wastewater.[4] The most obvious explanations for the escape of laboratory organisms are shortcomings in the handling of infectious liquid biological waste (LBW) and inadequate inactivation procedures. Biosafety concerns are not limited to bacteria. LBW may harbor various genetically modified or infectious organisms, including adenoviral or lentiviral vectors. Adenoviruses have been detected in wastewater both before and after clearance, with wastewater systems emitting aerosols containing significant concentrations of human adenoviruses.[5–8] These aerosols pose a risk to workers through inhalation or contact. Enveloped lentiviruses exhibit low stability in wastewater. Therefore, presumably infectious LBW containing lentiviruses poses a high risk to laboratory workers, especially when VSV-G pseudotypes are involved.[9,10] Laboratories that generate infectious LBW are responsible for its safe handling and disposal in accordance with local biosafety legislation.[11] In Switzerland, the accepted procedures for the inactivation of infectious LBW include autoclaving and chemical inactivation (CI) with some restrictions.[11,12] Autoclaving is considered the gold standard for the inactivation of laboratory waste and is the most reliable and easily controllable method for onsite inactivation.[13–15]

CI is a widely accepted method for the inactivation of LBW, not only in Switzerland but also worldwide, including the United

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https://doi.org/10.1016/j.jobb.2024.02.001
2588-9338/

States of America, Canada, Australia, New Zealand, India, and the European Union (EU). The World Health Organization Laboratory Biosafety Manual lists CI as an appropriate treatment method and is recommended for countries without their own biosafety regulations, such as many sub-Saharan countries.[16–22] According to these standards, CI methods need to be tested and should be validated on a regular basis to demonstrate their effectiveness in specific applications.[17,21] The biocidal chemical used must be carefully selected for each specific organism. Furthermore, the incubation time, concentration, and interfering factors such as organic matter, pH, temperature, and product stability must be considered to ensure effective inactivation.[12,17,23–25] In CI, the waste is not disinfected by a physical method, such as the sustained heat applied during autoclaving. Instead, the biocidal chemical is consumed by the organism during the inactivation process, which means that its concentration is not stable.[12,26] The killing kinetics are therefore non-linear, which means that time and dose cannot be extrapolated to estimate the effect of the inactivation process beyond the limits that have been tested.[12] In addition, CI is generally more difficult to control and more prone to handling errors than autoclaving.[17,27]

Nevertheless, according to a survey conducted in Switzerland in 2021, CI is still popular, particularly in the research sector (Office of Waste, Water, Energy, and Air of the Canton of Zurich, Switzerland, email communication, July 20, 2022). Reasons for the popularity of CI methods may be related to their perceived ease of use when validation is not considered, and lower space and infrastructure requirements than those of autoclaving.[28] For small laboratories, the purchase and maintenance of an autoclave can pose a financial burden.

Given the challenges outlined above, chemical disinfection may well pose a biosafety risk because inadequately inactivated LBW provides a potential escape route for infectious and genetically modified organisms.[1] In this study, an analytical method to examine the use of CI procedures in Swiss research laboratories was developed. On-site random sampling of presumably inactivated infectious LBW was conducted in 2018 and 2019. Because of the lack of *E. coli*-containing LBW, samples were tested for lentiviral vectors (LVV) and adenoviral (AdV) vectors to identify the presence of infectious viruses. Additionally, standard operating procedures (SOPs) were collected and reviewed. The knowledge of the laboratory staff of CI procedures was assessed with a questionnaire.

2. Material and methods

2.1. Field study design

For the sampling of LBW from laboratories, we focused on university and corporate research laboratories in the two leading life science cantons in Switzerland, Zurich and Basel-Stadt.[29] The local authorities responsible for the enforcement of the Containment Ordinance[11] were contacted, Kontrollstelle für Chemie- und Biosicherheit KCB Basel-Stadt and the Office of Waste, Water, Energy, and Air of the Canton of Zurich. Within these authorities, laboratories were selected for sampling and whether they would be notified in advance was decided (Fig. 1). A survey consisting of 12 questions about CI (Appendix) was distributed to the laboratories before or during the visit, irrespective of whether samples could be collected. In addition, the questionnaire was also distributed at a research institute in the canton of Aargau, but no LBW or SOPs were collected there. If LBW was present during our visit, we collected one waste sample per laboratory and obtained the SOP for inactivation to verify the laboratory's theoretical inactivation procedure (Fig. 1). We then analyzed the LBW samples for infectious particles, checked whether the SOPs contained technical problems, and evaluated the questionnaire to assess the laboratory workers' knowledge of CI procedures (Fig. 1).

2.2. Cell culture

Adherent human HEK 293 T (DMSZ: German Collection of Microorganisms and Cell Cultures GmbH, ACC 635) and HEK 293 (DSMZ, ACC305) cells were cultivated under standard cell culture conditions (37 °C, 5 % carbon dioxide) with Dulbecco's modified Eagle's medium (DMEM, Merck) supplemented with 10 % fetal bovine serum (FBS, Merck) and 1 % L-glutamine–penicillin–streptomycin solution (GPS, Merck). The cells were split twice a week using Dulbecco's phosphate-buffered saline (1 × PBS, Merck) and trypsin–EDTA solution (Merck).

2.3. Preparation of lentiviral (HIV-1) vectors

Packaging plasmids pMDLg/pRRE (Addgene plasmid # 12251; https://n2t.net/addgene:12251; RRID: Addgene_12251) as well as the VSV-G expression envelope plasmid pMD2.G (Addgene plasmid #12259; https://n2t.net/addgene:12259; RRID: Addgene_12259) were gifted by Didier Trono. The GFP-expressing transfer plasmid pLL3.7 was gifted by Luk Parijs (Addgene plasmid #11795; https://n2t.net/addgene:11795; RRID: Addgene_11795).

LVV were produced by transfecting HEK 293 T cells with 3.5 µg pMD2.G, 3.5 µg pRSV-Rev, 3.5 µg pMDLg/pRRE, and 10 µg pLL3 mixed with 50 µL X-tremeGENE™ 9 DNA transfection reagent (Merck) in 950 µL serum-free DMEM. After 4 h, the cells were washed with 1 × PBS and fresh medium was added. The next day, the cells were checked for GFP expression. Supernatants were collected and pooled 1–3 d post-transfection. The pooled virus solution was centrifuged and the supernatant was sterile filtered (0.45 µm, Whatman® Puradisc 30, Merck) to remove cell debris. Titers were determined using the HIV-1 p24 ELISA Kit (Abcam) or the Fluorescence Titering Assay for Lentivirus.[30] Aliquots (1 mL) were stored at − 80 °C. LVVs with a titer of 5×10^4 transduction units per milliliter (TU/mL) were used as a control.

