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Simulating a reference medium for determining bacterial growth in hospital wastewater for Raman spectroscopic investigation

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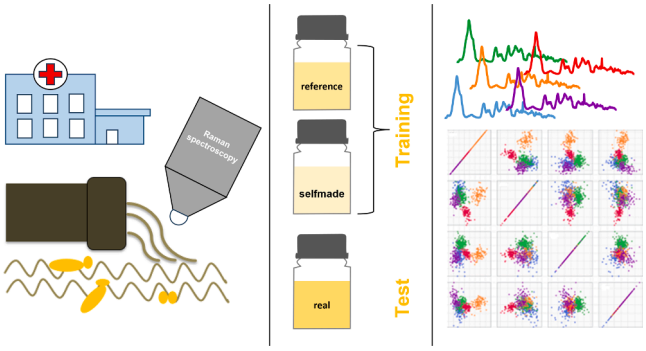
HIGHLIGHTS

- Wastewater has a complex composition and varies over time.
- Raman spectroscopic identification of bacteria is a phenotypic approach.
- For simulating wastewater as a matrix for bacterial growth different simulated wastewater types were tested.
- Only the combination of the two simulated wastewaters achieved satisfactory results in the Raman spectroscopic identification of bacteria from real wastewater.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater is a very complex and diverse medium, which despite low nutrient density still harbors bacteria. Especially the wastewater from hospitals contains a high germ load. However, wastewater is also very variable and differs not only from day to day, but also from house to house. Since wastewater is always changing and medium has an impact on Raman spectra of bacteria, it is necessary to find a surrogate material in which bacteria can be cultured to mimic a real hospital wastewater sample. In this study, we investigate two different artificial wastewaters for their abilities as a good alternative to real wastewater from the Jena University Hospital and to serve as a reference material for bacterial cultivation with subsequent Raman measurement. Each of the artificial wastewater on its own was not suitable to be used as a reference medium. Only the combination of the two simulated wastewaters achieved satisfactory results in the Raman spectroscopic identification of bacteria from

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real wastewater. These results could be used later in new experiments as a reference dataset to identify bacteria from real hospital wastewater samples.

1. Introduction

Wastewater is a complex mixture of different load and pollutant groups, such as dissolved and undissolved as well as easily or hardly degradable substances, plant nutrients, heavy metal compounds and salts. According to the Wastewater Discharge Act, Section 1, Article 2, "wastewater is water, the properties of which have been changed by domestic, commercial, agricultural or other uses, and the water drained together with it during dry weather conditions (polluted water), as well as water running off and collected from built-up or paved or asphalted surfaces following precipitation (rainwater). Liquids released and collected from facilities designed for the treatment, storage and depositing of waste shall also be deemed to be polluted water" [1]. According to the Ordinance on Requirements for Discharges into Waters (AbwV), Annex 1, hospital wastewater falls under domestic and communal wastewater [2]. A large part of the hospital wastewater is generated in bed and social areas, e.g., for washing, showering, toilet flushing and cooking. In addition, wastewater is generated in economic and technical areas as well as in medical functional areas, such as operating rooms, intensive care units and wards, laboratories, pathology or in the pharmacy. In addition to its general characteristics, hospital wastewater is also comparable to other municipal wastewater in terms of infection potential [3].

However, hospital wastewater can also be a potential reservoir for nosocomial infectious agents [4]. In particular, facultative pathogenic Gram-negative bacteria, such as *Escherichia coli*, as well as enterococci and non-fermenters, such as *Pseudomonas aeruginosa*, can find ideal conditions for their survival and spread in hospital wastewater and form the basis for persistent outbreaks in hospitals [5]. Therefore, monitoring of hospital wastewater is suitable as an additional tool for infection prophylaxis [6–8].

A general topic in identifying bacteria from environmental samples, such as wastewaters, is that the exact microbial species composition in the sample is unknown or changes depending on the sampling or sampling site [9]. In addition, only a fraction of the bacteria can be cultivated [10]. Due to these complications, a culture-independent method for the identification would be preferred. The constantly changing composition of wastewater can influence the growth of bacteria. Temperature, pH as well as the availability of water and oxygen, but also of nutrients such as carbon, phosphorus, nitrogen and trace elements have a decisive influence on the growth of bacteria [11].

One of these possible methods to monitor bacteria in wastewater is Raman spectroscopy. Raman spectroscopy as a representative of vibrational spectroscopy depends on the interaction of molecular vibrations with light [12]. Due to this phenotypic method, biological samples such as bacteria reveal the complete biochemistry within the cell [12,13].

Table 1
Composition of self-made wastewater (SM), .

Component	Concentration / mg/l
Peptone	17.4
Yeast extract	52.2
Milk powder	116.2
Starch	122.0
Sunflower oil	29.0
Ammonium acetate, CH_3COONH_4	79.4
Monopotassium phosphate, KH_2PO_4	23.4
Magnesium hydrogen phosphate, $MgHPO_4{\cdot}3H_2O$	29.0
Urea	91.7
Ammonium chloride, NH_4Cl	12.8
Iron(II)sulphate, $FeSO_4{\cdot}7H_2O$	5.8

adapted from [31]

Since bacteria can adapt genotypically and phenotypically to many environmental conditions, these changes also show up in the resulting Raman spectra [9]. Examples of bacteria adapting to changing environmental conditions and then also changing the appearance of their spectra can be found in many areas of microbiological sampling or cultivation [14]. Influences that change the spectra due to sample handling can be found in transport, storage [15], sample preparation [16], fixation or isolation of bacteria [17–20]. Especially in environmental samples, there are many influences since many parameters are not known or cannot be controlled. One example is when bacteria are embedded in matrix, which makes Raman-compatible isolation necessary [17]. In addition, the content of CO_2 in the ambient air can cause changes in the bacterial metabolism, which are apparent in Raman spectra [21]. Also, the composition of nutrients within the medium can change and influence the bacteria and their corresponding Raman spectra [22,23]. A study that carried out Raman spectroscopy on waterborne bacteria was confronted with the problem of a very low concentration of bacteria and therefore carried out various filtration steps to concentrate the bacteria. Thereby, the resulting Raman spectra obtained from filtered biomass can be very different from non-filtered biomass [24].

