

Exposure to Vancomycin causes a shift in the microbial community structure without affecting nitrate reduction rates in river sediments

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Abstract

Antibiotics and antibiotic resistance genes have shown to be omnipresent in the environment. In this study we investigated the effect of Vancomycin (VA) on denitrifying bacteria in river sediments of a Waste Water Treatment Plant (WWTP), receiving both domestic and hospital waste. We exposed these sediments continuously in flow-through reactors to different VA concentrations under denitrifying conditions (nitrate addition, anoxia) in order to determine potential nitrate reduction rates and changes in sedimentary microbial community structures. The presence of VA had no effect on sedimentary nitrate reduction rates at environmental concentrations, whereas a change in bacterial (*16S rDNA*) and denitrifying (*nosZ*) community structures was observed (determined by PCR-DGGE). The bacterial and denitrifying community structure within the sediment changed upon VA exposure indicating a selection of a non-susceptible VA population.

Key words: nitrate reduction; Vancomycin; community structure; *nosZ*; bacterial antibiotic resistance; flow-through reactors.

16 ***Introduction***

18 The use of pharmaceuticals, their disposal and consequent presence in the environment has
20 raised concern regarding its ecological impact (Santos et al., 2010). Among these pharmaceuticals,
22 antibiotics are of special interest as the intensive use in human and veterinary medicine has resulted
24 in the emergence of bacterial antibiotic resistance, nowadays considered as a worldwide public
health problem. The environmental occurrence of antibiotics ranging from ng L^{-1} in surface water
to $\mu\text{g L}^{-1}$ in Waste Water Treatment Plant (WWTP) and hospital effluents, and up to mg L^{-1} in the
vicinity of discharges of pharmaceutical plants, has received attention over the past decade (Brown
et al., 2006; Hernando et al., 2006; Oberle et al., 2012; Tamtam et al., 2008).

26 The effect of environmental levels of antibiotics on microorganisms and their role in the
28 maintenance and dissemination of antibiotic resistance genes in the environment is still poorly
understood. Chronic or significant contamination by one or several antibiotics could exert a
selective pressure on the microbial community and favour the growth of antibiotic resistant or non-
susceptible bacteria (Davies et al., 2006; Kohanski et al., 2010). Although antibiotic concentration
in surface water is lower than the minimal inhibitory concentration (MIC, around 1 mg L^{-1}), it has
been reported that sub-inhibitory or sub-lethal concentrations ($0.25\text{--}0.9 \times \text{MIC}$) can trigger
bacterial DNA transcription or are involved in mutagenesis (Davies et al., 2006; Gullberg et al.,
2011; Kohanski et al., 2010). Moreover, this chemical contamination is accompanied by a supply
of antibiotic-resistant faecal bacteria, mainly released via WWTP effluents or by run off or
leaching from soils. This input of genes from allochthonous bacteria could be involved in the spread
and development of antibiotic resistance genes within autochthonous microbial communities. The

presence of resistance genes may enhance the environmental resistome, *i.e.* all genes encoding
antibiotic resistances within the microbial community, which could in turn be retro-transferred to
potential pathogens (Baquero et al., 2008).

Beyond the problem of the emergence of antibiotic-resistant bacteria, the presence of
antibiotic compounds in the environment can also affect diversity and activity of microbial
communities involved in biogeochemical cycling (Kümmerer 2009; Thiele-Bruhn 2003). The
presence of antibiotics in soils and sediments has indeed shown to affect denitrification rates
(Costanzo et al., 2005; Kotzerke et al., 2008). However, most of these studies were carried out
using high, therapeutic concentrations (mg L^{-1} or mg kg^{-1}), which is in contrast with the
environmentally observed concentrations ($\text{ng-}\mu\text{g L}^{-1}$). Recently, Underwood et al. (2011)
demonstrated the inhibiting effect of environmentally relevant Sulfamethoxazole concentrations
on denitrifying abundance and community structure as well as on nitrate reduction rates in
groundwater enrichments.