2.4. Preparation of adenoviral (AdV) vectors

Ad5ΔE1-GFP was gifted by Markus U. Ehrengruber.[31] To amplify the virus, 50 % confluent HEK 293 cells were infected with 333 µL Ad5ΔE1-GFP (1.5×10^8 plaque-forming units per milliliter, PFU/mL) in 15 mL of cell medium. After 3 h, 10 mL of fresh medium was added, giving a total volume of 25 mL. The cells were monitored for 4 d to confirm infection, which was indicated by cell detachment. Detached cells and supernatant were harvested, and virus particles were released from the cells through three freeze–thaw cycles. Cell debris was then removed through centrifugation and filtration (0.45 µm, Sterilflip filter, Millipore). The titer was determined by qPCR[32] and 1 mL aliquots were stored at −80 °C. AdV vectors with a titer of 1×10^8 PFU/mL were used as a control.

2.5. Collection, detoxification, and concentration of samples

Cytotoxic disinfectants were detoxified using Sephadex filtration (LH20, Merck) according to the EU standard EN14476: 2013 + A1:2015. Sephadex columns were prepared one day prior to sampling or performing a detoxification test. A 22 % suspension of Sephadex in 1 × PBS was prepared and autoclaved. Sephadex suspension (10 mL) was transferred to a Sterilflip filter (0.45 µm) and then the excess liquid was removed using a vacuum pump to form a column. Either 30 mL of collected samples (Fig. 2) or equal volumes of test suspensions containing Virkon S (Lanxess, 1 % and 2 %), Dismozon Plus (Hartmann, 0.4 % and 1 %), and mixtures of sodium hypochlorite (Carl Roth) and Pursept AF (Merck)

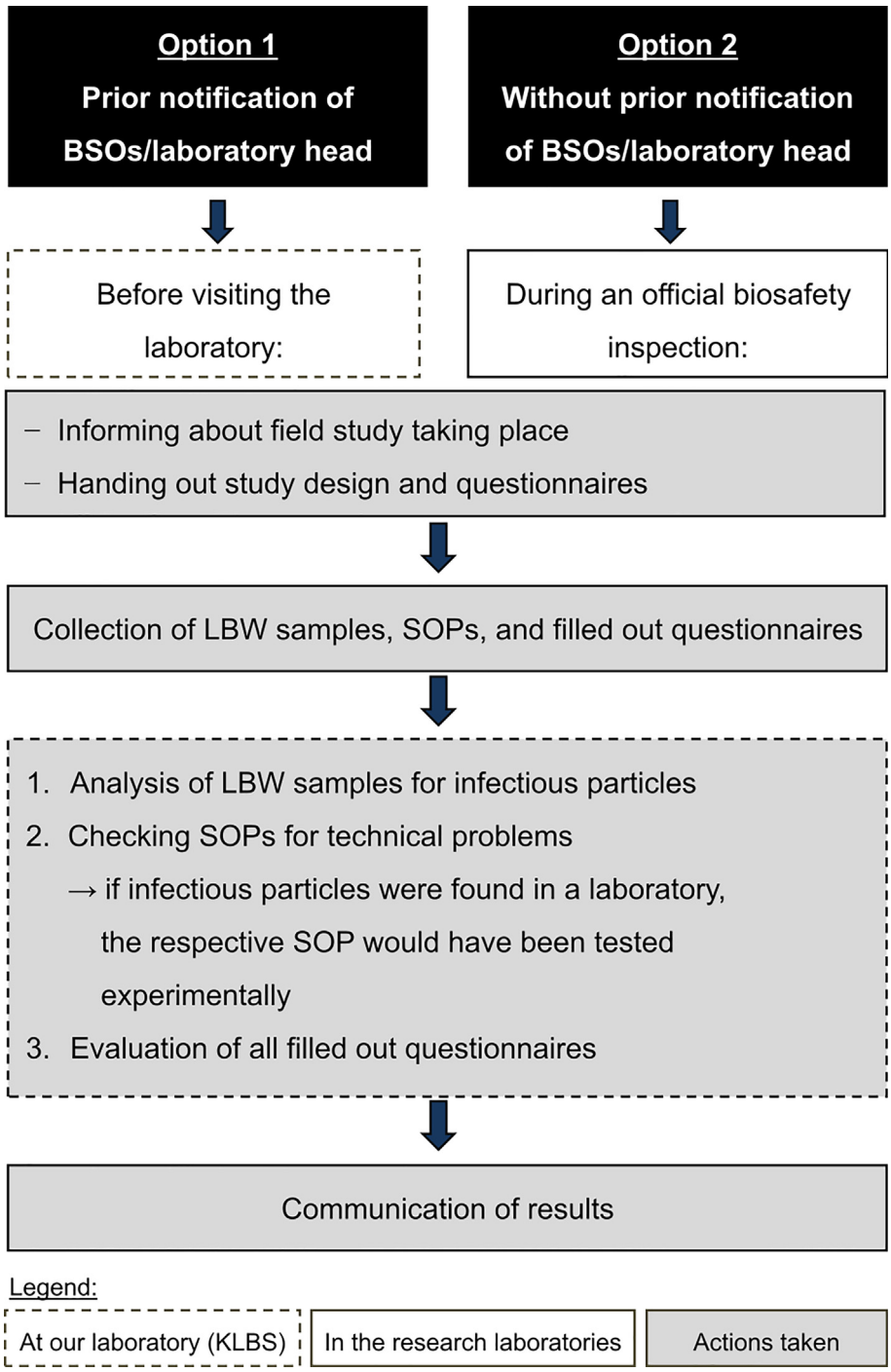

Fig. 1. Field study design. Depending on the decision of the local authority officials, Option 1 or Option 2 was initiated, i.e. either the respective BSO/laboratory head was informed prior to our visit and the questionnaire was handed out beforehand (Option 1), or our visit coincided with an official inspection (Option 2). In the latter case, information and questionnaires were handed out during the visit and questionnaires were completed by the laboratory staff during the visit.

at concentrations 2 %/1.5 %, 3 %/2.3 %, and 4 %/2.3 % were applied to the Sephadex columns. All disinfectant test suspensions were prepared using cell culture waste collected during routine cell passaging in our laboratory to account for the bioburden present in samples collected in the field study. Samples of presumably inactivated LBW (30 mL) were collected immediately at the end of the incubation period (as specified in the laboratory SOP). All but four samples (Table 1) were detoxified directly on site by vacuum filtration through Sephadex columns (Fig. 2). The remaining four samples had to be thawed prior to filtration because they were frozen by the sampled laboratories.