In the example of wastewater, the composition of nutrients is not defined and changes daily and in addition from house to house. Raman spectroscopy has already been used in the field of wastewater to answer issues such as monitoring of wastewater treatment [25] or the distribution of biofilms [26] to name a few. Since the composition of the wastewater and the influence on the Raman spectra is not known, a preliminary study is necessary to investigate these influences in more detail. As a second question, we wanted to investigate whether an artificial wastewater can be used to build up a dataset that can later be used to predict spectra from a real wastewater sample. For this purpose, two artificial wastewaters were used in this work to cultivate bacteria and build a model of bacterial Raman spectra, while the data of the bacteria cultivated on a real wastewater sample was used for identification.

2. Material and methods

2.1. Comparison of different types of wastewaters and cultivation of bacteria

As a maximum care facility, Jena University Hospital has 30 clinics and polyclinics and 25 institutes. It provides 1411 planned beds (including intensive care units) and 294 day hospital places for the treatment of patients. The Lobeda facility is the largest campus [27]. There, wastewater and rainwater are collected and discharged separately in one sewer each [28]. The wastewater volume at the Lobeda site for the period 2010 to 2016 averaged 400 m^3/day [29].

The hospital wastewater (WW) was taken as a qualified random sample according to DIN 38402-A11 [30] on a Friday morning at 8:00 a.m. at the transfer shaft (indirect discharger) of the Jena University Hospital, Lobeda site. The homogenized sample was filled into two 1 l polypropylene bottles and transported refrigerated at 4 °C.

WW was compared to two artificial wastewaters. The first one was prepared inhouse containing the compositions mentioned in Table 1 (self-made, SM) [31]. The second wastewater was purchased as a certified reference material (Sigma-Aldrich, SA).

To analyze wastewater samples, different parameters were characterized and compared to the bacterial growth. This includes physical parameters which were measured such as pH, conductivity, temperature, oxygen concentration, and chemical parameters, such as ammonia

nitrogen, chemical oxygen demand (COD), total phosphorus, Kjeldahl nitrogen (TKN), total bound nitrogen (Tnb), and sulfate. The measurement of relevant wastewater parameters of SM was carried out in the environmental laboratory of the Environmental Protection Department of the Jena University Hospital. The wastewater sample was homogenized using the RCT IKAMAG magnetic stirrer from IKA (Staufen, Germany). Standardized cell tests from Hach Lange (LCK 303 for ammonia; LCK 350 for phosphor; LCK 338 for total bound nitrogen, LCK 153 for sulfate, LCK 514 for COD) were used to determine the concentration of the wastewater parameters. The wastewater was digested for the measurement of chemical oxygen demand (COD), total phosphorus and total nitrogen by means of thermostat LT 200 (Hach Lange). To determine the dissolved salts, ammonium, nitrogen, and sulfate, the wastewater sample was filtered through a 0.45 μm cellulose acetate membrane filter. The photometric determination was carried out using a Hach Lange DR 2800 photometer (wavelengths 345 nm (LCK 338), 430 nm (LCK 153), 605 nm (LCK 514), 694 nm (LCK 303) and 880 nm (LCK 350)). The measurement of the wastewater parameters of WW was carried out by an external company as part of the indirect discharger sampling. The data for the composition of the reference material (SA) was taken from the certificate of analysis [32].

The first step in the microbiological experiments was the evaluation of the growth behavior of the bacteria on the different wastewaters. Since bacteria show different growth behavior on different nutritional media, it was necessary to evaluate the growth ability of the bacteria according to different artificial wastewaters. For the cultivation on solid plates Agar was added to each of the wastewaters.

In a pre-cultivation, bacteria were grown on complex media (Nutrition Agar (NA) or Tryptic Soy Yeast Agar (TSY), both were purchased from Merck) to create enough biomass. Later some colonies of bacteria were taken and suspended in water before they were inoculated on different wastewater media and cultivated at 37 °C for 18 h to compare the growth abilities on the different media (Figs. S1-S25, Supplementary Material).

For these experiments, typical wastewater bacteria were selected [33–36]. These were *Enterococcus faecalis UK003, Enterococcus faecium UK005, Acinetobacter baumannii UK011, P. aeruginosa UK007* and *E. coli UK014*. The respective strains were patient isolates and were provided by the Jena University Hospital.

2.2. Raman measurements

After it was proven, that bacteria do grow in all media, they were prepared for Raman measurements. Therefore, five to ten colonies of the bacteria were taken by an inoculation loop and suspended in water, centrifuged and the supernatant was removed. This washing step was repeated for three times in total and bacteria were spread on nickel foil. After the suspension was dried by room temperature, they were measured by Raman spectroscopy.