Vancomycin (glycopeptide family, VA) is among the older antibiotics of clinical use – for
almost 60 years. It is effective against most Gram-positive cocci and bacilli, inhibiting cell wall
synthesis (peptidoglycan) thus affecting dividing bacteria. Like most antibiotics vancomycin is
partially metabolized and discharged to sewage treatment plant or directly in water or soil (Dolliver
et al, 2008). The presence of vancomycin in treated effluents from hospitals in France has been
reported with concentrations ranging from 1.6 to $37.3 \mu\text{g L}^{-1}$ (Passerat et al, 2010; Dinh, 2012),
and at 29 ng L^{-1} in wastewater effluents (Zuccato et al, 2010). The removal of vancomycin
occurring during the activated sludge process or UV treatment has been estimated to 52% and

28%, respectively (Li and Zhang, 2011). This antibiotic has also been detected in surface water impacted by wastewater effluents with concentrations ranging from 0.44 to 5.17 ng L⁻¹ (Zuccato et al, 2010; Dinh et al, 2012). In Gram positive bacteria, such as enterococci, resistance to vancomycin is due to the presence of eight operons (*vanA*, *vanB*, *vanD*, and *vanM*, *vanC*, *vanE*, *vanG*, and *vanL*), that encode enzymes involved in (1) production of modified peptidoglycan precursors and (2) removal of the vancomycin-binding target (Lebreton et al, 2011). The actual origin of the genes responsible for high-level vancomycin resistance in enterococci has been linked to soil *Paenibacillus* spp. (Guardabassi et al, 2005).

Denitrification, the anaerobic reduction of nitrate (NO₃⁻) to gaseous nitrogen (N₂), is a key process in the biogeochemical nitrogen cycle and the primary biological pathway by which biologically fixed or synthetic added nitrogen is converted to a gaseous form and removed from ecosystems. Denitrifying bacteria are facultative anaerobes and a phylogenetically diverse group of microorganisms found in the domain *Archaea* and *Bacteria*, being either Gram-negative or Gram-positive (Cheneby et al., 2000; Philippot 2002). The enzyme involved in the last step during denitrification, nitrous oxide reductase (*nosZ*) converting nitrous oxide (N₂O) to N₂, has been widely used as a molecular marker for this process (Scala and Kerkhof, 1998), targeting mainly the Gram-negative bacteria (Jones et al., 2011; Philippot 2002). Possible negative effects of antibiotics, or more specifically VA, on denitrification rates in riverine sediments might affect the natural transformation of nitric pollution into inert gas. Furthermore, to the best of our knowledge, resistance of denitrifiers towards VA, including possible acquisition of resistance genes from pathogens towards this functional group, has not been explored.

Therefore, the goal of this study was to determine the effects of VA on nitrate reduction rates and structure of the denitrifying and whole bacterial community structure in river sediments. In addition to this we investigated the resistance towards VA among denitrifying enrichments from the same sediments. To this end, we used sediments exposed to different VA concentrations, collected near a Waste Water Treatment Plant (WWTP) receiving both domestic and hospital waste (effluent WWTP up to 8 $\mu\text{g L}^{-1}$ VA, Dinh, 2012). Nitrate reduction rates in sediments were determined using flow-through reactors (Laverman et al., 2006) allowing continuous supply of VA for 3 weeks. To determine the effect of VA on the total bacterial (*16S rDNA*) and the denitrifying (*nosZ*) communities, we compared the community structure in VA amended sediments using the PCR-DGGE approach (Muyzer et al., 1993; Throbäck et al., 2004). Furthermore, we investigated the resistance towards VA of denitrifying enrichments by an adaptation of the Minimal Inhibiting Concentrations (MIC) approach.

Material and Methods

Study site

The sediments used for this study were collected from the Charmoise River, a small tributary of the Orge River (North of France) in February 2010 (sediment incubations) and February 2012 (MIC enrichments). The site in the Charmoise River was chosen due to its vicinity to a Waste Water Treatment Plant, receiving both hospital and domestic effluents of Fontenay-lès-Briis (Seine basin, France). The hospital fuelling the WWTP contains 363 beds and the glycopeptide (mainly vancomycin) use in 2010 was 1.495 kg (T. Dinh, pers. comm.) The WWTP has a capacity of 5000 equivalent inhabitants and treats 1000 m³ waste per day and discharges its drain via a 50 m long channel in the Charmoise River. Sediments (0-1 cm depth) for our study were sampled 10 meters upstream and 10 meters downstream of the WWTP output.