In the case of sodium hydroxide (NaOH) as a chemical disinfectant (Sample 3), HEPES (0.125 M)/HCl (0.5 M) solution was used to neutralize NaOH before the waste was filtered through the Sephadex column. The Sephadex eluate was directly used in the bioassay or after further concentration using polyethylene glycol (PEG). For

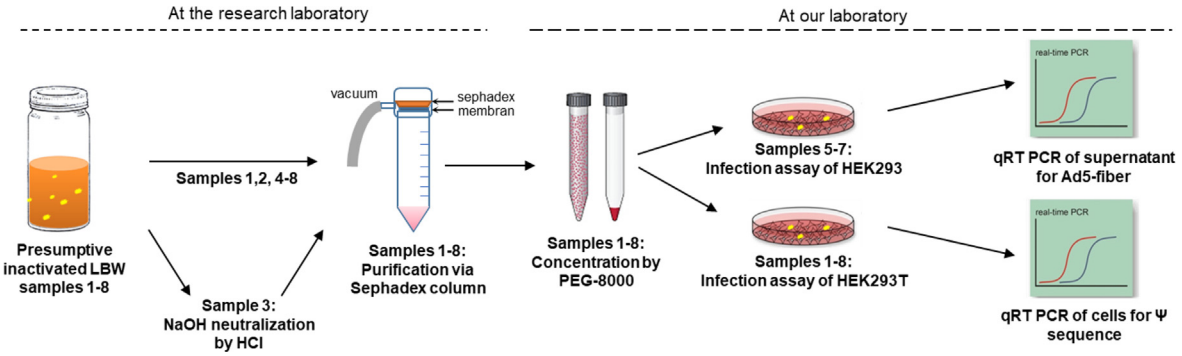

Fig. 2. Schematic representation of the processing of liquid biological waste (LBW) samples 1–8. Depending on the reported disinfectant used and viral content of the LBW samples (adenovirus or lentivirus), neutralization and purification were performed in slightly different ways as shown. While all samples except sample 3, could directly be filtrated using a Sephadex column sample 3 had to be neutralized using HCl. After concentrating the samples via PEG-8000 precipitation. The following infectivity test also differed depending on the virus being tested (adenovirus in HEK293 and qPCR for Ad5 gene from supernatants, lentivirus in HEK293T and qPCR of Ψ sequence from cell pellets). The location where the individual steps were performed is indicated by the top bar.

Table 1
Liquid biological waste (LBW) samples collected, their origin (Swiss canton, Organization), their reported viral content, the disinfectant used to inactivate infectious viruses and the procedures used by the laboratories.

Sample	Organization	Canton	Sampling announced?	Type of LBW and viral content	Disinfectant	Active ingredient	Inactivation procedure
1	A	Basel-Stadt	Yes	Lentivirus (cell culture waste), frozen sample	Virkon® S	Potassium peroxymonosulfate	1) Pre-poured Virkon® S in waste bottle. 2) Add waste until filling levelFinal concentration 1%, overnight incubation
2				Lentivirus (FACS waste), frozen sample	Dizmozon® Plus	Magnesium monoperoxyphthalate hexahydrate	1) Add waste below filling level 2) Mix with Dismozon® PlusFinal concentration 0.4%, overnight incubation
3				Lentivirus (cell culture waste), frozen sample	NaOH	Sodium hydroxide	1) Add waste below filling level 2) Mix with NaOH.Final concentration 1M, >4 h incubation
4				Lentivirus (cell culture waste), frozen sample	Virkon® S	Potassium peroxymonosulfate	1) Pre-poured Virkon® S in waste bottle 2) Add waste until filling levelFinal concentration 1%, overnight incubation
5 6 7	B	Zurich	No	Lentivirus and maybe Adenovirus (cell culture waste)	Mixture of bleach and Pursept® AF	Sodium hypochloride, quaternary ammonium compound	1) Pre-poured Pursept® in waste bottle 2) Add waste below filling level 3) Mix with than add bleachFinal concentration Purcept 2.3%, final concentration bleach >3%. Wait until waste changes color from pink to transparent.
8	C	Basel-Stadt	Yes	Lentivirus (cell culture waste)	Virkon® S	Potassium peroxymonosulfate	1) Pre-poured Virkon® S in waste bottle 2) Add waste below filling level 3) Mix with fresh Virkon® SEnd concentration 1%, >4 h incubation.

the PEG concentration, detoxified samples were mixed with 15 mL of polyethylene glycol-8000 (PEG-8000, Merck) and refrigerated overnight at 4 °C to precipitate and concentrate the viral content. Thereafter, the samples were centrifuged at 10,000 × *g* for 30 min. The resulting pellet was resuspended in 1 mL of complete cell culture medium to be used in a virus detection bioassay.

2.6. Bioassay for infectious adenovirus particles

In each well of six-well poly-L-lysine plates (Greiner Bio-One, Huberlab), 100 µL of detoxified LBW or mock sample was mixed with 1.9 mL of HEK 293 and HeLa (approximately 120,000 cells, i.e., at 80 % confluence) and incubated overnight under standard cell culture conditions. The next day, the medium was replaced and another 200 µL of the supernatant was taken 3 h later (i.e., sample day one, D1). Infected cells were incubated for a further week, with additional 200 µL samples taken on day three (D3) and day seven (D7).

Virus DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen). For the detection of adenoviral vector Ad5-fiber gene sequences, qPCR was performed using a TaqMan Fast Universal PCR MasterMix and StepOnePlus™ Real-Time PCR system (Applied Biosystems™, Thermo Fisher Scientific) as previously described.[33] Serial dilutions of the Ad5ΔE1GFP plasmid[34] containing the Ad5-fibre sequence were used as a quantification standard.[33] The limit of detection for this assay was determined to be 200 copies per sample and 100 µL (i.e., 2000 copies/mL). Statistical analyses were performed using R and RStudio.[35] *P*-values were calculated using the one-way ANOVA post-hoc Tukey test.