Raman spectroscopic investigations of single cells were performed using a Raman platform (BioParticle Explorer, rap.ID, Berlin, Germany) equipped with a frequency-doubled (532 nm), solid-state diode-pumped Nd:YAG laser (LCM-S-11-NNP25, Laser-export Co. Ltd., Moscow, Russia). An Olympus MPL-FLN-BD 100× objective (Olympus Corporation, Tokyo, Japan) focused the Raman excitation light onto the sample with a spot size of below 1 μm before the light irradiated the sample with a laser power of 10 mW. After the Rayleigh scattering was removed, the backscattered light diffracted by a single-stage monochromator with a 920-line/mm grating (HE 532; Horiba Jobin Yvon, Munich, Germany). After that the light was collected by a thermoelectrically cooled charge-coupled device (CCD) camera (DV401-BV; Andor Technology, Belfast, Northern Ireland) with a spectral resolution of 10 cm^{-1}. Before measurement of the bacterial samples, 4-acetamidophenol (4-AAP) was measured for the calibration of the wavenumber-axis. Each bacterial sample was measured under ambient conditions in three independent batches. For each sample 50 spectra of single cells were measured. To ensure comparable S/N ratios *A. baumannii, P. aeruginosa* and *E. coli* were measured for 5 s at 50% laser intensity for all wastewaters, *E. faecium* was measured for 5 s at 10% intensity. *E. faecalis* was measured for 5 s at 10% for self-made and hospital wastewater while the intensity was increased to 50% for the reference wastewater.

2.3. Data pre-processing

Raw spectra contain many artefacts that need to be cleaned up before data analysis. The pre-processing was done by means of Ramanmetix [37] to eliminate perturbations like spectral artefacts, cosmic rays, fluorescence and others. After cosmic spike removal [38], further artefacts were deleted by clipping the spectra in the range of 350–3150 cm^{-1}. For the calibration 4-acetamidophenol was measured at the beginning of each measurement day and used to calibrate the wavenumber-axis [39]. In the next step, a sensitive nonlinear iterative peak (SNIP) clipping algorithm was used for baseline correction and elimination of fluorescence. After baseline correction, the data were normalized. Here, a vector normalization was applied by dividing the spectra to the square root of sum of the squared spectral intensities. The last step of pre-treating the data was the removal of the silent region between 1800 cm^{-1} and 2600 cm^{-1}.

2.4. Classification of each growth condition

The pre-processed data were used to build a model to classify the bacterial spectra within each single wastewater. Here, a combination of a Principal Component Analysis and a Linear Discriminant Analysis (PCA-LDA) was used. Hereby, the number of PCs was optimized to 20 and a random 10-fold cross validation was used.

These steps of data analysis were done for all three wastewaters independently, but also the two artificial wastewaters (self-made and Sigma-Aldrich) were additionally combined as a single dataset. After the bacterial spectra cultivated on different wastewaters were classified, the data of the different wastewaters were compared. Therefore, self-made wastewater (SM), Sigma-Aldrich (SA) and the combination of both was used to build the PCA-LDA model and the data from the real wastewater sample was projected into this model to test the identification.

3. Results and discussion

Wastewater is a very diverse medium, which can differ not only between days but also between different sampling sites. Therefore, wastewater from municipal areas differs from industrial areas or clinical environments. Due to the different composition of the medium and their respectively nutrients, also the microbial community is highly variable.

In particular, hospital wastewater can harbor pathogenic bacteria and act as a source of nosocomial infections, but also as an early warning system for their outbreak [6–8]. Therefore, a fast, reliable, and cultivation-free tool for analyzing these samples is needed and presented here [9,10].

3.1. Evaluation of the influence of individual wastewater parameters on bacterial growth

In this study, we addressed questions of how different wastewater media affect both the growth and Raman spectra of bacteria. Furthermore, the question arose whether an artificial wastewater can be used to create a reference database for bacterial Raman spectra, with which spectra of bacteria from a real wastewater sample can be identified.

To answer the first part, wastewaters were analyzed, used as a solid medium for bacteria and their growth behavior was evaluated. For this purpose, a stock solution of bacteria was cultivated on the different media. After 18 h of incubation at 37 °C, the growth was evaluated. Two media were chosen as a positive reference towards the wastewaters.

Table 2
Concentration of representative wastewater parameters of the self-made wastewater (SM) and the wastewater from the Jena University Hospital (WW).

Wastewater Parameters	SM	WW
pH value	7	8
Conductivity / μS/cm	235	1030
Temperature / °C	20	18
Oxygen concentration / mg/l	4	–
Ammonia nitrogen / mg/l	35	16
Chemical oxygen demand (COD)/ mg/l	628	1200
Total phosphorus / mg/l	15	6
Kjeldahl nitrogen (TKN) /mg/l	–	62
Total bound nitrogen (Tnb) / mg/l	76	78*
Sulfate / mg/l	LLOQ**	53

*calculated from the total inorganic nitrogen (ammonia nitrogen, nitrate, nitrite) and the organic Kjeldahl nitrogen.
**LLOQ: lower limit of quantification.

Since these are complex media, it was obvious that the bacteria received all growth nutrients and the complete culture plate was overgrown by bacteria. Surprisingly, both enterococci did not show growth on NA, therefore TSY was also used.

Apart from the enterococci, growth was observed on the medium from the self-made wastewater, but it was significantly lower than in the complex media. The reference material as medium already looked very opaque, so that no colonies were visible to the naked eye. Also, for the brownish wastewater sample from the hospital, no bacterial growth was visible by naked eye. With both artificial wastewaters (SM and SA), no growth could be observed optically, but enough bacteria could be taken from the plates so that the prepared sample could be measured by Raman spectroscopy (images of the plates are shown in the Supplement Material, Figs. S1-S25). In contrast to a complex medium, which is designed to fulfill all requirements for bacterial growth, this is not guaranteed for the different wastewater samples.

In addition to temperature and pH, the availability of water and oxygen also have a decisive influence on the specific growth of bacteria. Nutrients, such as carbons sources, phosphorus, nitrogen as well as microelements, also play a major role in the growth of bacteria [11]. For the evaluation of the influence of the wastewater on the bacterial growth as well as on their metabolism, which can be detected by means of Raman spectroscopy, relevant wastewater parameters were measured for the self-made wastewater (SM) and the hospital wastewater (WW) (see Table 2) and the biodegradation of these wastewaters was calculated.