Turbidity, oxygen concentrations, temperature and pH of the overlying water at the time of sampling were determined using a multi parameter sensor (YSI 6600 V2-4). The sediment moisture content was calculated from determining the weight loss of a known quantity of sediment (5 cm³) after drying at 105°C for 24 hours. Vancomycin (VA) was extracted from the sediment with methanol (Tamtam et al., 2011) and analyzed by an LC-MS/MS system, for details regarding this method see Dinh et al. (2011).

110 *Determination of nitrate reduction rates*

112 Sediments were placed in flow-through reactors (FTRs) and continuously supplied with
 113 nitrate (5 mM NaNO₃) and different VA concentrations (0, 1, 200, 1000 µg L⁻¹) by a peristaltic
 114 pump (Gilson, France) with a flow rate of 4 ml h⁻¹. All treatments were run in triplicates for a
 115 period of three weeks at 20°C (± 2°C) in the laboratory in the dark to minimize the
 116 photodegradation of the antibiotics as well as to prevent photosynthesis in the sediment. To assure
 117 anoxic conditions, the inflow solutions were purged once a day with nitrogen gas (30 minutes) to
 118 remove all traces of oxygen. The outflow was collected once a day and stored at 4°C until analysis
 of NO₃⁻. Concentrations of nitrate (NO₃⁻) were analyzed with a Dionex ICS 3000 ion
 chromatograph using an auto sampler AS50.

120 Nitrate reduction rates were calculated over the period 7 to 24 days using the following
 equation:

$$122 \quad R = \frac{\Delta C * Q}{V} \quad (\text{Eq. 1})$$

124 Where ΔC is the difference of NO₃⁻ between inflow and outflow solution (nmol L⁻¹), Q is
 125 the volumetric flow rate (mL h⁻¹), V volume of the reactor (cm³). Nitrate reduction rates are used
 126 throughout the text, as only net nitrate reduction rates were measured. Note that the rates
 determined are potential rates as nitrate is supplied to the sediment, whereas carbon used during
 this process is derived from the sediment. For further details regarding the FTR approach see
 128 Laverman et al. (2006) and Yan et al. (2013).

130 ***Minimal Inhibiting Concentration of the Environmental microbial community (MIC-E)***

In order to determine the effect of VA on the denitrifying community, we used a
 132 denitrifying medium amended with different VA concentrations. A bacterial suspension of the
 sediment was prepared by suspending 5 cm³ of fresh sediment in 45 mL saline solution (2 % NaCl
 134 w/v; 0.3 % MgCl₂ w/v) and vortexed for 1 minute (2500 rpm). Sterile Hungate tubes with Durham
 tubes containing medium for denitrifiers (1.5 mM KH₂PO₄, 1.5 mM K₂HPO₄, 5.0 mM NH₄Cl, 4.0
 136 mM KCl, 1.0 mM CaCl₂·2H₂O, 2.5 mM MgCl₂·6H₂O, 0.10 g L⁻¹ of yeast extract, 5.0 mM KNO₃,
 5 mM Na-Succinate, 5 mM Na-Acetate, 5 mM Lactate, 1.0 mL resazurin at 0.5 g L⁻¹ and 1.0 mL
 138 of SL9 solution (Tschech and Pfennig 1984), pH adjusted to 7.0-7.5 using a 10M NaOH solution)
 were then inoculated with these suspensions (1 mL suspension in 9 mL medium). The denitrifying
 140 enrichment after 3 days of growth was diluted in saline solution to achieve an optical density (OD)
 of 0.01 at 580 nm. One mL of this suspension was then injected in a Hungate tube containing a
 142 Durham tube and 9 mL of medium with VA (at different concentrations), and 1 tube per
 experiment without VA serving as a control. Each experiment was conducted in triplicate, and
 144 tubes were incubated at 30°C for 6 days. The range of VA concentrations tested was: 0; 4; 8; 16;
 32; 64; 128; 256 mg L⁻¹. Growth of denitrifiers was determined according to turbidity and gas
 146 production inside the Durham tubes. As a MIC is defined being a Minimal Inhibiting
 Concentration for pure culture, the concentration of VA that inhibits the enrichment will hereafter
 148 be indicated as a MIC-E (the minimal inhibiting concentration of an enrichment or environmental
 community).

