Dynamic laser speckle and fuzzy mathematical morphology applied to studies of chemotaxis towards hydrocarbons

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Abstract: The movement of the microorganisms towards a higher concentration of the chemical attractant is called positive chemotaxis and is involved in the efficiency of chemical degradation. Several studies are focused in this field related to genomics, and towards demonstrating chemotactic responses by bacteria, but there is little information related to the activity and morphology of their response. In this work, we use a recently reported dynamic speckle laser method, to process images and to distinguish motile surface patterns per area of colonisation by applying image processing techniques called fuzzy mathematical morphology (FMM). The images of bacterial colonies are usually surfaced, with vague edges and non-homogeneous grey levels. Hence, conventional image processing methods for shape analysis cannot be applied in these cases. In this paper, we propose the application FMM to solve this problem. The approach given was effective to segment, detect and also to describe colonisation patterns.

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1 Introduction

In the last 40 years, bacterial chemotaxis has been an area of increasing attention for researchers. This interest has been powered by increasing experimental insight into the behaviour of bacteria, both at the population and on the individual scales (Tindall et al., 2008). Bacteria use a well-defined biochemical network to communicate the detected temporal difference of the extracellular environment at the membrane receptors to the flagellar motors (Tindall et al., 2008). A combination of runs and tumbles allow bacteria to explore and respond to environmental changes. Therefore, understanding the behaviour of chemotactic bacterial populations is interesting since bacteria behave independently, but populations exhibit collective behaviour in the form of colonies and biofilms, which can have substantial impact upon industry and medicine (Davey and O'Toole, 2000).

Hence, studying and understanding the morphology and distribution of motility clusters on a nutritive surface would greatly facilitate the prediction of bacterial behaviour in the natural environment.

Chemotaxis is a behavioural response exhibited by flagellated bacteria moving directly towards or away from chemicals concentration gradients in the environment (Pandey and Jain, 2002; Parales and Harwood, 2002). Some authors attribute to the bacterial chemotaxis towards toxic substances an important role in the fate of pollutants in the environment (Parales and Harwood, 2002). Chemotaxis towards hydrocarbons has already been studied in bacteria able to degrade naphthalene (Grimm and Harwood, 1997), and BTEX compounds (benzene, toluene, ethylbenzene and xylenes; Parales et al., 2001). In addition, chemotaxis of an isolated hydrocarbon-degrading bacterium towards a pure alkane has also been reported (Lanfranconi et al., 2003). Some chemotactic *Pseudomonads* have been described with more hydrophobic pollutants, such as high-molecular-mass PAHs (Ortega-Calvo et al., 2003). Van Beilen et al. (2001) mention preliminary evidence of chemotactic response of *Pseudomonas aeruginosa* towards hexadecane. The focus on our work here was to consider the *Pseudomonas sp* isolated in our laboratory (Murialdo et al., 2003) and capable of metabolising chlorophenols (Wolski et al., 2006) for chemotactic morphology studies. These bacteria also showed hydrocarbon degradation and chemotactic responses to these pollutants (Nisenbaum et al., 2013). The exploration for safe and efficient methods to remove environmental pollutants has its major driving force in the search for novel biosurfactant-producing, chemotactic and multiple toxic compounds-degrading microorganisms. This research highlights the importance of selecting microorganisms with specific motile or chemotactic affinities in order to optimise pesticide bioremediation.