Various inhibitory or non-degradable ingredients in the wastewater may influence the suppression of colony formation. Real wastewater in particular consists of a wide spectrum of diverse constituents, ranging from readily degradable organics, such as carbohydrates, proteins, to growth-inhibiting substances, such as heavy metals and hydrogen sulfide, among others [11].

Temperature affects the physical behavior of molecules and the rate of chemical and enzyme catalyzing reactions, and is considered one of the most important environmental factors for the growth and existence of bacteria [40]. Apart from enterococci, all bacteria grew at 37 °C during the 18 h cultivation on the complex media, also on the wastewaters. This indicates that this temperature and the time is optimal for the growth of these bacteria, except for enterococci.

In addition to the temperature, oxygen is also necessary for the decomposition of organic substances and the conversion of nutrients. The more polluted a wastewater is, the higher the oxygen consumption due to bacterial respiration resulting from the biological degradation of organic matter. Low concentrations of oxygen, in turn, can inhibit the growth of aerobic bacteria [40]. In order to evaluate this influence, a dissolved oxygen content at SM of 4 mg/l was determined as an example; no measured values were provided for WW. In general, an oxygen deficiency and thus an influence of aerobic bacteria is present for water below a content of about 9 mg/l at 20 °C [40]. Nevertheless, for bacterial cultivation on Agar plates, the concentration of oxygen remains the same over the time of incubation. Furthermore, the pH value of the wastewater influences cell growth. For SM, an almost neutral pH of 7 and for WW a slightly basic pH of 8 could be measured. The majority of microorganisms prefer a slightly acidic pH of 5 to 9 [11]. This is also true for the bacteria studied. Their lower growth on WW may be due to the slightly increased pH.

Salts as well as other osmotically active substances can reduce water activity, i.e. the availability of water for the bacteria. Osmotic pressure is also created, in which water is drawn out of the bacterial cell and the cell is in danger of drying out, which inhibits growth [11]. To determine the dissolved salt content in the effluents, electrical conductivity was measured. Conductivity of 235 μS/cm was determined for SM and 1030 μS/cm for WW. The increased conductivity at WW is due to the high variability and concentration of salts in real wastewater caused by the input from various sources, such as patient rooms, kitchens, and research facilities. Exemplary anions measured in WW were sulfate (53 mg/l) and chloride (120 mg/l). For SM, a concentration of sulfates below the limit of quantification from 40 to 150 mg/l was determined. Inhibitors of bacterial growth include chlorinated organic solvents as well as hydrogen sulfide and cyanides. Hydrogen sulfide is formed by the reduction of sulfate. Primarily as an undissociated form, it has an inhibitory effect on bacterial growth [40]. In the form of sulfate and sulfide, sulfur can be taken up by bacteria and used as a protein building block and in metabolic reactions [11].

Nitrogen is required for the formation of nucleic acids, proteins and other cell components [11]. In wastewater, nitrogen occurs organically, in the form of proteins and urea, but also inorganically as ammonium, nitrate, nitrite and as elemental nitrogen. From a pH above 9, ammonium is converted to ammonia, which interferes with bacterial growth [37]. To determine the nitrogen content of wastewater, total bound nitrogen (76 mg/l) was measured for SM. For WW, Kjeldahl organic nitrogen (62 mg/l) and total inorganic nitrogen (16 mg/l) were measured and added (78 mg/l). The content of total bound nitrogen is comparable (with in 97%) in the two wastewaters (effluents) SM and WW. For bacteria, phosphorus is another essential nutrient, especially for their building material and energy metabolism [11]. In wastewater, phosphorus occurs mainly as phosphate derived from the degradation of nucleic acids and, to a lesser extent, from detergents. In high concentrations, phosphorus and inorganic nitrogen compounds can lead to increased oxygen depletion in water, which impairs bacterial growth [40]. Total phosphorus was determined in both effluents, consisting mainly of inorganic dissolved orthophosphate and only a small amount of dissolved or bound organic phosphates. A concentration of 15 mg/l was determined in SM and 6 mg/l in WW. Since phosphorus can be present in municipal wastewater at a maximum concentration of 13 mg/l, there should be no effect on cell growth in the measured SM and WW effluents.

Information on the oxidation of readily biodegradable compounds as well as poorly biodegradable natural products and xenobiotics is provided by the sum parameter chemical oxygen demand (COD) [41]. In this experiment, COD was measured to estimate the availability of oxidizable substances and the resulting advantages and disadvantages for growth and amount of oxygen required for degradation. The COD of 628 mg/l measured for SM and the COD of 1200 mg/l measured for WW showed that the hospital wastewater contains more organic compounds that should be degraded by high oxygen consumption. Actually, this should result in increased growth in the hospital wastewater, but this was not the case. Possibly the reason lies in the carbon sources in this medium, which are difficult to degrade and thus inhibit growth. The COD:N:P ratio was calculated to evaluate the biodegradation and the resulting biomass increase. For aerobic degradation in the wastewater treatment plant, it should be 200:5:1 [42]. The ratio of COD:N:P for SM was 200:24:5 and the ratio of COD:N:P for WW was 200:13:1. Therefore, WW was nearly comparable to an optimal nutrient supply for aerobic