2.7. Bioassay for infectious lentivirus particles (HIV1)

HEK 293 T cells were seeded in six-well poly-L-lysine plates and incubated under standard cell culture conditions (approximately 200,000 cells). The next day, when the cells had reached 50 %–80 % confluence, the medium was replaced with 1.8 mL of fresh medium and incubated for 1 h before 200 µL of detoxified LBW or test sample was added per well and incubated for a further 4 h. The cells were washed twice with 1 × PBS before fresh cell medium (2 mL) was added. During the following two weeks, the cells were split three times under standard cell culture conditions before harvesting.

Cell DNA was extracted using the QIAamp® DNA Mini Kit. The DNA was then analyzed for lentivirus-specific sequences by qPCR

using the TaqMan Fast Universal PCR MasterMix and StepOnePlus™ Real-Time PCR systems. For the detection and quantification of stably integrated lentiviral sequences (HIV-1), we used the primers and probe for the HIV-Ψ sequence[33,36] and the housekeeping gene RNaseP was used for the reference of the HEK 293 T cell number.[37,38] Serial dilutions of the LL3.7 plasmid containing the Ψ sequence were used as quantification standards as previously described.[33] The limit of detection for this assay was 400 copies per sample and 200 µL (i.e., 2000 copies/mL). The statistical analyses between Sephadex-filtered and non-filtered samples were performed using R and RStudio.[35] *P*-values were calculated using a Welch two-sample *t*-test.

3. Results

3.1. Verification of testing procedure

Ideally, the chemical disinfectant should be present in excess in the LBW so it is not completely consumed during CI. It was therefore necessary to detoxify the disinfectant from the LBW to avoid a false negative result in the bioassay. For this purpose, a Sephadex detoxification procedure was adapted and tested for the disinfectants used in the sampled laboratories of Organization A, B, and C (Table 1; 1 % Virkon S, 0.4 % Dismozon Plus, and 1 M NaOH was used by Organization A and C and a 3 %/2.3 % mixture of sodium hypochlorite and Pursept AF was used by Organization B). While incubation of HEK 293 T cells with untreated 1 % Virkon S for 4 h resulted in cell death within 24 h, Sephadex-filtered Virkon S at concentrations of 1 % and lower did not appear to harm the cells (Fig. 3A). Detoxification of Virkon S at concentrations above 1 % was unsuccessful, because approximately 50 % of the cells died within 24 h. This may be caused by the impaired filtration capacity of Sephadex at high concentrations of Virkon S. Cells treated with 0.4 % Dismozon Plus survived until the next day, showing signs of stress, but recovered within 24 h, whereas 1 % Dismozon Plus resulted in cell death within the same period. At both 0.4 % and 1 % Dismozon Plus, Sephadex filtration was able to completely remove their disinfecting and cytotoxic properties (Fig. 3A). The CI treatment used by laboratories from Organization B (Table 1) consisted of a combination of 2.3 % Pursept AF pre-placed in a bottle and "at least" 3 % bleach (final concentration) added to the full bottle. In our test, HEK 293 T cells treated with the Sephadex-filtered disinfectant mixture for 4 h showed marked signs of cell stress within 24 h. Only when the cells were treated with a lower concentration than specified in the laboratory SOP (1.5 %/2% of Pursept/bleach), did they recover and were still alive after 7 days, including cell splitting 4 days after the treatment (Fig. 3A). Finally, Sephadex filtration could remove the disinfecting and cytotoxic properties of 1 M NaOH (data not shown), which was used by Organization A in addition to Virkon S and Dismozon Plus, when the NaOH was neutralized with an equimolar amount of HCl before filtration (Fig. 3B).

To ensure that Sephadex purification did not affect the infectivity of the viruses presumably present in the samples, HEK 293 T and HEK 293 cells were infected with Sephadex-filtered and untreated LVV and AdV vectors, respectively. HEK 293 T pellets (for lentivirus) and HEK 293 supernatant (for adenovirus) were harvested according to the bioassay procedure and the respective viral sequences were quantified by qPCR. While Sephadex filtration had no effect on the infectivity of AdV vectors, it decreased the amount of infectious LVV particles by 10.4 times (Fig. 4A and B).

To process larger volumes of waste and increase the sensitivity of our tests (in particular, to counteract the loss of lentiviral infectivity), 30 mL of the collected samples was concentrated to 1 mL by gel filtration using PEG-8000. PEG polymers non-covalently bind to virus particles, causing them to form a pellet when centrifuged. In a preliminary test, we prepared a mock sample by mixing 1 mL of LVVs with 30 mL of DMEM medium. The mock sample was filtered through a Sephadex column and the virus was concentrated using PEG-8000. The concentrated virus solution was subjected to an HEK 293 T cell infection assay followed by qPCR to compare the amount of integrated lentiviral sequences in the cell DNA PEG-8000 concentrated LVV with that of the non-concentrated LVV. Our tests showed that cells infected with the PEG concentrate had an infection rate that was about seven times higher than that of the cells treated with non-concentrated LVV (Fig. 4C).

3.2. Sample collection in the field study

Samples of presumed inactivated LBW were collected from eight laboratories in three different organizations (Table 1). The collection of five of these samples (No. 1–4 and 8) was planned with the institute's biosafety officer or laboratory head (Option 1, Fig. 1) to ensure that there would be inactivated LBW available at the time of sampling. These samples were reported to contain only LVVs. The remaining three samples (No. 5–7) were taken without prior warning during biosafety inspections at the same research sites as where Wichmann et al.[1] collected wastewater samples. Because the sampling of samples No. 5–7 was unannounced, their contents could not be specified, but it was assumed that they may contain LVV and AdV vectors. The most common disinfectant in this study was 1 % Virkon S (three out of eight samples