The chemotactic response is based on the created gradient because of the metabolisation of the carbon source and the consequent movement of the bacteria (Wolfe and Berg, 1989). Different assays have been developed to show qualitative bacterial chemotaxis in semisolid and liquid mediums (Jin and Hereld, 2009; Murialdo et al., 2009). In all of them, the spatial bacteria accumulation is observed at different times using white light. Parales and Harwood (2002) reported that the presence of chemotactic response produces a sharp chemotactic ring of the bacteria that grows and moves outwards from the inoculums which is visualised in the swarming dish. In these assays, low concentrations of bacteria can hinder the visualisation of the chemotactic bacterial accumulation. We have recently developed a new method for chemotaxis determination, based on dynamic speckle activity segmentation in swim assay (Murialdo et al., 2009). This method allows differentiation of diverse degrees of motility in bacteria. Bacterial growth on hydrocarbons as sole sources of carbon and energy lead to slow bacterial growth and low cell yields (Johnsen et al., 2002). A dynamic speckle laser method has been recently tested by our group (Nisenbaum et al., 2013) with toxic compounds metabolisable by bacteria, but the morphology and the activity of the chemotactic colony were ignored. Therefore, the goal of this work is to distinguish motile surface patterns per area of colonisation by applying image processing techniques, in particular fuzzy mathematical morphology (FMM; Bloch and Maitre, 1995; Bloch, 2005) to the laser speckle chemotaxis images. The images of bacteria colonies are usually textured, have fuzzy edges and non-homogeneous grey levels. Hence, conventional image processing methods for shape analysis cannot be applied in these cases (Serra, 1982, 1988; Gonzalez and Woods, 2002). In this paper, we propose to apply FMM to solve this problem.

2 Experimental

2.1 Bacterial strains and growth media

The strain of *Pseudomonas* sp. used in this study was isolated from soil and capable of degrading chlorophenols (Murialdo et al., 2003; Wolski et al., 2006; Durruty et al., 2011a, 2011b). Microorganisms were stored and grew as detailed in a previews work (Nisenbaum et al., 2013).

2.2 Biomass growth

Biomass growth in presence of hydrocarbons determined by turbidity at OD_{600} with a UV–visible spectrophotometer (Shimadzu, UV1601PC). Culture in MS was supplemented and incubated as explained by Nisenbaum et al. (2013). Strain growth curves were expressed as $OD_{600} f(t)$.

2.3 Chemotactic assays

These assays were done as Nisenbaum et al. (2013) reported. Chemotactic rings were documented photographically by using the 'bucket of light' described by Parkinson (2007). Following this approach, we used a stock culture of *Pseudomonas* sp., which is known to have the ability to degrade HC (Nisenbaum et al., 2013). Cells growing in the in exponential phase were collected and centrifuged for 2 min at 2000 g to separate impurities. After centrifugation at 3000 g for 20 min the microorganisms were resuspended in motility buffer (MB) (0.1 mM EDTA and 10 mM K_3PO_4, pH 7) previously vortexed to achieve good aeration. Tubes with bacteria where incubated at 150 rpm at 20°C with no carbon source for 24 h before the chemotactic assays.

The swim assay was carried out to measure the determinate chemotactic response towards different pure hydrocarbons and mixtures (kerosene). Three microlitres of the previously prepared cells where inoculated in the centre of a Petri dish with agar 0.2% (p/v) in MB, triton-X 0.01% (v/v), and 0.025% (p/v) LB (positive control) or 0.25% (v/v) of hydrocarbons. The negative control was carried out without any carbon source in the agar. Dishes were incubated at 20°C in a wet chamber and were examined every 24 h. Photographs or videos were recorded with a CCD camera, using white light or speckle laser as illumination sources. The biospeckle method was implemented as described by Murialdo et al. (2009). An expanded and attenuated HeNe laser 632.8 nm and 30 mW illuminated the plate under study from the bottom through a ground glass diffuser. A CCD camera connected to a frame grabber registered a sequence of 8 bits images and 768×572 squared pixels, and stored it into the computer. A constant 4-Hz sampling frequency was used, and the camera integration exposure time was set to 40 ms. A pseudo-coloured image was obtained after a processing stage, where the higher energy regions correspond to higher bacterial intensity movement (Sendra et al., 2005).

2.5 Fuzzy mathematical morphology

FMM combines the power of mathematical morphology (MM) with the fuzzy set theory. In order to distinguish motile surface patterns per area in dynamic speckle images (Figure 1(a)), FMM operators were applied.