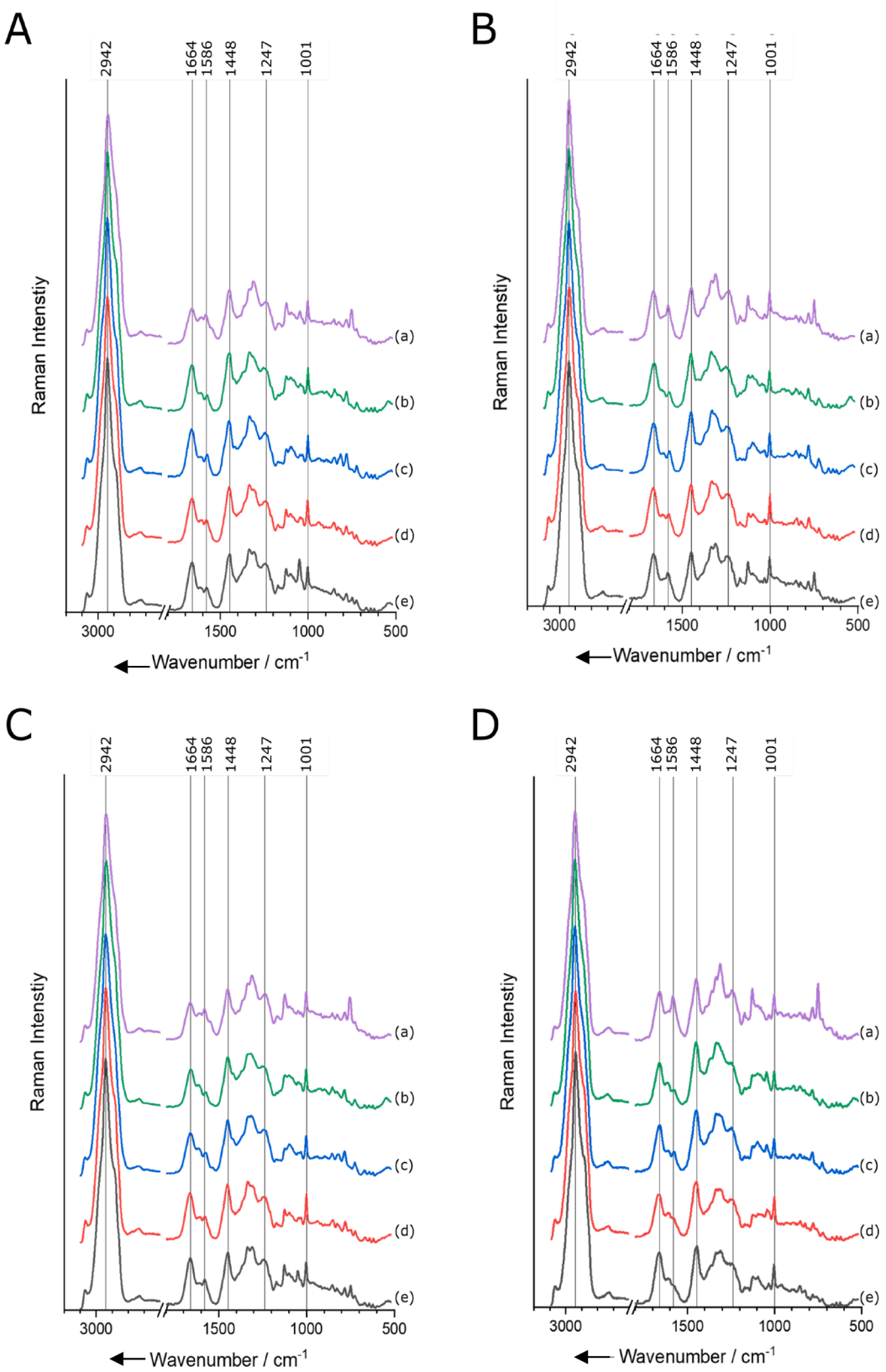

Fig. 1. Mean Raman spectra of bacteria grown on different wastewaters. (a) *P. aeruginosa,* (b) *E. faecium* (c) *E. faecalis,* (d) *E. coli,* (e) *A. baumannii.* A: self-made wastewater (SM), B: reference material from Sigma-Aldrich (SA), C: combination of both artificial wastewaters (SM + SA), D: real hospital wastewater sample (WW).