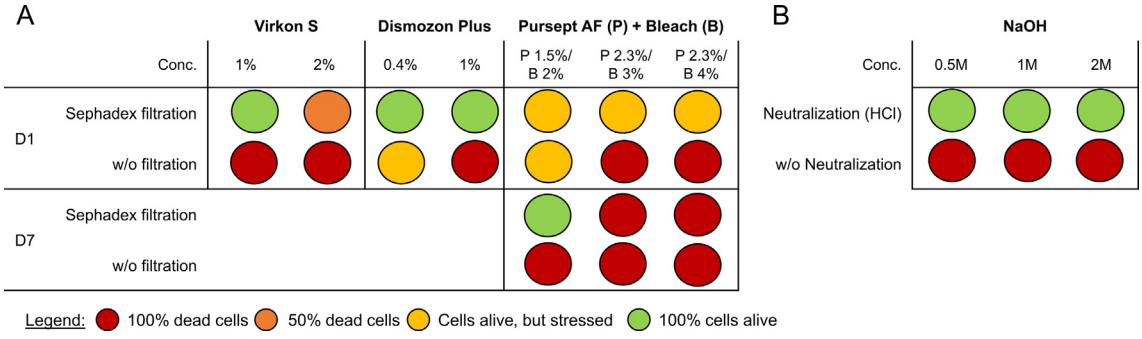

Fig. 3. Schematic overview of cytotoxicity tests with disinfectants used in the laboratories visited. Mock waste suspensions consisting of standard cell culture waste and disinfectants (Panel A: Virkon S, Dismozon Plus, Pursept AF/Bleach; Panel B: NaOH) at different concentrations (conc.; as indicated in first row; P: Pursept AF, Bl: Bleach) were added to HEK293T cells and incubated for 4 h before the medium was replaced by fresh medium. Test suspensions were either used directly or after filtration through Sephadex (A) or suspensions neutralized with HCl before Sephadex-filtration were compared with non neutralized ones (B). The next day, the cells were checked for viability and classified as "all cells alive and healthy looking" (green), "cells alive, but showing signs of stress" (yellow), "50 % of the cells dead" (orange), "all cells dead" (red). Tests with Pursept AF mixed with Bleach were monitored beyond day 1 after treatment (D1), until day 7 (D7) to observe potential recovery of stressed cells (D1 classified as orange).

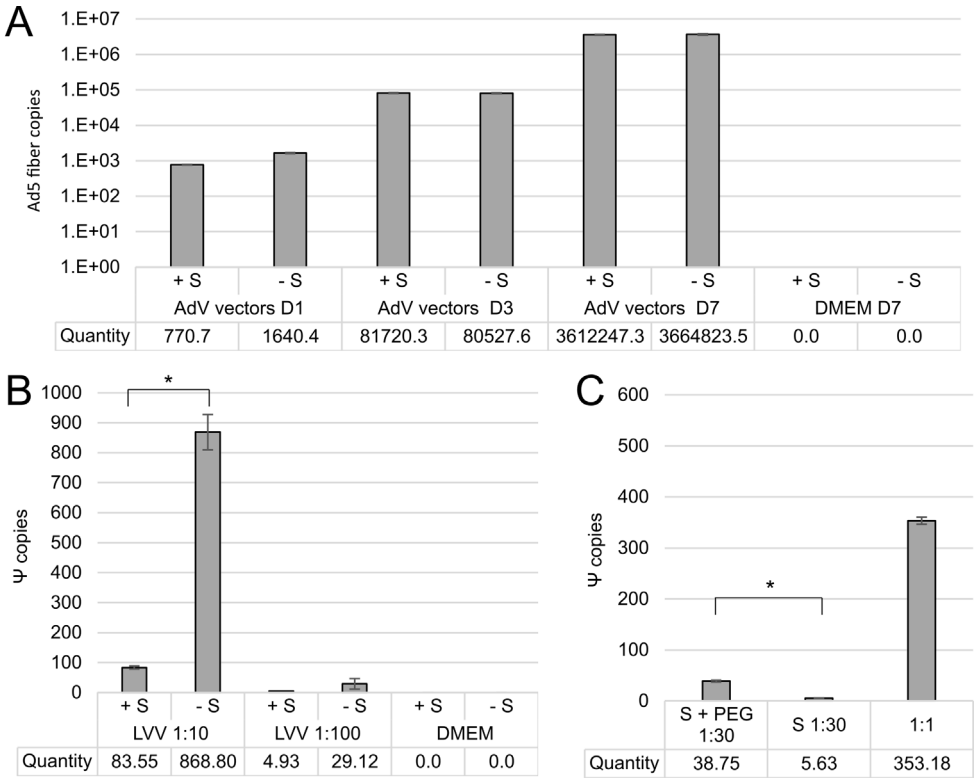

Fig. 4. Analysis of the effect of Sephadex filtration and PEG precipitation on virus infectivity. (A) In a bioassay, Sephadex filtered (+S) vs. non-treated AdV vectors (-S) preparations were used to infect HEK293 cells. The qPCR results are given as number of Ad5-fiber gene copies measured in the cell supernatant on day 1 (D1), day 3 (D3) and day 7 (D7) post infection. DMEM served as negative infection control. *p*-values of the respective filtered and non-filtered samples showed no significant differences. (B) Sephadex filtered (+S) vs. non-treated lentiviral (HIV1) vector (-S) preparations (at two different dilutions of the stock, 1:10 and 1:100) were used in a bioassay to infect HEK293T cells. The qPCR results are given as number of HIV-Ψ DNA sequence copies integrated into the cell genomes measured 2 weeks post infection. DMEM was used as negative infection control. (C) In a bioassay, 1:30-diluted LVV preparations which were either Sephadex-filtered and PEG concentrated (S + PEG) or only Sephadex-filtered (S) were used to infect HEK293T cells. Undiluted and untreated lentiviral (HIV1) vector preparations served as positive control. As in (B), the qPCR results of the cell pellets 2 weeks after infection are shown as number of HIV-Ψ DNA sequence copies. In all panels, A-C, the exact numbers are denoted below the graph. One asterisk (*) indicates *p* value smaller than 0.05 ($p < 0.05$).

from two different organizations, Table 1). In three laboratories, all belonging to Organization B, samples containing a mixture of Pursept AF (2.3 %) with bleach ("at least" 3 % according to the SOP) were collected (No. 5–7). Sample No. 2 and 3 were treated with Dismozon Plus (0.4 %) and NaOH (1 M), respectively. Four of the samples, all from Organization A, were frozen by the laboratories prior to sampling.

