Simultaneous Gram and viability staining on activated sludge exposed to erythromycin: 3D CLSM time-lapse imaging of bacterial disintegration

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Abstract

The effect of erythromycin on activated sludge bacteria according to their Gram type was investigated with 3-dimensional Confocal Laser Scanning Microscopy (CLSM) time-lapse imaging. The fluorescent stains SYTOX Green and Texas Red-X conjugate of wheat germ agglutinin stained dying bacteria and Gram + bacteria respectively. Time-lapse imaging allowed an understanding of the staining mechanism and the measurement of the death rate. In presence of erythromycin (10 mg/L), Gram + bacteria had a higher mortality rate than the Gram – bacteria. This result suggested that antibiotics in wastewater could change the activated sludge bacteria composition, according to their Gram type by selecting the bacteria which are the least sensitive to the antibiotics. However bacterial death was followed by bacterial disintegration leading to a decrease in the fluorescence. Results suggested that the viability indicators based on membrane integrity should be used with a correct sampling method, which can give the initial quantity of living bacteria.

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Introduction

After consumption and partial metabolization (Lienert et al., 2007), antibiotics for human use and their metabolites are excreted into wastewater and transported to urban wastewater treatment plants (WWTPs) by sewage. In untreated urban wastewater antibiotics are measured from trace to approximately 2 µg/L. Macrolide antibiotics erythromycin and roxithromycin are among those that are measured in the largest concentrations (Göbel et al., 2005; Kärthikyan and Meyer, 2006; Gulkowska et al., 2008; Xu et al., 2007; Spongberg and Witter, 2008; Lin and Tsai, 2009). In hospital wastewater antibiotic concentrations range from 0.3 µg/L to 87 µg/L and fluoroquinolones are measured with the highest concentrations (Hartmann et al., 1998; Brown et al., 2006). It has to be noticed that in many facilities hospital wastewater is also treated into urban WWTPs without any specific pre-treatment. Runoff from livestock farms and antibiotic use in fish farming are two other sources of contamination (Cole, 2009). For detailed reviews of these topics, see Kümmerser (2001, 2009a,b). Drug manufacture effluents should normally be treated before being discharged into the aquatic environment or sent to an urban WWTP for polishing. They are the most concentrated effluents that WWTPs have to treat. As an example, Larsson et al. (2007) measured a minimal concentration of 28 mg/L of ciprofloxacin in an industrial effluent.

WWTPs were originally designed in order to remove organic pollution and nutrients (nitrogen, phosphorus). In contrast, they are not expected to eliminate the total load of micropollutants. This is particularly true for bioactive compounds such as antibiotics (Joss et al., 2006). Antibiotics are only partially removed, erythromycin being one of the antibiotics with the lowest removal rate (Rosal et al., 2010) and one of the most frequently detected antibiotics in surface and ground water, including in untreated drinking water sources (Focazio et al., 2008; Lin and Tsai, 2009). In addition to this poor removal rate, antibiotics cause two other subjects of concern in WWTPs. Whether WWTPs could be reactors that favour bacterial resistance emergence and stability is currently in discussion (Baquero et al., 2008; Al-Ahmad et al., 2009). Moreover, antibiotics can reduce the capability of WWTP to remove organic pollution (Al-Ahmad et al., 1999; Louvet et al., 2010a) and inhibit nitrification (Halling-Sørensen, 2001). Erythromycin is reported as one of the antibiotics that inhibit the most pollution removal (Avella et al., 2010). This inhibition was measured at µg/L concentration range (Louvet et al., 2010b; Fan et al., 2009). Erythromycin inhibition on nitrification depends on sludge origin (Louvet et al., 2010a) but the reason is still unknown. Therefore there is a need to measure and understand the variation of the time-kill activity of the antibiotics on activated sludge. One hypothesis is that the erythromycin activity on activated sludge depends on the amount of Gram + bacteria. Indeed, macrolides usually exhibit potent activity against Gram-positive bacteria but can also affect Gram-negative bacteria (Brysikier and Butzler, 1997). As cultivable bacteria measured as Colony Forming Unit represent only 1–15% of the total activated sludge bacteria (Wagner et al., 1993), the traditional plate
count method is inadequate to address the count of viable bacteria in activated sludge samples (Amann et al., 1995). On the contrary the fluorescent Viability Kit BacLight™ based on membrane integrity was found to be a valuable tool (Stocks, 2004; Lopez et al., 2005; Louvet et al., 2010b). However this method does not give information about bacterial Gram type. That is why in this study a simultaneous Gram and viability staining protocol was used to observe the fate of activated sludge flocs bacteria in the presence of erythromycin. Compared to Fluorescent In Situ Hybridisation (FISH), the method presented in this paper has the advantage of enabling time-lapse imaging of living bacteria without changing the structure of the flocs (no dehydration, centrifugation or washing step needed). Three-dimensional time-lapse Confocal Laser Scanning Microscopy (CLSM) imaging was conducted during a 9 h exposure time to erythromycin. The results were compared to the behavior of activated sludge in a control microscopic chamber over 12 h. To our knowledge this is the first report of the use of the ViaGram™ Red+ Bacterial Gram Stain and Viability Kit (Molecular Probes, Eugene, OR, USA) on activated sludge bacteria. Combination of this kit with FISH has been shown to be valuable in the assessment of the survival rate of fecal bacteria in drinking water (Savichtcheva et al., 2005). Our results suggest that the ViaGram™ Red+ Bacterial Gram stain could be a useful tool. However bacterial death was followed by bacterial disintegration and we discuss this limitation concerning the viability measurement based on fluorescent membrane indicators.