A grey level image is a function defined on a subset of $\mathbb{Z}^2$ into the natural range $[\![0, L-1]\!]$, where L is a natural number. We need to fuzzify the image to apply the operators of FMM. Therefore, grey level images f can be fuzzified to fit in the [0, 1] range, via the function $g : \{0,1,2,\ldots,255\} \to [0,1]$:

$$g(x) = \frac{x}{255}.$$

It is important to note that this does not mean that the image represents a fuzzy membership function for some object; this is just a way to model the grey level image to be able to apply morphological operators defined via fuzzy set operations. Images can be defuzzified by applying $h : [0,1] \to \{0,1,2,\ldots,255\}$ defined by

$$h(x) = [255.x],$$

where $[.] : \mathbb{R} \to \mathbb{Z}$ represents the function:

$$[a] = \sup\{k \in \mathbb{Z} \,/\, k \le a\}.$$

In what follows, μ and ν denote two fuzzy sets with $\mu : U \subset \mathbb{R}^2 \to [0,1]$ and $\nu : U \subset \mathbb{R}^2 \to [0,1]$ membership functions, where the first is a grey level image and the second is the structuring element. The membership functions are obtained by applying fuzzification function on each of the grey level images. Under these considerations, the definition of the basic operations of the FMM is the following:

Fuzzy morphological dilation of an image μ by a structuring element ν defined by (Bloch and Maitre, 1995):

$$\delta_\nu(\mu)_{(x)} = \sup_{y \in U}\left[t\big(\mu(y), \nu(y-x)\big) \right],$$

where $t(a, b)$ is a t-norm (Dubois and Prade, 1980).

Fuzzy morphological erosion of an image μ by a structuring element ν is defined by (Bloch and Maitre, 1995):

$$\varepsilon_\nu(\mu)_{(x)} = \inf_{y \in U}\left[s\big(\mu(y), c(\nu(y-x))\big) \right],$$

where $s(a, b)$ is a s-norm and $c(a)$ is the fuzzy complement (Klement et al., 2004).

Fuzzy opening and closing are defined in the same way as in the MM. The fuzzy opening of an image μ by a structuring element ν is defined by (Bouchet et al., 2010):

$$\gamma_\nu(\mu) = \delta_\nu\big(\varepsilon_\nu(\mu)\big).$$

The fuzzy closing of an image μ by a structuring element ν is defined by

$$\phi_\nu(\mu) = \varepsilon_\nu\big(\delta_\nu(\mu)\big).$$

The basic filters opening and closing can be combined and applied in successively with an increased structuring element, resulting in Alternating Sequential Filters, formally defined as the Alternating Sequential Filter 'OC' (*opening-closing*) of n iterations:

$$n\nu - \gamma\phi(\mu) = \gamma_{n\nu}\big(\phi_{n\nu}(\ldots\gamma_\nu(\phi_\nu(\mu)))\big).$$

The algorithm to distinguish motile surface patterns per area of colonisation can be summarised as follows:

Step 1: A pre-processing is applied to the original image by Alternating Sequential Filter 'OC' using the operations given by Arithmetic Mean-Based Compensatory Fuzzy Logic (Bouchet et al., 2011; Figure 1(b)).

Step 2: Resulting image of the previous step is binarised with the Otsu method (Otsu, 1979; Figure 1(c)).

Step 3: Area opening and area closing operators are applied to remove any pore (i.e., background connected component) with an area less than a threshold. Connectivity is given by the structuring element (square structuring element 3×3) (Serra, 1988).

Step 4: Alternating Sequential Filter 'OC' for binary image are applied to the image result of the previous step (Serra, 1988; Figure 1(d)).

Step 5: In order to delimit motile surface, the morphological gradient is calculated (Serra, 1988; Figure 1(e)).