3.3. Analysis of inactivated liquid BSL2 waste samples

To detect infectious LVVs still present in the presumed inactivated LBW, HEK 293 T cells were infected with aliquots of sample No. 1–8. For the adenovirus bioassay, samples No. 5–7 were also added to both HEK 293 and HeLa cells. Again, HEK 293 T cells (for lentivirus/HIV-1 detection) and HEK 293 and HeLa supernatants (for adenovirus detection) were harvested and analyzed for specific lentiviral (Ψ-HIV packaging signal sequence) or adenoviral (Ad5-fiber gene) sequences via q-PCR. Large amounts of adenoviral or lentiviral sequences were not detected in any of the samples (Table 2 and 3). We encountered lower cell numbers in the infection assays of the Pursept/bleach LBW samples (No. 5–7), as indicated by 20 %–80 % higher Ct values for the RNaseP gene for samples No. 5–7 compared with those of the other samples (Table 2). Therefore, samples No. 5–7 have a higher chance of giving false negative results than the other samples.

3.4. Analysis of collected standard operating procedures

To verify the theoretical process of the CI procedures, we collected not only physical samples but also the related SOPs from

Table 2
Bioassay results for infectious lentivirus (HIV1) in the liquid biological waste (LBW) samples 1–8. Results are given as copy number of lentiviral Ψ sequence (mean of two technical duplicates ± SD) and RNaseP Sequence in Cts (mean of two technical duplicates ± SD) obtained by qPCR measurements of DNA from HEK293T cell pellets. DMEM was used as negative control. nd: not detected.

Nr.	Disinfectant	Ψ-copiessample	RNaseP Ct sample		RNaseP Ct DMEM	
1	Virkon S	Nd	22.30	±0.09	20.40	±0.07
2*	Dismozon Plus	nd	22.61	±0.02	22.59	±0.17
3	NaOH	nd	26.06	±0.05	26.06	±0.01
4*	Virkon S	nd	21.72	±0.04	22.59	±0.17
5†	Bleach, Pursept AF	nd	39.94	±1.06	21.67	±0.23
6†	Bleach, Pursept AF	nd	27.00	±0.01	21.67	±0.23
7†	Bleach, Pursept AF	nd	26.51	±0.01	21.67	±0.23
8	Virkon S	nd	23.67	±0.01	23.50	±0.01

* and † marked samples that were analyzed on the same day/in the same experiment.

Table 3
Bioassay results for infectious adenovirus (Ad5) in the liquid biological waste (LBW) samples 5–7. Results are given as copy number of Ad5 fiber gene (mean of two technical duplicates ± SD) obtained by RT-qPCR measurements of RNA in HEK293 cell supernatants. nd: not detected.

	time point			
Nr.	Disinfectant	Day 1	Day 3	Day 7
5	Bleach, Pursept AF	nd	nd	nd
6	Bleach, Pursept AF	nd	nd	nd
7	Bleach, Pursept AF	nd	nd	nd

the laboratories. If the waste samples contained detectable levels of infectious particles, they would have been verified by analytical means. The review of the SOPs involved checking the process in general, the type of disinfectant used (e.g., whether it is approved as a type-2 biocidal product for the organism in question), the controls in place to ensure a safe process, the safe and environmentally sound disposal of the inactivated LBW, and whether efficacy testing was performed prior to the first use of the method and/or on a regular basis.

All the SOPs had at least one deficiency (Table 4). None of the SOPs included regular checks to ensure a successful inactivation process. One of the SOPs exhibited five other shortcomings, including a lack of documentation about when the incubation with the disinfectant starts and the disinfectant concentration used. This meant that there was no assurance that the CI process had been carried out correctly. This method also involved mixing two different disinfectants; Pursept AF, a quaternary ammonium compound, was mixed with sodium hypochlorite (bleach). Mixing these two active ingredients is strongly discouraged as it can lead to the formation of explosive compounds and toxic gases. Luckily, in this case, the risks are negligible because the pH of Pursept AF makes dangerous reactions unlikely. However, mixing of disinfectants is not considered good practice as they can interact and weaken the inactivation effect. In addition, Pursept AF must be disposed of as hazardous waste because of its environmental impact. Despite this, the SOP stated that the inactivated LBW can be disposed of down the sink.

More positively, all SOPs prescribed an excess of chemicals and a much longer incubation time (often overnight) than the respective manufacturer deemed necessary for surface disinfection. Because there is no approval process for liquid inactivation, surface disinfectants are officially allowed to be used for CI in Switzerland.

Table 4
Number of collected protocols of chemical inactivation (SOPs) in the research laboratories and the identified shortcomings.

Organization	Number of SOPs	Identified shortcomings
A	3	1) No procedure to check when incubation time is completed mentioned. 2) No validation to indicate if inactivation was successful or not is mentioned in SOP (but could be mentioned elsewhere)
B	1	1) No procedure to check when incubation time is completed mentioned. 2) Mixing of two different disinfectants (mixing Pursept AF active ingredient, which is an quaternary ammonium compound and bleach could lead to the formation of explosive compounds and toxic gases) 3) No measuring cup for Javel (on site observation) 4) Incorrect disposal of Pursept AF (compulsory as hazardous waste, not down sink) 5) Pursept AF is not approved as biocide class 2 (but could be registered at the Federal Office of Public Health (BAG) by manufacturer, lab/organization should check with BAG before usage) 6) No validation to indicate if inactivation was successful or not is mentioned (but could be mentioned elsewhere)
C	1	1) No validation to indicate if inactivation was successful or not is mentioned in SOP (but could be mentioned elsewhere)

3.5. Evaluation of collected questionnaires

To determine the knowledge of laboratory staff of CI and the attention paid to its use, they were asked to complete a 12-question survey (Appendix). We received 27 completed questionnaires from ten different laboratories in three different organizations (A, B, D; Table 5). The questionnaires included responses from 35 laboratory staff members and gave up to 49 answers (multiple responses were possible for some questions). No LBW samples were obtained from Organization D and no questionnaires were handed out in Organization C.

The first two questions of the survey dealt with the relevance of CI in the responding laboratories and the organisms handled (Fig. 5). Within the last seven days prior to the survey, only seven out of 35 laboratory members reported having worked with lentiviruses, the most common organism being chemically inactivated in these laboratories (in 13 out of 49 responses; see Fig. 5A) followed by *E. coli* (12/49). Most work was done with eukaryotic cells (10/35) or *E. coli* (9/35), followed by lentiviruses (7/35), while some respondents had not worked with any organisms in the last week (Fig. 5A and B).

The next four questions probed the responsibilities within the laboratory organization and in-house training on CI. The majority of respondents (25/27) claimed that there was no dedicated person in charge of CI and the same number of respondents declared to not have received any training on the use of CI (25/27) (Fig. 5C and D). In addition, about one third (8/27) of the laboratory staff reported that no SOP was available or that they were not aware of one, although most named the biosafety officer as the person responsible for providing the manual.