Materials and methods

Sludge and wastewater

Activated sludge (mixed liquor) and wastewater were collected in the final clarifier recycle line and after grid removal, respectively, on an urban wastewater treatment plant (Nancy-Maxéville, France, 350,000 person-equivalents). The Nancy WWTP uses a hybrid system of activated sludge and biofilm on sand particles. In this plant, the daily average characteristics of the wastewater are as follow: chemical oxygen demand (COD): 260 mg/L; biological oxygen demand (BOD₅): 120 mg/L; Kjeldahl nitrogen (NTK): 30 mg/L; N-NH₄⁺: 18 mg/L. For time-lapse imaging the sludge was maintained for a maximum of 48 h in a batch aerated reactor, filled with 1 L of activated sludge and 2 L of wastewater.

Classical Gram staining

The Gram Hucker staining protocol (RAL, Martillac, France) was used. A smear of bacteria (100 µL) was deposited on a glass slide and thoroughly air dried. It was stained for 1 min in Crystal Violet solution, 1 min in iodine solution, washed for 10 s in ethanol and, finally, counterstained with safranin for 1 min. The glass slide was examined under oil immersion at 250x magnification with direct illumination with a DiaLux 20 (Leitz, Solms, Germany) microscope equipped with a 3CCD color camera Sony 3CCD EXWAVE HAD (Aries, Châtillon, France) and connected to a PC via a Meteor (Matrox, Dorval, Quebec, Canada) grabbing board.

ViaGram™ staining

ViaGram™ Red+ Bacterial Gram Stain and Viability Kit was used according to the manufacturer’s instructions (Molecular Probes, Eugene, OR, USA). This kit provides a fluorescent staining protocol that stains many Gram+ and Gram- bacterial species differentially and, at the same time, discriminates live from dead cells on the basis of plasmic membrane integrity. The kit contains three reagents: two nucleic acid stains for viability determination and a fluorescently labeled wheat germ agglutinin (Texas Red X_CWGA) for Gram-sign determination (red fluorescence for Gram+ bacteria). Bacteria with intact cell membranes stain fluorescent blue with 4,6-diamidino-2-phenylindole (DAPI), whereas bacteria with damaged membranes stain fluorescent green with SYTOX® Green nucleic acid stain. If there is no nucleic acid in the bulk water, the background of the images remains poorly fluorescent. Indeed, when bounding with polynucleotides, DAPI exhibits a considerable fluorescence enhancement (Kapuscinski and Wlodzimierz, 1979). This is also the case for SYTOX® nucleic acid stain according to Roth et al. (1997) and the manufacturer reference method. 4 µL of SYTOX Green and DAPI solution was mixed with 4 µL of Texas Red X_CWGA into 1 mL of tap water (for the erythromycin reactor tap water was spiked with erythromycin to a concentration equal to 20 mg/L). 100 µL of undiluted mixed liquor was mixed with 100 µL of the tap water containing the dyes into a microscope slide well. This short staining protocol allowed direct observation of the original structures of flocs and the time-lapse microscopy. No centrifugation, washing or fixation steps were needed. Microscopic observations started 15 min after staining.

