Proteomics reliability for micropollutants degradation insight into activated sludge systems
Gianluigi Buttiglieri, Neus Collado, Nuria Casas, Joaquim Comas and Ignasi Rodriguez-Roda

ABSTRACT
Little information is available on pharmaceutical trace compounds degradation pathways in wastewater. The potential of the proteomics approach has been evaluated to extract information on activated sludge microbial metabolism in degrading a trace concentration of a pharmaceutical compound (ibuprofen). Ibuprofen is one of the most consumed pharmaceuticals, measured in wastewater at very high concentrations and, despite its high removal rates, found in different environmental compartments. Aerated and completely mixed activated sludge batch tests were spiked with ibuprofen at 10 and 1,000 μgL⁻¹. Ibuprofen concentrations were determined in the liquid phase: 100% removal was observed and the kinetics were estimated. The solid phase was sampled for proteomics purposes. The first objective was to apply proteomics to evaluate protein profile variations in a complex matrix such as activated sludge. The second objective was to determine, at different ibuprofen concentrations, which proteins followed pre-defined trends. No newly expressed proteins were found. Nonetheless, the obtained results suggest that proteomics itself is a promising methodology to be applied in this field. Statistical and comparative studies analyses provided, in fact, useful information on biological reproducibility and permitted us to detect 62 proteins following coherent and plausible expected trends in terms of presence and intensity change.

Key words | biodegradation, differential in gel electrophoresis (DIGE), ibuprofen, pharmaceuticals, protein profiles

INTRODUCTION
Large amounts of micropollutants, among them, pharmaceutical active compounds (PhACs), are administered in large quantities to humans or animals and their residues continuously discharge into the sewer system. If not fully metabolized in wastewater treatment plants (WWTPs), which are nowadays not designed to remove them completely, they can arrive in the environment (Barceló & Petrovic 2007). The real effect of PhACs traces on ecosystems and human health, directly via drinking water and/or indirectly via the food chain, is not yet completely elucidated and no legislation is yet present. Nonetheless PhACs can have deleterious effects on aquatic organisms (Santos et al. 2010) and it is essential to further understand their fate and increase their removal rates. In case of reasonably polar compounds, e.g. most PhACs, with a low affinity for adsorption to sludge, a significant percentage of removal relies on biodegradation performed by microbial communities (Carballa et al. 2004). Nonetheless, little information is available and further research is required to entirely define PhAC degradation pathways within WWTP facilities.

The target compound, ibuprofen, is one of the most used active principal ingredients in pharmaceutical formulations with an estimated annual consumption in developed countries of several thousands of tons (Buser et al. 1999; Carballa et al. 2008). It is highly biodegradable in aerobic conditions in WWTPs and with literature references for biodegradation reaction rates (Joss et al. 2006; Collado et al. 2013b). Despite this, it can still be found in secondary effluents at relevant concentrations (3.6 μgL⁻¹ average; Verlicchi et al. 2012).

Activated sludge is a very dynamic and also chaotic system. Changes may happen to populations for several reasons and it is difficult to interpret and follow clear patterns. Usually, activated sludge systems are controlled with parameters such as sludge age, sludge loading, organic removal, etc.
Proteomics is a technique based on the study of microbial community proteins. Its central assumption is that the fingerprints of expressed proteins are specific to the conditions experienced by the organisms. Its goal is to observe differences or changes in protein expression levels in biological processes (Lopez 1999). The high potential of proteomics has been successfully demonstrated in pure strains and also, in very few cases, on activated sludge systems to find and follow target proteins (Collado et al. 2013) or on phosphorous removal in conventional wastewater treatment systems (Wilmes et al. 2008).

To the authors’ knowledge, no information is available on proteomics applied to PhAC trace biodegradation in activated sludge. Protein identification from a complex matrix like activated sludge remains a big challenge in particular for the biodegradation of organic compounds at very low concentrations (e.g., trace PhACs) even more so if a complete target removal pathway (biological and/or chemical) is missing.