After relevance and responsibility, the disinfectants used and their handling in laboratories were queried. Twelve different disinfectants were reported, with Incidin Plus being the most common (11/40), followed by Virkon S (6/40), Pursept (5/40), bleach (4/40), and NaOH (3/40; Fig. 5G). For the LBW samples we received, only the last four could be covered. One third of the laboratory members did not know whether the disinfectant agent used in their labora-

Table 5
Overview of the number of questionnaires handed out and collected from the laboratories of Organization A, B, D in the Swiss Cantons indicated. Note: No questionnaires could be handed out to Organization C.

Organization	Number of questionnaires collected	Canton	Number of laboratories surveyed	Number of LBW samples collected
A	3	Basel-City	3	4
B	13	Zurich	7	3
C	0	Basel-City	0	1
D	11	Aargau	unknown	0

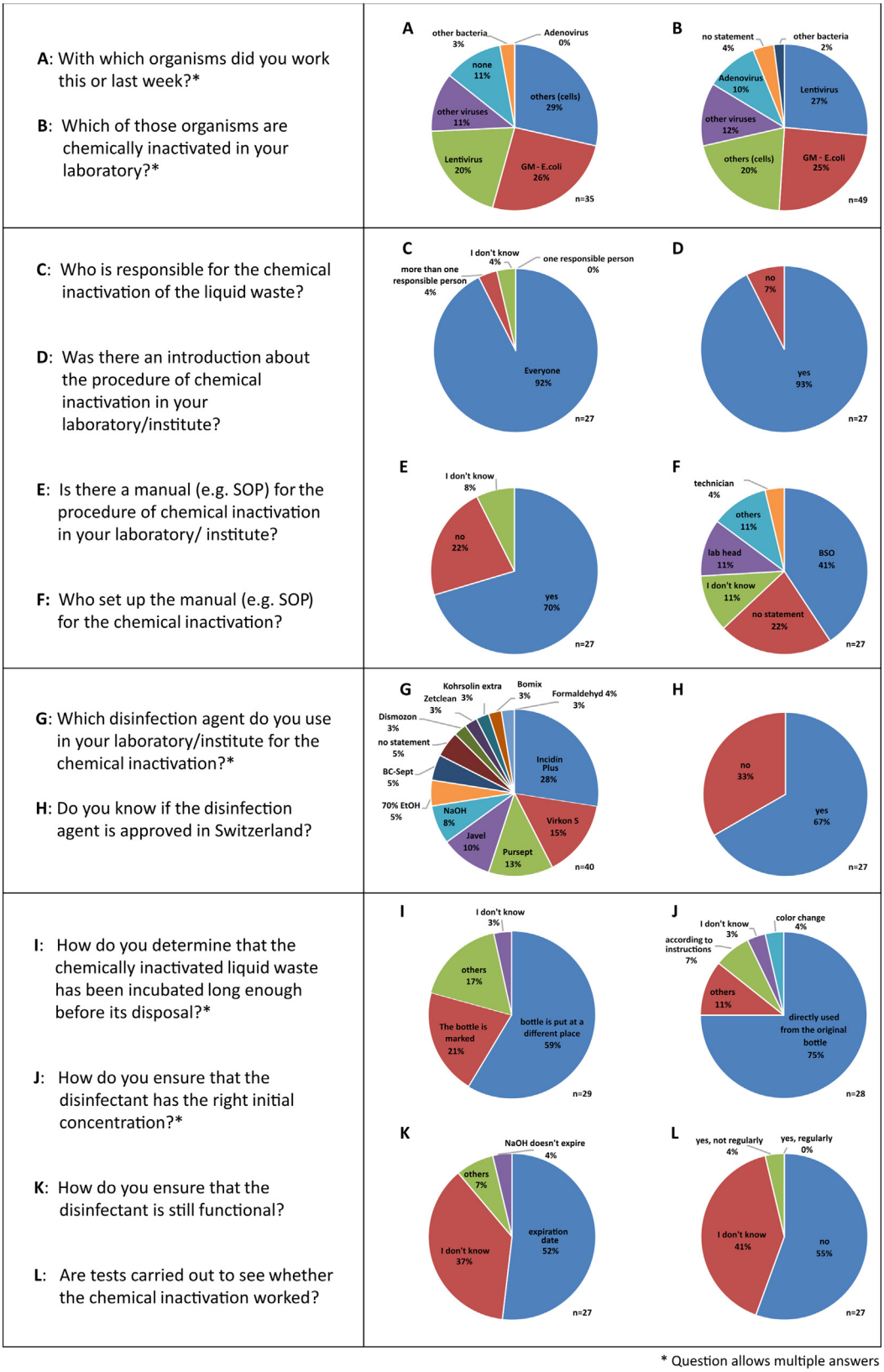

Fig. 5. Overview of the questionnaire results. The questions are listed on the left, while the corresponding results are depicted on the right, in pie charts as percentages of answered received. The number of responses received for each question is indicated at the bottom right of each pie chart.

tory was registered in Switzerland as a type-2 biocidal product (Fig. 5H).

Finally, we wanted to determine if there were controls in place to confirm successful CI. While all but one respondent were able to identify methods to ensure the correct incubation time and final concentration of the disinfectant (Fig. 5I and J), ten people out of 27 did not know how to check that the disinfectant was still functional (Fig. 5K). Furthermore, only one out of 27 respondents indicated that the effectiveness of the CI was sporadically checked, whereas the remaining respondents either reported that no checks were carried out (15/27) or that they did not know of any checks (11/27) (Fig. 5L).