Confocal Laser Scanning Microscopy (CLSM)

Images series were obtained with a FV10i confocal inverted microscope. Three laser sources (405 nm (22 mW), 473 nm (15 mW) and 559 nm (18 mW)), were used to excite DAPI, SYTOX Green and Texas Red X_CWGA. The bandwidths of the detected fluorescence have been optimized for each channel to the maximum emission (400–440 nm for blue channel (DAPI), 440–540 nm for the green channel (SYTOX Green) and 520–620 nm for the red channel (Texas Red X_CWGA)). All regular acquisitions were collected sequentially (405 nm/473 nm/559 nm) to avoid potential cross-talking (Fig. 1). 512 x 512 pixels regular images were obtained with a 60- objective, with a 0.231 µm x 0.231 µm pixel size. The 60- objective lens combined with an optical zoom permitted to grab images with an increased resolution of 0.041 µm/pixel. A constant 1 µm step size in the vertical direction was used. Images were stored as 12 bits/pixel TIFF files and analyzed using the procedures developed with Visilog 6.2 software (Noësits, Saint Aubin, France).

Time-lapse observations were performed with sludge and wastewater sampled in the batch reactor. The first experiment was done with a chamber that contained 10 mg/L erythromycin. The next day another run was performed in a control chamber without erythromycin. The two time-lapse sequences were conducted with exactly the same microscope settings excepted for the number of step size in the vertical direction (114 images per stack for the control chamber versus 126 images per stack for the chamber with erythromycin). The Lab-Tek™ II Chamber Slide™ System was used. These non-fluorescent microscope slides contain 8 chambers (square wells, working volume: 0.2–0.5 mL). The chamber was placed in an incubator with a 37 °C temperature and a humidity level higher than 90%. Water was automatically supplied to the water-immersion inverted objective. During the experiments the scan was continuously running.

Image analysis

Each set of three images (corresponding to the red, green and blue channels, each channel corresponding to the fluorescence of a probe as stated in Table 1) was analyzed according to the following automated procedure:

- The mode of the intensity histogram of each channel image was determined: it corresponds to the average intensity of the background.
- Each channel image was segmented between background and bacteria using an entropy-based method (Hannah et al., 1995) in
the range defined by the mode and 255 (highest possible intensity in an image).

To monitor the background intensity change over time, the distribution of the background pixels according to their intensity was studied in the three color channels.

**Results and discussion**

**Validation of the staining with high resolution observations**

The high resolution images permitted to validate the staining of the bacteria by the probes and check the background fluorescence that could be due to unlinked probes or probes linked with other compounds than bacteria themselves. In activated sludge, bacteria are aggregated into flocs in which the main components are the exopolymeric substances (EPS) that contain proteins, sugars and lipids. Wheat germ agglutinin was used to stain EPS (Strathmann et al., 2002). That is why it was necessary to validate the fact that Texas-Red-Xcwga only stains the Gram+ bacteria. According to the observations, Texas Red-Xcwga targeted bacteria specifically and did not stain EPS (Fig. 2). Due to the motion of bacteria between the three sequential scans, some blurring occurred and it was not possible to verify if Texas Red-Xcwga was linked specifically to membranes. The day of sampling the Nancy WWTP sludge presented numerous Gram+ filamentous bacteria (Fig. 3). The Sludge Volume Index was 205 mL/g. The comparison of confocal images with classical Gram staining images showed that the largest filamentous bacteria that are mainly Gram+ bacteria in Nancy WWTP were

![Fig. 1. Fluorescence and excitation and emission spectra of the three dyes used in the staining.](image1)

![Fig. 2. High resolution 3D maximal projection CLSM image of an activated sludge floc in a control microscopic chamber (Nancy WWTP). Viability and Gram type were assayed using the ViaGram™ Red Gram Stain and Viability Kit. Blue: DAPI, all nucleic acids; green: SYTOX Green, nucleic acids of dying bacteria; red: Texas Red-Xcwga; Gram− bacteria surface.](image2)