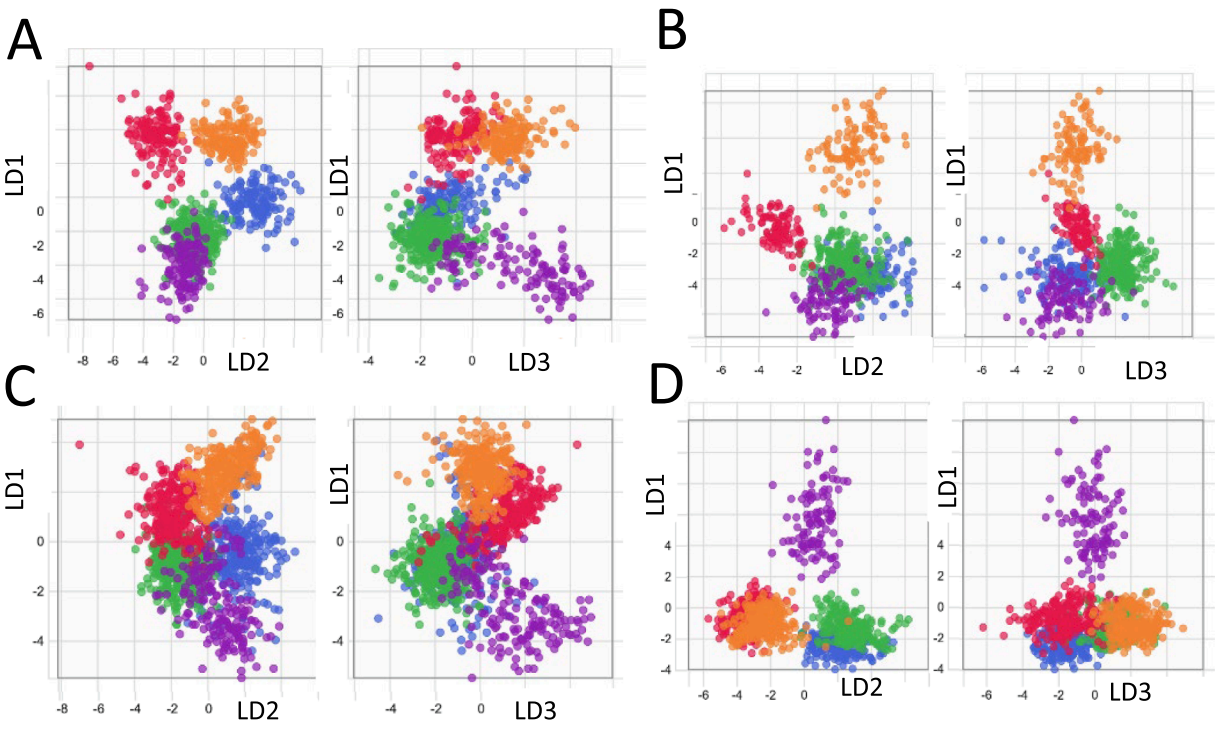

Fig. 2. PCA-LDA results of classification of bacteria grown on different wastewater. Purple: *P. aeruginosa*, green: *E. coli*, red: *E. faecalis*, orange: *E. faecium*, blue: *A. baumannii*. A: self-made wastewater (SW), B: reference material from Sigma-Aldrich (SA), C: combination of both artificial wastewaters (SW + SA), D: real hospital wastewater sample (WW).

degradation.

3.2. Mean Raman spectra of bacteria according to different wastewaters

Since Raman spectroscopy is a phenotypic method, not only the whole biochemistry of the cell is depicted but also many factors can influence the spectra [12]. One example of these influencing factors is the different composition of media and nutrition for the bacteria [22]. As it was already mentioned before, the composition of wastewater samples differs from day to day and from sampling site to sampling site. Therefore, bacteria grown in different wastewaters can show different Raman spectra. For the detailed analysis of three different wastewater samples, we could show some differences. Hence, we were wondering, if these differences can also be displayed in the Raman spectra of the respective water samples. A second question showing up was, if an artificial wastewater can be used as a substitute for a real hospital wastewater to build a reference dataset. This replacement could solve the problem of the changing sample medium.

Fig. 1 shows the mean Raman spectra of the bacteria in a single wastewater medium, namely self-made wastewater (SM), a reference purchased from Sigma-Aldrich (SA) and a real wastewater sample from the hospital (WW). For a comparison of artificial wastewaters and real hospital wastewater samples also the data obtained of bacteria grown in both artificial wastewaters (SM and SA) were combined and mean Raman spectra were calculated. The selection of bacteria is composed of common representatives of the microbiome from wastewater samples [33–36]. The selected representatives are *P. aeruginosa, E. faecium, E. faecalis, E. coli*, and *A. baumannii*. Raman spectroscopy is a phenotypic method which depicts the whole biochemistry of a cell. Therefore, a bacterial spectrum consists of the most abundant signals from components within a cell. Those signals represent ν(C–H) at 2942 cm^{-1}, amide I at 1664 cm^{-1}, DNA at 1586 cm^{-1}, δ (CH_2/CH_3) at 1448 cm^{-1}, amide III at 1247 cm^{-1} and phenylalanine at 1001 cm^{-1} [12].

The spectra of individual bacterial species differ only slightly. The spectra of *P. aeruginosa* and *A. baumannii* show increased signals in the area of 1584 cm^{-1} and in the range of 1416–1314 cm^{-1}, which represent an enhanced cytochrome content [43]. Furthermore, *P. aeruginosa* exhibits just a low intensity in the amide I signal when grown in self-made wastewater (SM). For all bacteria, the signal of the DNA is very low. This could correlate with the slow growth of the bacteria on the different media (also see at photos of plates in the Supplementary Material, Figs. S1-S25). Another interesting aspect in the evaluation of the spectra is the ratio between the relative intensity of the peaks representing DNA and amide I [15,44]. In preparation for cell division, the amount of DNA must be doubled and also the amount of proteins increase to provide the components for the new cell. So, one can deduct changes in cell growth according to the changes of relative intensity for those two peaks. Especially in the spectra of *E. faecium*, a higher division rate can be observed (Fig. 1 A and D, spectra b). DNA molecules incorporate nitrogen and so the increased growth rate of this species can also be supported by the fact that more nitrogen was found in the reference water than in the real hospital wastewater (see Table 2). Otherwise, no further differences could be observed by naked eye. To observe the impact of the different wastewaters on the spectra of a bacterial species, chemometric methods must be applied to highlight changes in the spectra, which are too small to be seen by eye.

Therefore, a first step was to classify the bacterial spectra within a single wastewater in a PCA-LDA model. The results are shown in Fig. 2 (LD 1—3, more LDs are shown in the Supplementary Material, Fig. S26) and in Table 3. In the graphical visualization, each dot represents a single cell spectrum. Ideally, the model shows a small intraspecies variation but a large interspecies variation, so that the data are well separated. The dataset of bacteria grown in SM (Fig. 2A) received a good separation of the species. The classification of species cultivated on SA wastewater (Fig. 2B) showed an overlap of *P. aeruginosa* and *E. coli* for LD 1 and 2 and these data could not be separated accurately. The same observation is done for the classification of both artificial wastewaters combined (SA + SM, Fig. 2C) but here, it was the second LD, which can separate them. This overlap might be due to the increased amount of cytochrome, which was already observed in the mean Raman spectra

Table 3
Classification models of bacteria grown on different artificial and real wastewater samples.