4. Discussion

Research laboratories regularly accumulate infectious LBW containing bacteria from plasmid preparations and viral vectors in cell cultures. At first sight, it seems convenient to chemically inactivate LBW directly in laboratories. For example, 40 % of the laboratories surveyed in the canton of Zurich reported that they use CI in addition to autoclaving, and 9 % even use CI exclusively (Office of Waste, Water, Energy, and Air of the Canton of Zurich, Switzerland, email communication, July 20, 2022). Unlike autoclaving, CI is not a standardized method. Therefore, laboratory-specific procedures must be tested for effectiveness when established, and their effectiveness should to be monitored routinely.[17,21] Manufacturer information on dose and incubation time can be used as a reference for CI procedures. Although most commercial disinfectants refer to surface applications, the required standard tests for disinfectants in the EU always include tests in liquids similar to waste inactivation.[39–41] For these reasons, the Swiss authorities tolerate the use of disinfectants registered as type-2 biocidal products for the inactivation of LBW, particularly liquid cultures, at biosafety levels 1 and 2 without prior efficacy testing. If the disinfectant does not meet this requirement, the efficacy of a CI process must be demonstrated experimentally. At biosafety levels 3 and 4, validation of the CI procedure must be performed with all types of disinfectants. Demonstrating the efficacy of CI procedures for a range of applications as they occur in research laboratories and installing measures to control them in routine use are complex tasks. These tasks are further complicated if the LBW contains vector particles and viruses whose infectivity can only be tested in cell culture and if the waste may still contain disinfectant with cytotoxic properties. The disinfectant must, therefore, be removed or neutralized without affecting the infectivity of the virus, as this would lead to false negative results in bioassays. Depending on the disinfectant used, complete detoxification may not be fully achievable. For example, in this study, the complete removal of cytotoxic properties from culture waste treated with Pursept AF 2.3 % mixed with 3 % and 4 % bleach (as required by one of the SOPs of Organization B) was not achieved, thus impeding the efficacy pre-test. We also encountered a lower cell count when analyzing the samples containing this disinfectant mixture, indicating residual cytotoxicity in these samples after Sephadex filtration.

Given these challenges, chemically inactivated LBW could likely still contain infectious organisms at the time of disposal. Improper handling of LBW, particularly incomplete inactivation, has been suggested as a major cause of the escape of genetically modified *E. coli* into the laboratory wastewater from a Swiss research site. Over a two-year period, 45 % of the analyzed wastewater samples contained viable genetically modified *E. coli*.[1] Another study showed that interspecific transfer of laboratory plasmids occurred in laboratory wastewater.[4] Driven by these findings, we aimed to analyze random LBW samples for the presence of infectious organisms and investigate CI methods.

We mainly focused on the cantons of Basel and Zurich because they have the largest life science sectors in Switzerland, with 18 % and 14 % of all persons employed in the sector, respectively.[29] Unfortunately, we had to exclude most other regions in Switzerland because of our limited sampling radius. This was necessary to ensure the highest possible concentration of remaining infectious agents and avoid the possibility of false negative results.

We managed to collect LBW containing LVVs (HIV-1) and/or AdV vectors from eight laboratories in three organizations, one of which was where Wichmann and colleagues conducted their wastewater study and found viable genetically modified *E. coli*.[1] The study was limited by the challenge of obtaining LBW samples at the right time when experiments with infectious organisms had been taking place and the LBW was considered to be inactivated and was about to be discarded. Therefore, we could not influence which type of organisms (*E. coli* or other viral vectors) the LBW samples contained. To increase the number of samples, sampling was announced in some instances (five of the eight samples), which may have made the staff treating the LBW more cautious than usual. On several occasions, when sampling visits were not announced or when staff accidentally disposed of the freshly inactivated LBW, no LBW could be collected. Because of these logistical problems, some samples were frozen prior to analysis, which may well have decreased the amount of potentially infectious viruses in these samples.

We observed several shortcomings in the technical knowledge and training of laboratory staff on CI of LBW (e.g., 37 % did not know how to ensure that the disinfectant still worked and only 7 % had received an introduction to the method) and potential problems in the laboratory SOPs (e.g., few contained tests for efficacy). Nevertheless, no infectious virus could be detected in any of the collected LBW samples, considering the detection limit of 2000 virus copies per milliliter of LBW. Whilst we acknowledge that the number of LBW samples collected is very small and that the freezing of some of the samples did further lower the chance of detecting infectious viruses, we conclude that CI of LBW containing infectious LVV and AdV vectors does not result in the systematic release of considerable amounts of infectious virus into the environment in Switzerland. Our assumption is that in most cases, chemical disinfectants are added in excess and for a longer incubation period than necessary. Furthermore, research with LVV and AdV vectors is conducted according to BSL2 regulations, in contrast to work with *E. coli* (which is generally BSL1). This difference may also explain the contrast of our results with those of the earlier study by Wichmann et al.,[1] who found viable genetically modified *E. coli* in 45 % of the laboratory wastewater samples collected. Further studies on liquid waste from BSL1, particularly bacterial waste, could shed light on whether insufficient CI is more common in BSL1 or in bacterial waste. Other independent studies conducted by local laboratories to test the reliability of CI under real laboratory conditions in different countries may yield varying results. These studies could further emphasize the importance of providing adequate training to laboratory personnel on CI, and would help to exclude or identify ineffective CI as a potential escape route for bacteria and viruses from laboratories into the environment in these countries.

5. Conclusion

Some deficiencies in technical knowledge and training on CI were identified through analysis of SOPs and an assessment of laboratory staff of the investigated laboratories in Switzerland. This study found no evidence of the systematic release of chemically inactivated LBW containing infectious lentiviruses and adenoviruses into the environment by Swiss research laboratories. However, we believe that autoclaving should be the preferred

method for inactivating liquid waste over CI because it is more reliable, more robust, and less prone to handling errors because it requires less training.

Conflict of 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.

CRediT authorship contribution statement

Alina C. Teuscher: Investigation, Validation, Formal analysis, Visualization, Writing – original draft. **Charlotte Ruhnau:** Investigation, Validation, Formal analysis, Methodology, Conceptualization. **Nicole Stöcklin:** Investigation, Validation, Formal analysis, Methodology. **Fabienne Wichmann:** Investigation, Validation, Formal analysis, Methodology. **Evelyn Ilg Hampe:** Writing – review & editing, Supervision. **Claudia Bagutti:** Writing – review & editing, Supervision, Project administration, Conceptualization, Funding acquisition.

Acknowledgments

We thank the Federal Office of Public Health (FOPH) of Switzerland for financially supporting this study. We thank the Office of Waste, Water, Energy, and Air of the Canton of Zurich for supporting this study and providing the results of their 2021 survey. We also thank the Chemical and Biological Safety Inspectorate Basel-Stadt (Kontrollstelle für Chemie und Biosicherheit KCB) for their assistance with selecting sample sites in Basel, and Susanne Biebinger and Dirk Hamburger for their helpful comments on the manuscript. We are grateful to Gregor McCombie for his careful editing of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jobb.2024.02.001.

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