![Fig. 3. Classical Gram staining of activated sludge flocs (Nancy WWTP).](image3)

### Table 1: ViaGram™ Red+ Bacterial Gram Stain and Viability Kit mode mechanism.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Target</th>
<th>Fluorescence color</th>
</tr>
</thead>
<tbody>
<tr>
<td>4′,6-Diamino-2-phenylindole (DAPI)</td>
<td>Nucleic acids (all bacteria)</td>
<td>Blue</td>
</tr>
<tr>
<td>SYTOX Green</td>
<td>Nucleic acids (permeabilized bacteria)</td>
<td>Green</td>
</tr>
<tr>
<td>Texas Red-X conjugate of wheat germ agglutinin</td>
<td>Gram+ bacteria surface</td>
<td>Red</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Gram+</th>
<th>Gram−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live bacteria</td>
<td>Blue</td>
<td>Blue + red</td>
</tr>
<tr>
<td>Dying bacteria</td>
<td>Blue + green</td>
<td>Blue + green + red</td>
</tr>
<tr>
<td>Dead bacteria</td>
<td>Blue + green or no fluorescence</td>
<td>Blue + green + red or no fluorescence</td>
</tr>
</tbody>
</table>
stained by Texas Red-Xcwgsa, confirming the validity of the staining (Figs. 3, 4B, and 5A,B).

Lower magnification was also interesting to observe heterogeneity into the distribution of activated sludge bacteria. In addition to areas where Gram+ and Gram− bacteria were thoroughly mixed other areas were observed where Gram+ bacteria were strongly clustered such as in the center of Fig. 4A. Such observations are difficult with classical Gram staining where Gram+ and Gram− bacteria are superimposed into dense flocs and are difficult to discriminate (Pandolfi and Pons, 2004). In the presence of 10 mg/L erythromycin there were more bacteria stained by SYTOX Green than in control. This was an indication of the validity of the SYTOX-Green staining to mark bacteria with damaged membrane. 10 mg/L erythromycin concentration has previously been shown to cause the death of part of heterotrophic and autotrophic bacteria of the Nancy WWTP sludge (Louvet et al., 2010a). It was even possible to zoom on zones where there were no more bacteria stained only with DAPI and where green fluorescence was strongly predominant (Fig. 4B). Those results are in agreement with Roth et al. (1997) that monitored the Escherichia coli susceptibility to some antibiotics using SYTOX Green. In contrast, Lebaron et al. (1998) showed that the SYTOX Green staining of starved population of E. coli and Salmonella typhimurium underestimates the fraction of dead cells and that its application to natural samples should be considered with caution. In order to monitor more closely the behavior of the dye, time-lapse observations combined with automated image analysis quantification were performed.

**Time lapse observations**

Time-lapse observations lasted for 12.5 h in the control microscopic chamber and for 9.5 h in the microscope containing erythromycin. This time-lapse protocol allowed an accurate measurement of changes in the fluorescence of probes. Indeed, this protocol significantly reduces the error due to sampling, as changes into the same bacteria population could be observed. In the microscopic chamber containing 10 mg/L erythromycin the bacterial population observed during the 9 h of observation remained the same. In the control microscopic chamber some additional bacteria entered the field of vision and led to a small increase in the fluorescence between the second and third hour of the experiment (Fig. 7). The bacterial population observed during the 9.5 h that followed (t = 3 h to t = 12.5 h) remained the same. The dynamics with respect to time using the ViaGram staining were quantified by the total number of bacteria (DAPI stain), the number of dying bacteria (SYTOX Green stain) and the proportion of Gram+ bacteria (Texas Red stain). Results showed that after death the bacteria were disintegrated. A decrease in the blue, the red and the green fluorescence was measured in both chambers. The decrease in the blue fluorescence in the chamber that contained erythromycin was faster than in the control which showed the effect of the antibiotic. In the erythromycin chamber the same bacteria were observed during the 9.3 h. Another evidence of the bacteria disintegration was the increase in the background fluorescence. The blue and green background fluorescence increases during the first 15 min of contact with erythromycin (Fig. 6A and B). DAPI and SYTOX Green both stained dying bacteria but there was a leakage of nucleic acids after the loss of membrane integrity (Table 2).