Self-made (SM)								
Predicted	[1]	[2]	[3]	[4]	[5]	Sensitivity	Specificity	Accuracy
True								
A. baumannii [1]	132	6	0	9	2	0.887	1	
E. coli [2]	0	301	0	0	5	0.972	0.918	
E. faecalis [3]	0	4	144	0	0	0.973	0.995	
E. faecium [4]	0	0	4	151	0	0.974	0.987	
P. aeruginosa [5]	0	38	0	1	91	0.702	0.986	
Whole model						0.901	0.977	0.919
Sigma-Aldrich (SA)								
Predicted	[1]	[2]	[3]	[4]	[5]	Sensitivity	Specificity	Accuracy
True								
A. baumannii	114	10	1	1	13	0.82	0.962	
E. coli	10	225	3	0	1	0.944	0.961	
E. faecalis	0	1	100	4	0	0.98	0.985	
E. faecium	1	0	6	95	0	0.941	0.997	
P. aeruginosa	10	6	0	0	71	0.835	0.976	
Whole model						0.904	0.976	0.908
Artificial wastewater (SM + SA)								
Predicted	[1]	[2]	[3]	[4]	[5]	Sensitivity	Specificity	Accuracy
True								
A. baumannii	222	16	8	21	21	0.771	0.955	
E. coli	12	468	60	1	4	0.872	0.941	
E. faecalis	7	25	218	2	1	0.862	0.933	
E. faecium	1	1	21	234	0	0.908	0.98	
P. aeruginosa	53	19	4	1	140	0.682	0.98	
Whole model						0.819	0.958	0.835
Hospital wastewater (WW)								
Predicted	[1]	[2]	[3]	[4]	[5]	Sensitivity	Specificity	Accuracy
True								
A. baumannii	126	5	0	12	0	0.847	0.983	
E. coli	1	160	0	2	0	0.927	0.949	
E. faecalis	0	0	231	31	0	0.938	0.973	
E. faecium	2	5	0	165	0	0.829	0.957	
P. aeruginosa	0	6	0	3	107	0.972	1	
Whole model						0.902	0.972	0.864

(Fig. 1 spectra (a) and (e)).

The LDA of the bacteria cultivated on a real wastewater sample from the hospital showed a slightly different pattern (Fig. 2D). Here, the first observation is done for *E. coli* and *A. baumannii*. These species were well separated from the other clusters. But also *P. aeruginosa* and *A. baumannii* were well separated. Fortunately, in all conditions, the two enterococci were separated, although they are close relatives. All classification models performed very well with an accuracy above 83% (Table 3).

The classification of spectra obtained after a cultivation in self-made wastewater (SM) showed an overall sensitivity of 90.1%, specificity of 97.7% and accuracy of 91.8%. Each single species had a sensitivity higher than 70.2% and a specificity over 91.8%. Even, if some spectra of *A. baumannii* were falsely classified as *E. coli*, these are particularly good results and the spectra of the species from the self-made wastewater (SM) showed a good classification. The visualization of the linear discriminants (LDs) showed, that even the first two LDs were sufficient to separate the single clusters of species. *E. coli* and *P. aeruginosa* needed the third LD to be separated from each other. For the classification of data obtained from a cultivation in reference wastewater from Sigma-Aldrich (SA), the overall sensitivity is 90.4%, specificity is 97.6%, and accuracy was 90.8%. In this case, *A. baumannii* showed the lowest

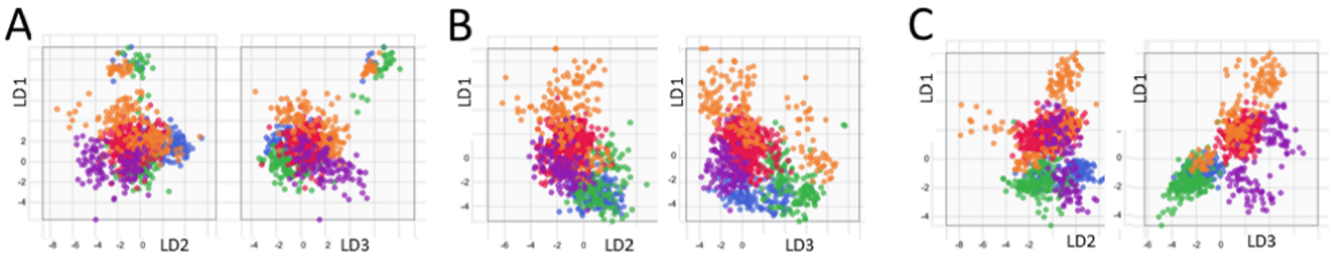

Fig. 3. PCA-LDA results of the identification. Artificial wastewaters were used as a training dataset, while data from real wastewater were used as a test dataset. Purple: *P. aeruginosa*, green: *E. coli*, blue: *A. baumannii*, red: *E. faecalis*, orange: *E. faecium*. A: Self-made wastewater (SM) as training and hospital wastewater (WW) as test, B: Wastewater Sigma-Aldrich (SA) as training and hospital wastewater (WW) as test, C: combination of self-made wastewater (SM) and Sigma-Aldrich (SA) as training, hospital wastewater (WW) as test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Confusion matrix of the identification of hospital wastewater (WW). Artificial wastewaters were used as a training dataset, while data from real hospital wastewater (WW) were used as a test dataset. SM: Self-made wastewater, SA: reference material from Sigma Aldrich.