150

Molecular analysis

152 Bacterial and denitrifying community structures in the sediments (3 weeks) subjected to
different vancomycin concentrations were determined by PCR and DGGE analyses. DNA was
154 extracted from sediment using the 'Powersoil™ DNA Isolation Kit' (MoBio). In order to study
the whole bacterial community structure, PCR amplification with GoTaq (Promega, Madison, WI
156 - USA) of a 180-bp fragment of *16S rDNA* was conducted using the primer sets and conditions
described by Muyzer et al. (1993) (R518 5'-ATTACCGCGGCTGCTGCTGG and F357
158 5'CCTACGGGAGGCAGCAG with a 40-bp GC-clamp attached to the 5' end). Primer annealing
took place at 55°C, and PCR reaction was 30 cycles long. To characterize the N₂O reducing
160 bacteria, PCR amplification with MyTaq (BioLine, London, UK) of a 411-bp fragment of the *nosZ*
gene was conducted using the *nosZ*-F (5'-CGYTGTTCMTGGACAGCCAG; (Kloos et al., 2001)
162 and *nosZ*1622Rb (5'-CGCRASGGCAASAAGGTSCG; (Throbäck et al., 2004) primer set, with a
33-bp GC-clamp attached to the 5' end of the latter primer. Primer annealing was conducted with
164 a touchdown from 62 to 55°C during 35 cycles followed by 10 cycles at 55°C for sediment
samples, and 45 cycles at 55°C for enrichment samples. The *16S rDNA* fragments will be referred
166 to throughout the text as the bacterial community, and the *nosZ* fragments will be referred to as
the denitrifying community.

168 After checking amplification and normalizing DNA quantity on a 2 % agarose gels, the
PCR products were then separated in relation to their sequence with the Denaturing Gradient Gel
170 Electrophoresis (DGGE) technique using the Ingeny phorU system (Ingeny, Goes, the

Netherlands) using a 9% (w/v) polyacrylamide gel (acrylamide:bisacrylamide ratio, 37.5:1) in 1X
TAE buffer (40 mM Tris acetate, 40 mM acetic acid, and 10 mM EDTA, pH 7.6). The denaturants
used were urea and formamide at respectively 33.6 % (g/v) and 32 % (v/v) for a 80 % denaturing
solution. The gradients used were 40-70 % denaturant for *16S rDNA* and *nosZ*, and the migration
was realized in 1X TAE at 62°C with a voltage between 130 and 160 V for optimum amperage of
70 mA per gel for 17h. After electrophoresis, gels were stained with 1X TAE buffer containing
1% Sight DNA Stain (Euromedex, Souffelweyersheim, France) and then photographed using a
UV imaging system. The DGGE gel images were then analysed with 'GelCompar, fingerprint and
gel analysis software II' version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) to compare
the lanes of migration (number and position of bands) using either the Jaccard or Bray-Curtis
coefficient and building a tree with the Unweighted Pair Group Method with Arithmetic mean
(UPGMA). Bacterial species richness was determined from the number of bands on DGGE
corresponding to the number of OTUs (operational taxonomic units)

Statistical analyses

The Kruskal-Wallis non parametrical test and Tukey pairwise comparison were used to
identify significant effects of antibiotic contaminated sediment *versus* uncontaminated sediment
on nitrate reduction rates. Significant differences were accepted at $p < 0.05$. All statistical analyses
were calculated using SigmaPlot Version 11.0 (Systat Software Inc, San José, CA, USA).