The level of SYTOX Green fluorescence was the result of two opposite processes: SYTOX Green fluorescence increased when bacteria were dying but it decreased when those bacteria were disintegrated. As a consequence, the level of SYTOX Green fluorescence depends on the rates of the reactions. As an example, the relative stability in the SYTOX Green fluorescence between 2 and 9 h in the erythromycin chamber was the result of the balance between death and disintegration (Fig. 7). This phenomenon has also been discussed by Savichtcheva et al. (2005). Our results confirm Lebaron et al.’s (1998) conclusion: the calculation of the number of dead bacteria only based on the fluorescence of SYTOX Green would lead to an underestimation. This is particularly true in the case of starved population where the hydrolysis of dead bacteria could occur due to the lack of substrate. During the same period there was also a decrease in the DAPI fluorescence in the erythromycin chamber when DAPI fluorescence was stable in the control microscopic chamber. The decrease in the blue fluorescence is a marker of the disappearance of the cells. A second evidence of these opposite reactions was the small increase in the SYTOX Green fluorescence intensity measured in the control chamber between...
Fig. 5. (A) 3D maximal projection CLSM image in the control microscopic chamber 15 min after staining, (B) control microscopic chamber 9 h 30 min after staining (the white arrow indicate a zone stained by SYTOX Green where the fluorescence decreased with time), (C) microscopic chamber with 10 mg/L erythromycin 15 min after staining, (D) Microscopic chamber with 10 mg/L erythromycin 9 h 30 min after staining. Viability and Gram type were assayed using the ViaGram™ Red+ Bacterial Gram Stain and Viability Kit.

Table 2
Results of image analysis at the beginning of the experiments and after 9.30 h for control microscopic chamber and chamber that contained 10 mg/L erythromycin.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th></th>
<th>Evolution (%)</th>
<th>10 mg/L erythromycin</th>
<th></th>
<th>Evolution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t = 15 min</td>
<td>t = 9 h 30 min</td>
<td></td>
<td>t = 15 min</td>
<td>t = 9 h 30 min</td>
<td></td>
</tr>
<tr>
<td>DAPI (fluorescence intensity (10^3 a.u.))</td>
<td>2739</td>
<td>2135</td>
<td>−22</td>
<td>4114</td>
<td>1949</td>
<td>−53</td>
</tr>
<tr>
<td>DAPI (10^3 pixels)</td>
<td>35</td>
<td>29</td>
<td>−15</td>
<td>43</td>
<td>21</td>
<td>−50</td>
</tr>
<tr>
<td>CytoX Green (fluorescence intensity (10^3 a.u.))</td>
<td>207</td>
<td>78</td>
<td>−62</td>
<td>358</td>
<td>80</td>
<td>−78</td>
</tr>
<tr>
<td>CytoX Green (10^3 pixels)</td>
<td>7</td>
<td>5</td>
<td>−36</td>
<td>7</td>
<td>2</td>
<td>−66</td>
</tr>
<tr>
<td>Texas Red-X (fluorescence intensity (10^3 a.u.))</td>
<td>594</td>
<td>157</td>
<td>−74</td>
<td>2525</td>
<td>519</td>
<td>−79</td>
</tr>
<tr>
<td>Texas Red-X (10^3 pixels)</td>
<td>15</td>
<td>6</td>
<td>−63</td>
<td>25</td>
<td>7</td>
<td>−71</td>
</tr>
</tbody>
</table>

8 and 12 h when, at the same time the number of green pixels increased drastically. That can be explained by a decrease in the fluorescence of the highly fluorescent green pixels when at the same time there were an increase in the number of dying bacteria. When lysis of damaged bacteria happens, the fluorescence of activated sludge flocs decreases immediately because some probes are released into the water. On the contrary, the number of detected green pixels does not decrease immediately. Indeed, even with
lower fluorescence intensity those pixels still have a fluorescence intensity higher than the threshold value. As an illustration, the white arrow indicates a bright fluorescent green zone at the beginning of the observation (Fig. 5A) and the same green zone at the end of the experiment (Fig. 5B). In this zone, the same number of pixels was detected during all the experiment but the green fluorescence intensity decreased. This explains why during the same period (t = 2 h to t = 9 h) the number of detected pixels increased more than the fluorescence intensity in both the control and the chamber with erythromycin (Figs. 6 and 7).