No proteins clearly attributed to any PhAC degradation route are yet known in activated sludge systems. The expression of many proteins is supposed to be loading dependent (Thompson et al. 2007). A higher protein expression can be induced by a higher substrate concentration (mg L⁻¹) leading, in theory, to easier protein detection and identification. This approach requires, nevertheless, a confirmation for in situ concentrations: at mg L⁻¹ level organic compounds may serve as primary substrate by inducing the expression of targeted proteins. This is not the case at μg L⁻¹ or lower concentration: completely different degradation pathways may be relevant in situ and experiments have to be repeated at lower content.

Moreover, in some cases degradation may happen co-metabolically, i.e. because the compounds are transformed by proteins expressed for other purposes (Cao & Loh 2009). In other cases, for example under starvation conditions, microorganisms may express housekeeping proteins, i.e. broad range proteins occasionally degrading other compounds. Hence, the probability of differentially finding and discriminating expressed proteins owing to micropollutant degradation against the proteins from sludge itself represents a challenge. Proteomic analyses are anyway expected to deliver more data on the overall activity of microbial communities and complement information on biomass activity.

The general aim of this work is to test proteomics reliability when dealing with activated sludge samples spiked with a pharmaceutical compound (ibuprofen), to identify which proteins are relevant to be considered for micropollutant degradation. Protein patterns of sludge at higher pharmaceutical load (1,000 μg L⁻¹) and at a lower and environmentally relevant load (10 μg L⁻¹) were compared and investigated. Afterwards, coherent and plausible trends over time of proteins were defined (related to their presence and changes in their intensity levels when degrading ibuprofen), and the proteins following these pre-established trends were sought.

**MATERIALS AND METHODS**

**Batch tests**

A set of laboratory biodegradation batch tests were performed with activated sludge from a municipal WWTP. Sets of 2 L glass bottles (in duplicate), were permanently aerated and automatically and continuously stirred. Experiments were run at room temperature (20 ± 2 °C) and with constant oxygen concentration, around 7.5 mg O₂ L⁻¹, so that dissolved oxygen was not a limiting factor. The suspended solid content was 1,000 mg total suspended solids (TSS) L⁻¹ with two ibuprofen concentrations (10 and 1,000 μg L⁻¹) investigated. Ibuprofen was provided by Sigma-Aldrich (St Louis, MO, USA). The sludge was taken from a conventional WWTP (Castell Platja d'Aro, NE Spain 35,000 m³ day⁻¹, 175,000 I.E.) with average solids concentration of around 1–2 g TSS L⁻¹. This sludge was aerated for a couple of hours before its use to minimize the amount of rapidly degradable organic matter. Sludge was further diluted to the appropriate final suspended solid concentration for each batch study. Samples were taken for ibuprofen analyses (liquid phase) and for differential in gel electrophoresis (solid phase).

**Analysis of ibuprofen**

Ten millilitres of aqueous samples, at times of 0, 60, 180, 300 and 1,440 minutes, were filtered through 0.45 μm glass fibre filters and kept in 20 mL glass vials with 1 mL of p-formaldehyde solution at 37% v/v to avoid any further degradation during its conservation period. All the samples were well homogenized and frozen at –20 °C until their analysis. Ibuprofen was analysed using an ultra performance liquid chromatography (UPLC) system (Waters Corp., Milford, MA, USA) coupled to a triple quadrupole–linear ion trap mass spectrometer (5500 QTRAP, Applied Biosystems, Foster City, CA, USA) working in negative ionization mode in multiple reaction monitoring mode. More details on analytical methodology can be found in Ferrando-Climent et al. (2012).
Differential in gel electrophoresis analyses

In this study, 100 mL volumes of activated sludge were sampled at times 0 (before spiking with ibuprofen and considered as a reference condition), 30, 60 and 300 minutes for both duplicate batch tests. After centrifugation (at 10,000 rpm for 10 minutes), the supernatant was discarded and the pellets were conserved at –80 °C. The solid phase of the samples was analysed for proteomics purposes, and differential in gel electrophoresis (DIGE) analyses was run. DIGE is a form of gel electrophoresis where up to three different protein samples can be labelled with fluorescent dyes prior to two-dimensional (2-D) electrophoresis (Alban et al. 2003). As commonly applied to experiments comprising several gels, an internal standard was prepared as a pool of all the samples (using Cy2 as a marker, Table 1) and included in each gel. This allows the measurement of the abundance of a protein in each sample relative to the internal standard. After electrophoresis, the gel is scanned with the excitation wavelength of each dye one after the other, so that each sample can be seen individually. More details can be found in Bech-Serra et al. (2006).