Figure 1   (a) Grey level images; (b) alternating sequential filter 'OC'; (c) Otsu binary image; (d) area opening, area closing and alternating sequential filter 'OC' for binary image and (e) gradient image

| (a) | (b) | (c) | (d) | (e) |
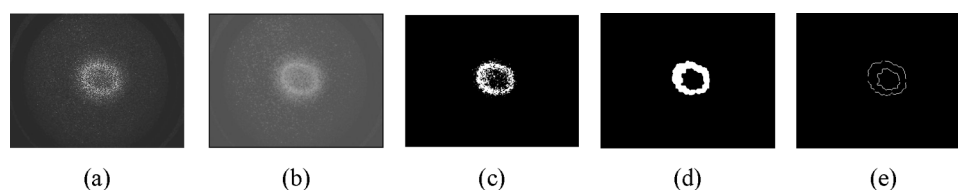
3   Results

Chemotaxis of *Pseudomonas sp* was qualitatively detected in swim assays as the creation of expanding rings of growth on semi-solid agar plates with n-hexadecane, 1-undecene, kerosene and 1-dodecene as a substrate. The swim assays were tested with white light (Figure 2(a)–(d)) and dynamic speckle laser (Figure 2(e)–(h)). The images obtained with dynamic speckle laser were subject to analyses by applying image processing techniques called FMM (Figure 2(i)–(l)). It can be observed that the algorithm locates the area of greatest bacterial activity. Table 1 shows the area of greatest bacterial activity knowing that the area of the complete image is 18.6 cm^2. This resembles the area colonised for the most mobile bacteria. It is also shown that the geometry of growth does not affect the ability of the algorithm to discriminate the desired area.

**Table 1**   Area of greatest activity calculated from Figure 2

Image 1	*Image 2*	*Image 3*	*Image 4*
0.5097 cm^2	0.4478 cm^2	0.3105 cm^2	0.1199 cm^2

Although with white light a spot of high density was seen from the point of inoculation, dynamic speckle laser showed to be effective in showing the same bacterial activity in all

the bacterial spreading (Figure 2). Hence, this spot indicated zones of death or immobile bacteria. All the laser speckle images added data regarding viability and mobility of the microorganisms to the information provided with white light photographs. The speckle images in Figure 2 allowed to detect chemotactic rings not visible with white light (Figure 2(a) and (e), (b) and (f), (g) and (h)). Beyond the point of inoculation using dynamic speckle laser it was possible to detect different zones of variable bacterial activity what was not feasible using white light. This may be related to the low density of mobile bacteria presented in the external area from the inoculation. The dynamic speckle laser image for 1-dodecene showed a remarkable intensity of the rings in the chemotactic area (Figure 2(e)). This is due more to the higher amount of mobile bacteria in the ring than to the successive part of the colony, allowing a discrimination of motility levels inside the colony. On the other hand, the white light images (Figure 2(a)) showed a high density spot in the source of inoculation, which is not visible with dynamic speckle laser (Figure 2(e)), meaning a colony of high density of non-motile or dead microorganisms (Figure 2(a) and (e)), also detected with FMM as a black hole in the centre of the image (Figure 2(i)).

Figure 2   Chemotactic responses of *Pseudomonas sp* in the swim assay method.
(a), (e), (i) 1-dodeceno; (b), (f), (j) hexadecane, (c), (g), (k) kerosene;
(d), (h), (l) 1-undeceno (a)–(d) pictures taken with white light; (e)–(h) original
dynamic laser speckle processed images; (i)–(l) FMM processed images