The antibiotic concentration used in the current work was 10 mg/L. Louvet et al. (2010a) reported that the erythromycin toxicity on activated sludge depends on the date of sampling of the mixed liquor and on the origin of the sludge. However, the authors reported that a 10 mg/L erythromycin concentration provides a significant inhibition whatever the sampling date or the origin of the sludge. This concentration might seem large with respect to the levels reported in urban wastewater. However, this concentration is in the same order of magnitude as antibiotic concentrations in drug manufacture effluents. The effect of erythromycin on bacteria has been showed at the μg/L concentration level (Fan et al., 2009; Louvet et al., 2010b). However this was with exposure times that are longer than the possible duration of CLSM time-lapse imaging experiments. At the beginning of the experiments, the general appearance of activated sludge flocs was the same in both chambers. Activated sludge floc diameters were about 20 μm. Flocs were linked together by Gram+ filamentous bacteria (Fig. 5A and C). This general appearance was in agreement with the classical Gram staining observation (Fig. 3). The percentages of dying bacteria in the control and the erythromycin chambers were also similar (21 and 17% respectively). In contrast, the percentage of Gram+ bacteria was sensibly lower in the control microscopic chamber (44% versus 58%) (Gram+ (%) = red pixels/blue pixels × 100). This difference could be explained by the heterogeneity of the distribution of Gram+ bacteria in the sludge (Fig. 4A) and the limitation of the observation zone. The main difference concerning the evolution of the staining was the drastic decrease in blue fluorescence in the erythromycin chamber (~53%) whereas in the control chamber it was moderate (~22%). In the control chamber, the death of bacteria was mainly measured after the first 5 h of imaging and this decay may be due to the lack of substrate and to the laboratory conditions. During the first 3 h, the death rate of the Gram+ bacteria was higher than the rate of the Gram− bacteria in both chambers (Fig. 9). During the second part of the experiment in the control chamber the death rate of Gram+ bacteria was lower than the death rate of the Gram− bacteria. In contrast the death rate of Gram+ bacteria continued to be higher than the death rate of Gram− bacteria in the erythromycin
chamber. This result is in agreement with the erythromycin spectrum of action (Bryskier and Butzler, 1997). This result suggests that antibiotics in the wastewater of pharmaceutical industries could influence the activated sludge bacteria composition by selecting the least sensitive bacteria. More investigations have to be done with lower antibiotic concentrations and with genetic tools to show if the antibiotics in urban wastewater could influence the composition of activated sludge.

Fig. 8. Evolution of surface of flocs during CLSM 3d time-lapse imaging. ▲ SYTOX Green, DAPI. Texas Red-Xcrga. (A) Control microscopic chamber and (B) microscopic chamber with 10 mg/L erythromycin.

Conclusion

The fluorescent stain Texas Red-X conjugate of wheat germ agglutinin stained Gram+ bacteria and its combination with the DAPI stain and CLSM permitted a good quality imaging of complex microbial aggregates such as activated sludge flocs. SYTOX Green stained dying bacteria. However, as the bacteria lysis and disintegration lead to a decrease in their fluorescence, the evolution of the DAPI stain whose fluorescence only decreases gave results more easily explainable than the SYTOX Green fluorescence. Indeed dying bacteria made the SYTOX Green fluorescence increase but bacterial disintegration made the SYTOX Green fluorescence decrease. Therefore bacterial viability should be preferentially based on the decrease of the fluorescence of one probe and the sampling method is important so as to know the initial quantity of living bacteria. In this study, exposure to erythromycin (10 mg/L) changed the activated sludge bacteria population because the Gram+ bacteria had a higher death rate than the Gram- bacteria. This result suggests that antibiotics in wastewater can change the composition of the activated sludge bacteria according to their Gram type by selecting the bacteria which are the least sensitive to the antibiotics.

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