Table 1 | Gel composition: each gel is composed of two samples (marked differently with Cy3 or Cy5) and the internal standard samples (present in any gel and marked with μCy2)

<table>
<thead>
<tr>
<th>Gel</th>
<th>Sample</th>
<th>Time</th>
<th>Dye</th>
<th>μL/gel</th>
<th>μL dye</th>
<th>μL lysine</th>
<th>Total volume (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 μg L⁻¹</td>
<td>0' – 1st</td>
<td>Cye3</td>
<td>6.04</td>
<td>1</td>
<td>1</td>
<td>8.04</td>
</tr>
<tr>
<td>2</td>
<td>1,000 μg L⁻¹</td>
<td>0' – 1st</td>
<td>Cye5</td>
<td>5.28</td>
<td>1</td>
<td>1</td>
<td>7.28</td>
</tr>
<tr>
<td>3</td>
<td>10 μg L⁻¹</td>
<td>0' – 2nd</td>
<td>Cye3</td>
<td>6.04</td>
<td>1</td>
<td>1</td>
<td>8.04</td>
</tr>
<tr>
<td>4</td>
<td>1,000 μg L⁻¹</td>
<td>0' – 2nd</td>
<td>Cye5</td>
<td>6.27</td>
<td>1</td>
<td>1</td>
<td>8.27</td>
</tr>
<tr>
<td>5</td>
<td>10 μg L⁻¹</td>
<td>30' – 1st</td>
<td>Cye3</td>
<td>5.54</td>
<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>6</td>
<td>1,000 μg L⁻¹</td>
<td>30' – 1st</td>
<td>Cye5</td>
<td>10.32</td>
<td>1</td>
<td>1</td>
<td>12.32</td>
</tr>
<tr>
<td>7</td>
<td>10 μg L⁻¹</td>
<td>30' – 2nd</td>
<td>Cye3</td>
<td>15.24</td>
<td>1</td>
<td>1</td>
<td>17.24</td>
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<tr>
<td>8</td>
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<td>30' – 2nd</td>
<td>Cye5</td>
<td>4.06</td>
<td>1</td>
<td>1</td>
<td>6.06</td>
</tr>
<tr>
<td>9</td>
<td>10 μg L⁻¹</td>
<td>60' – 1st</td>
<td>Cye3</td>
<td>7.62</td>
<td>1</td>
<td>1</td>
<td>9.62</td>
</tr>
<tr>
<td>10</td>
<td>1,000 μg L⁻¹</td>
<td>60' – 1st</td>
<td>Cye5</td>
<td>5.12</td>
<td>1</td>
<td>1</td>
<td>7.12</td>
</tr>
<tr>
<td>11</td>
<td>10 μg L⁻¹</td>
<td>60' – 2nd</td>
<td>Cye3</td>
<td>5.50</td>
<td>1</td>
<td>1</td>
<td>7.50</td>
</tr>
<tr>
<td>12</td>
<td>1,000 μg L⁻¹</td>
<td>60' – 2nd</td>
<td>Cye5</td>
<td>7.45</td>
<td>1</td>
<td>1</td>
<td>9.45</td>
</tr>
<tr>
<td>13</td>
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<td>300' – 1st</td>
<td>Cye3</td>
<td>11.18</td>
<td>1</td>
<td>1</td>
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<tr>
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<td>Cye5</td>
<td>4.47</td>
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<td>300' – 2nd</td>
<td>Cye3</td>
<td>5.41</td>
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<td>1</td>
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</tr>
<tr>
<td>16</td>
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<td>300' – 2nd</td>
<td>Cye5</td>
<td>4.19</td>
<td>1</td>
<td>1</td>
<td>6.19</td>
</tr>
</tbody>
</table>

Each experiment was done in duplicate (indicated in the table as ‘1st and 2nd’).