| (a) | (b) | (c) | (d) |

| (e) | (f) | (g) | (h) |

| (i) | (j) | (k) | (l) |
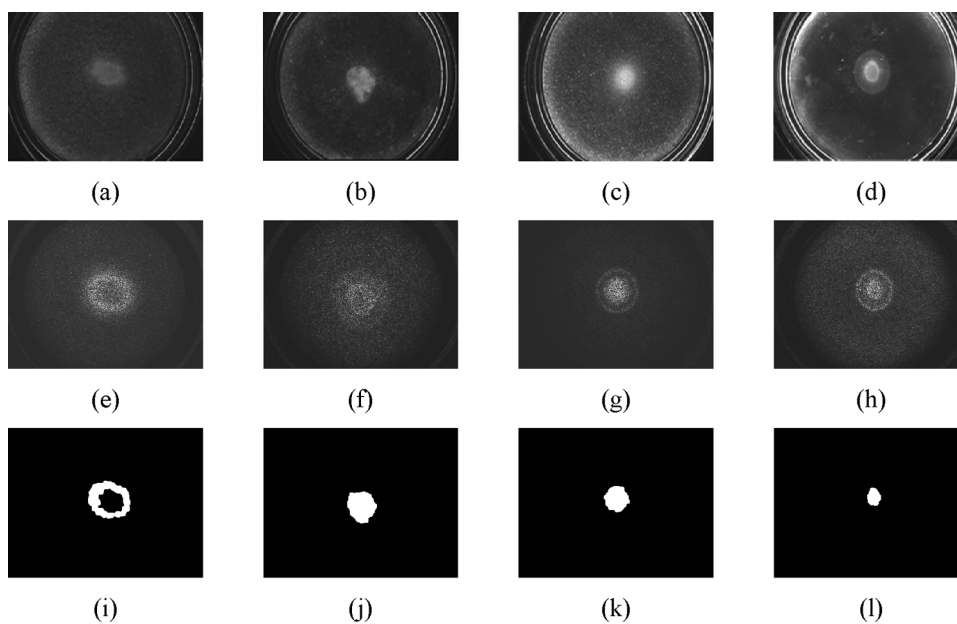
4   Conclusions and discussion

The chemotactic images obtained recently with speckle laser (Murialdo et al., 2009, 2012), were applied in this work for the first time to investigate chemotaxis morphology in presence of non-polar pollutants as attractants. We showed the effectiveness of using two complementary techniques with white light and dynamic speckle laser plus the FFM

analysis for chemotactic morphology studies. The chemotactic bands considered by Ford and Cummings (1992) in *Pseudomonas putida* showed changes in the bacterial density of the bands due to loss of bacteria, which could produce changes in the morphology. Owing to the hydrocarbon toxicity, it is possible that these compounds generate a situation of stress in the microorganisms, and consequently modify their activity and colony morphology. Heipieper et al. (1994) have described lipophilic molecules responsible for the alteration on the structure and fluency of the cell membrane when these molecules are attached to the membrane. In this work, we observed clusters of different motility which could depend on the available substrate for cell growth. Pfeffer (1888) has shown aggregation of chemotactic bacteria in regions of high attractant concentration. It is possible that carbon sources of low solubility or low availability activate the membrane chemoreceptors involved on the chemotactic response towards metabolisable substrates.

Some authors (Alpkvist et al., 2004) described a new mathematical model for chemotactic bacterial colony growth. In this model they studied the morphology of a chemotactic bacterial colony, which grows in the direction of increasing nutrient concentration. They showed results with rich heterogeneous morphology involved in chemotactic responses. Although, the images of bacterial colonies are usually surfaced, they have vague edges and non-homogeneous grey levels. Therefore, standard image processing methods cannot be applied. In this work we have proposed the application of FMM to solve this problem. We distinguished motile surface patterns per area of colonisation by applying image processing techniques in particular FMM. The given approach given was effective to segment, detect and also to describe non-completely spatially random colonisation patterns. The FMM developed algorithms were fast and precise, allowing to analise the pattern spacing and orientation between bacteria groups in order to manipulate biofilms development and thereby control the degradation kinetics.

Also, we could accurately identify the bacterial cluster area of highest motility bacteria clusters. Nutrient uptake was shown to depend on the uptake rate and on the fast or slow swimming cells (Tindall et al., 2008). This stresses the importance of putting more emphasis in studying the morphology of chemotactic response, which could be involved in a faster biofilm formation and the consequent biodegradation. Additional work is being carried out and we expect to extend this technique to the segmentation of different activity levels and thus to simultaneously estimate areas with different density of motile microorganisms.

Acknowledgements

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