Results

Site characteristics

The different characteristics for the two sampling sites, upstream and downstream of the WWTP outlet can be found in Table 1. Overall, pH, temperature, conductivity and oxygen concentrations in the overlying water were similar for the two sites. In contrast, the turbidity was higher in the downstream waters with the input of suspended solids by the treated waste water. VA concentrations were below the detection limit (5 ng g^{-1} , Dinh et al., 2011) in the upstream sediments, whereas on average 62 VA ng g^{-1} was detected in the downstream sediments. Species richness, *i.e.* the number of operational taxonomic units (OTUs), was not different between the upstream and downstream site for either the total or the denitrifying bacterial communities (Table 1). However, the structure of the total and denitrifying bacterial communities were different between both sites (65 and 20% similarity in band pattern, Table 1).

Effect of VA on nitrate reduction rates and community structure

An overview of the nitrate reduction rates in the upstream and downstream sediments amended with different VA concentrations can be found in Table 2. Average nitrate reduction rates (NRR) in upstream sediments were $156 (\pm 16) \text{ nmol NO}_3^- \text{ cm}^{-3} \text{ h}^{-1}$ and $165 (\pm 21) \text{ nmol NO}_3^- \text{ cm}^{-3} \text{ h}^{-1}$ in downstream sediments. No significant differences in NRR were observed for the different VA concentrations compared to the control in the upstream sediments (Kruskal-Wallis, $p > 0.05$). A

small, but significant decrease in nitrate reduction rates (Tukey Test, $p < 0.05$) was observed in

the downstream sediments supplied with $1000 \mu\text{g L}^{-1}$ VA compared to the control.

The number of total bacterial (*16S rRNA*) OTU's observed in the upstream and downstream sediments decreased after the 3 week exposure to nitrate in the anaerobic flow-through reactors, regardless of treatment with vancomycin (Fig 1.). The number of OTU's for the un-treated controls (no vancomycin) were unaffected or reduced by 16% for the upstream and downstream sediments, respectively. In the downstream community, there was a trend of a decreasing number of OTUs with increasing vancomycin treatment. For the upstream and downstream communities, samples clustered by vancomycin treatment, low vancomycin treatments were more similar to the un-treated control than the high vancomycin treatments (Figure 1). In the upstream sediments the addition of vancomycin changed the bacterial community structure as shown by a decrease in similarity of the bacterial communities; from 73% similarity between the 0 and $1 \mu\text{g L}^{-1}$, to 63% between 0 and 200 and only 54% similarity between 0 and $1000 \mu\text{g L}^{-1}$ vancomycin. For the downstream sediments a similar change in community was observed with the non-vancomycin amended sediments showing a 65% similarity with 1 and $200 \mu\text{g L}^{-1}$ and 59.5% similarity with $1000 \mu\text{g L}^{-1}$. The corresponding dendrograms show that the change in bacterial community structure is similar in the upstream (53% similarity with structure of all other communities) and the downstream (59.5%) sediments.

The richness of the denitrifying community represented by the N_2O reducing bacteria (*i.e.* *nosZ* gene) in the sediments supplied with and without VA is shown in Fig. 2. The number of OTUs, varied between 5 and 7 in these sediments. Overall the comparison between the DGGE

patterns of *nosZ* in initial sediments and those incubated for 3 weeks with and without VA in both upstream and downstream sediments, exhibits little variation in richness of the denitrifying bacterial community. On the contrary, in the downstream sediments the initial *nosZ* diversity was very distinct (30.5% similarity) from the incubated sediments (Fig. 2), showing 75.5% similarity between them. In the upstream sediments, a 54% and a 37% similarity between the control and respectively the lowest ($1 \mu\text{g L}^{-1}$) and highest (200 and $1000 \mu\text{g L}^{-1}$) vancomycin concentrations indicates a shift in community structure. Overall, a 75% similarity between non-amended and vancomycin amended sediments indicate a minor impact of vancomycin on the community structure.