Protein expression and statistical analysis and software

After scanning, the gels were analysed with the Progenesis Samespots V4.3 Software (Nonlinear Dynamics, Newcastle upon Tyne, UK) for normalization of the gels and statistical analysis (for protein expression and abundance studies). Accurate gel alignment accounted for gel distortions and positioned all spots in exactly the same location. Spot detection produced a complete data set because all gels contained the same number of spots, with each of the spots matching on all gels. The automatic analysis consequently, led to 100% matching and no missing values (i.e. not detected or matched incorrectly) and this allowed the application of valid multivariate statistical analysis. Among the tests included were principal component analysis (PCA) and power analysis.

Differences between the two concentrations and the replicates were validated by PCA: this measured how the data clustered within the experiments; gel data can be plotted in a bi-dimensional graph determining whether samples had the grouping expected (if the spots appear close to each other they are likely to have similar expression profiles) or if there were any outliers in the data. Differences among matched spot intensities were statistically validated by performing an analysis of variance (at a 5% significance level) ascertained by a normal distribution. A fold of at least 1.5 was applied as a threshold to consider two proteins as significantly different in terms of expression. The power analysis indicates whether enough replicates have been run for valid results. This depends on the sample size and can calculate the effect of running a different number of replicates. The power threshold was 80% as generally accepted.

The results were presented in interactive dendrogram trees, where similar expression profiles cluster together, according to how strongly correlated the spots were: highly correlated clusters appeared nearer the bottom of the dendrogram.

RESULTS AND DISCUSSION

Ibuprofen removal and reaction rate constants

The removal efficiencies obtained from the set of experimental combinations, at different initial ibuprofen concentrations, are presented in Figure 1. A complete removal of ibuprofen was observed for both cases in 24 hours. Higher removal rates were obtained at lower ibuprofen concentration (50% removal after 1.5 hours for 10 μg L⁻¹ and around 3 hours for
1,000 μg L⁻¹). Pseudo-first-order kinetics was applied and the obtained ibuprofen degradation rate values were 17.44 and 4.76 L g TSS⁻¹ day⁻¹, for 10 and 1,000 μg L⁻¹, respectively. More data on removal and kinetics can be found elsewhere (Collado et al. 2013b).

**Statistical analysis and comparisons between different ibuprofen concentrations**

PCA was performed on each spot belonging to every 2-D gel, to achieve information on spot variability and how they clustered considering replicates and tested conditions. The spots were, as expected, widely dispersed (Figure 2) because they represented a high number of proteins (housekeeping or expressed for any other reason, including ibuprofen degradation). At each test condition (same sampling time and same ibuprofen concentration), the spots belonging to the same protein in 2-D gels, but relative to different duplicates, were expected to be close to one another. In some cases, such spots (e.g., at 10 μg L⁻¹ – t = 0 and 1,000 μg L⁻¹ – t = 30’, Figure 2) were significantly different. In many other cases (e.g., the few coloured spots not marked in Figure 2), they were much more homogenous and similar. However, it is apparent that working with duplicates can lead to information which can sometimes be difficult to generalize.

As a second step, the 2-D gel spots belonging to the same sampling time (but at different ibuprofen concentration) were compared, also taking into account the duplicates. Some proteins, significantly different for the two spiked concentrations, were found at each of the sampling times (Figure 3). These proteins were few compared to the total number of around 1,500 spots. Furthermore, none of them appeared to be present and significantly different at more than a sampling time. The intersections of the Venn diagrams were always empty and, consequently, these proteins can be considered unrelated to ibuprofen degradation (running for more than two sampling times).

No significant difference was expected, in terms of number, because the only presumed difference between the systems was ibuprofen concentration. Conversely, the proteins eventually found to be significantly different could have been linked to diverse ibuprofen degradation pathways (e.g., co-metabolism vs. induced degradation). Proteomics has been occasionally applied in activated sludge systems to follow target proteins (Collado et al. 2013a) or directly to degradation (Wilmes et al. 2008) but not clearly attributed to pharmaceutical trace degradation. Nonetheless, up-regulation of proteins in the presence of low levels of ibuprofen has been found for some identified proteins in pure cultures from a bacterial strain, isolated from activated sludge (Almeida et al. 2013). The details of such pathways and how they are regulated remain largely unknown.