SM								
Predicted true	[1]	[2]	[3]	[4]	[5]	Sensitivity	Specificity	Accuracy
A. baumannii [1]	79	1	2	61	0	0.52	0.932	
E. coli [2]	13	109	5	34	2	0.583	0.952	
E. faecalis [3]	24	53	58	123	4	0.435	0.821	
E. faecium [4]	4	0	58	109	1	0.607	0.683	
P. aeruginosa [5]	0	2	37	13	64	0.542	0.988	
Whole model						0.537	0.875	0.521
SA								
Predicted true	[1]	[2]	[3]	[4]	[5]	Sensitivity	Specificity	Accuracy
A. baumannii [1]	48	63	0	0	32	0.326	0.973	
E. coli [2]	0	159	2	2	0	0.994	0.777	
E. faecalis [3]	18	72	135	26	11	0.656	0.84	
E. faecium [4]	0	37	51	84	0	0.479	0.964	
P. aeruginosa [5]	5	2	43	3	63	0.534	0.948	
Whole model						0.598	0.900	0.617
SM + SA								
Predicted true	[1]	[2]	[3]	[4]	[5]	Sensitivity	Specificity	Accuracy
A. baumannii [1]	100	40	0	0	3	0.709	0.979	
E. coli [2]	13	148	2	0	0	0.91	0.906	
E. faecalis [3]	4	1	160	97	0	0.716	0.869	
E. faecium [4]	0	35	29	106	2	0.627	0.868	
P. aeruginosa [5]	0	0	47	5	64	0.547	0.998	
Whole model						0.702	0.924	0.713

sensitivity of 82.0% while all species had a specificity over 96.2%. Since nearly no spectrum was misclassified, this is a good example of a good separation of the data during the classification. For the visual separation of the data, the first three LDs were sufficient to separate most of all spectra.

The classifications of both artificial wastewaters alone showed remarkable results. However, if both cultivation conditions were taken together (SA + SM), specificity stayed high at 95.8% while sensitivity droped to 81.9%, and accuracy to 83.5%. Nevertheless, this was still a particularly good classification. Mostly, *P. aeruginosa* was responsible for the drop of the accuracy. This species showed a low sensitivity of 68.2% and an overlap with *A. baumannii*.

For the classification of bacteria cultivated on the real hospital wastewater (WW) the overall sensitivity was 90.2%, specificity 97.2%, and accuracy 86.4%. Both enterococci were overlapping a bit but were still well separated. This resulted in sensitivities of 93.8% for *E. faecalis*, 82.9% for *E. faecium.* The same can be said for *A. baumannii* (sensitivity 84.7%) and *E. coli* (sensitivity 92.7%).

Even due to these results of the classification, we could show an influence of different kinds of wastewater towards Raman spectra of bacteria. But since the classification of all different media was satisfying, we went on and explored the question to what extent an artificial wastewater could be used as a training dataset for bacteria from a real wastewater sample.

Therefore, single artificial wastewaters (SM, SA, SM + SA) were used to build a model and the data of the real wastewater sample from the clinics (WW) were projected into this model. The resulting results are shown in Fig. 3 and Table 4.

For the combination of self-made wastewater (SM) as training dataset and real hospital wastewater (WW) sample as test dataset, it showed low identification results. For all species, an identification was not possible because many spectra were misidentified as other species. This combination of data resulted in an overall accuracy of 52.1%. Due to this low rate of correct identification, it was not possible to use self-made wastewater (SM) as a substitute for a real wastewater sample.

The combination of reference wastewater material from Sigma-Aldrich (SA) showed slightly better results for the identification of real hospital wastewater (WW) samples. The overall accuracy was 61.7%. In this case, both enterococci did interfere with each other. Additionally, *A. baumannii* showed a very low sensitivity of just 32.6%. These results were slightly better than the model built with self-made wastewater (SM), but also this wastewater could not be used as a substitute to identify bacteria from real wastewater samples.

Since both artificial wastewaters were not suitable to used as a substitute for establishing a database of bacteria isolated from hospital wastewaters, the next step was to combine the data of both wastewaters (SA + SM) and also build a model, which was used to identify bacteria from real hospital wastewater (WW) (also see Fig. 3C).

Surprisingly, this result was more satisfying. The only species with a sensitivity below 60% was *P. aeruginosa*. This was due to the misidentification as *E. faecalis* from the same genus. Each single species was identified correctly with a sensitivity higher than 54.7% and a specificity over 86%.

Both artificial wastewaters on their own were not able to be used as a substitution for a real wastewater sample and to build a training model. But if both wastewaters were taken together, they made it up to a proper training dataset.

4. Conclusion

Since wastewater in general and hospital wastewater in particular do not have a universal composition, but rather its composition is constantly changing, these experiments revolved around the question of whether there is a arificial alternative to a real sample on which bacteria can be cultured and later measured using Raman spectroscopy. Since Raman spectroscopy is a phenotypic method, many parameters, like the nutrient composition of the growth medium can affect the bacteria and the resulting spectra. Therefore, not only the compositions of the various real and artificial effluents were analyzed, but also Raman spectra of the bacteria grown on them. As a first step, representative parameters were measured to characterize different wastewaters. Most of the parameters, which were measured in hospital wastewater and self-made wastewater, match and are comparable.

For the next step, a selection of bacteria was cultivated in different wastewaters and measured by means of Raman spectroscopy. Interestingly, the data of bacteria from each wastewater showed very good

classification, but very poor results when used as a training data set for the identification of bacteria grown on the real wastewater sample.

These results conclude in two main statements regarding to Raman spectroscopy: On the one hand, the analytical results of the effluents are very similar, but the Raman spectra are still very different. On the other hand, it shows that individual training datasets do not necessarily lead to good identification, but when taken together, the rate of correctly identified data increases due to a synergistic effect. Therefore, the analysis must be evaluated with independent data before a statement regarding its quality can be stated. These results are of great interest for future experiments where it is not possible to simulate a cultivation medium because the original sample is constantly changing.

CRediT authorship contribution statement

Christina Wichmann: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Jennifer Dengler:** Formal analysis, Investigation, Visualization, Writing – review & editing. **Marc Hoffmann:** Writing – review & editing, Supervision. **Petra Rösch:** Conceptualization, Writing – review & editing, Supervision, Project administration. **Jürgen Popp:** Writing – review & editing, 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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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