**Time course comparisons and defined intensity trends**

No new proteins were found; to get more information on the system (and potentially on pharmaceutical degradation), it was necessary to distinguish the candidate protein/s from the others, based on the assumption that some of the already

![Figure 2](https://iwaponline.com/wst/article-pdf/72/6/882/466287/wst072060882.pdf)
present proteins changed their pattern after ibuprofen dosage. Taking into account each of the two ibuprofen concentrations separately, the protein expressions were compared between the sampling times, two-by-two, to highlight all the significant expression dissimilarities over time. The aim of this step was to determine all the proteins that can be linked to any change in the biological activity. As an example in Figure 4, a protein is presented increasing its expression in the proteome after 30 minutes since the ibuprofen spike, coherent with ibuprofen degradation and in agreement with Almeida et al. (2013). Spot excision and identification would be necessary to check protein function and to correlate them to pharmaceutical degradation. Even so, the applied protocol allowed highlighting of all the potential proteins to be considered for further analysis.

A comparison between the intensities of the spots for each sampling time combination was performed and the obtained results are presented in Table 2. A relatively small number of proteins significantly modified their expression, indicating a general constancy of the proteome (as expected, with the ibuprofen spike and degradation being the only difference in the system). The trends during the whole duration of the found proteins were checked singularly. Those that did not follow a logical trend coherent with our hypotheses were not considered of interest, e.g., decreasing with time while degradation occurred. Ibuprofen is reported to have some chronic effects on vertebrate and invertebrates (Hayashi et al. 2008) but not a high toxic impact vs. bacteria (e.g., Vibrio fischeri) with EC₅₀ (toxic effects corresponding to 50% affectivity) ranging from 10 to 40 mg L⁻¹ (Farré et al. 2001; Ra et al. 2008). Toxicity can probably be disregarded in this context, in particular at 10 µg L⁻¹. Some of the excluded proteins might be linked to unexpected behaviour owing to ibuprofen but, most likely, not to ibuprofen biodegradation.

Finally, two specific protein intensity trends were defined:

- increasing from time 0’–30’ and then stable until the last sampling time (300’);
- increasing from time 0’–30’, then stable until 60’ and finally decreasing at 300’.

These two trends, among those possible, were coherent with the degradation of ibuprofen and in agreement with Almeida et al. (2013). The corresponding clusters were searched by means of dendrogram (Figure 5) and successfully found. A total of 28 and 34 proteins related to the first and second defined trend, respectively, were identified and used as candidates for further identification.

It was confirmed that the applied protocol is valuable even when working with activated sludge, handling high numbers of proteins and microorganisms. Moreover, the
sensitivity was high enough to differentiate protein intensity over time.

CONCLUSIONS

Proteomics reliability dealing with activated sludge spiked with a pharmaceutical compound (ibuprofen) was investigated over time and at different concentrations. Aerated and completely mixed activated sludge batch tests were spiked at two ibuprofen concentrations (10 and 1,000 μg L⁻¹/C⁰). A complete removal was observed. The obtained degradation rates were 17.44 and 4.76 L g TSS⁻¹/C⁰ day⁻¹, for 10 and 1,000 μg L⁻¹/C⁰, respectively. Proteins expressions were compared between the two ibuprofen concentrations at each sampling time (few significantly different proteins were found: 40 at time 0, 8 at time 30', 10 at time 60' and 8 at time 300') and during the time course at each concentration separately. Statistical analyses provided useful information on biological reproducibility and replicate necessity. No newly expressed proteins were found. Nonetheless, the applied statistical and comparative study allowed the detection of 62 proteins following two pre-defined coherent and plausible expected trends in terms of presence and up-regulation intensity change. The obtained results suggested that proteomics itself is a promising methodology even if more effort is required in such complex matrixes.

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Figure 5 | On the top: the dendrogram for 10 μg L⁻¹, with the defined clusters highlighted by means of colour; on the bottom, the corresponding time course of the proteins belonging to these two clusters.
REFERENCES


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