Vancomycin resistance of the culturable denitrifying bacteria

In order to investigate to what extent denitrifiers were resistant to vancomycin we determined the MIC of denitrifying Enrichments (MIC-E) towards VA of a culturable denitrifying subpopulation. Denitrifying enrichments were grown on denitrifying medium containing different VA concentrations in order to determine the concentration of VA that inhibited growth of this enrichment. The VA concentrations tested were above the known MIC of pathogenic bacteria (8 mg L^{-1}), allowing determination of MIC of the denitrifying community from a medical perspective. Figure 3 shows the number of positive denitrifying growth among the triplicate cultures, at the different VA concentrations in time (5 days). Growth after one day showed that the downstream denitrifying communities were most sensitive towards VA with inhibition observed (MIC-E) at 8 mg L^{-1} , compared to 64 mg L^{-1} for the upstream enrichments. However from 2 days, all

enrichments were able to grow at VA concentrations up to 256 mg L⁻¹ (Fig. 3), suggesting that at

least one strain was able to grow, albeit at lower growth rates for the upstream sediments.

Discussion

In this study, we investigated the effect of VA on the total bacterial and denitrifying communities in river sediments upstream and downstream of a WWTP effluent. The sediments downstream of the WWTP receive the effluent of the treatment plant that treats both domestic and hospital waste waters and have shown to be contaminated by several antibiotics including VA (Dinh et al., 2011). To our knowledge, there are only a limited number of studies that report the effect of antibiotics on the structure and function of bacterial communities in river sediments and in particular on the activity of denitrifiers. Our study shows that nitrate reduction rates were unaffected in these river sediments, except at therapeutic concentrations in the downstream sediment. In similar river sediments, nitrate reduction rates were unaffected by a chronic exposure to tetracycline at environmentally relevant concentrations (0.5, 20 and 10,000 µg L⁻¹; Roose-Amsaleg et al. (2013) or to concentrations (< 1 mg L⁻¹) of flumequine and sulfamethoxazole (Yan et al 2013). In contrast, exposure to erythromycin, clarithromycin and amoxicillin at 1 mg L⁻¹ led to the decrease in denitrification rates in sediments (Costanzo et al., 2005). In groundwater, Ahmad et al (2014) also reported an inhibition of the activity of denitrifying bacteria in the presence of sulfamethazine (0.01 mg L⁻¹) and chlortetracycline (1.0 mg L⁻¹). As VA only affects Gram positive bacteria, the lack of inhibition in our study can be related to (i) the initial presence of Gram negative denitrifiers (ii) by the initial occurrence of non-susceptible bacteria (Wright, 2003) or (iii) a shift

in initial bacterial communities chronically exposed to microbial and chemical contamination from the treated input of the WWTP favoring non susceptible bacteria (Rizzo et al., 2013).

In upstream and downstream sediments, VA contamination induced a change in the diversity of the total microbial community. At low and intermediate VA concentrations (up to 200 $\mu\text{g L}^{-1}$), the sedimentary bacterial community structures show a minor shift. Changes in the bacterial community structure were observed at the highest VA concentration (1000 $\mu\text{g L}^{-1}$), approaching therapeutic concentrations. Antibiotics, even those designed to be broad-spectrum drugs, have their selective effects on various groups of microbes. The selective antibiotic effects alter the relative abundance of microbial species (Ding et al., 2010). A change in bacterial community structure upon the application of antibiotics has been observed in soil (Hammesfahr et al., 2008; references in Ding et al., 2010). In this study the modification of the structure of microbial communities could be explained by the disappearance of susceptible strains whereas active but non-growing Gram positive bacteria persisted, and maintenance or development of non-susceptible or resistant bacteria. Moreover, the persistence of bacteria in the presence of 1000 $\mu\text{g L}^{-1}$ of VA can also be explained by the degradation of this antibiotic by heterotrophic denitrifiers as previously shown for sulfamethoxazole (Nodler et al., 2014).

The impact of antibiotics on denitrifying bacteria has been assessed mainly through the quantification of genes encoding the nitrate, nitrite, and nitrous oxide reductases and diversity analysis of these genes by DGGE (Kleineidam et al., 2010; Ollivier et al., 2010; Hammesfahr et al., 2008). Ollivier et al (2010) reported that the abundance of *nosZ* was affected by sulfadiazine but the impact on the transcript level was less pronounced, suggesting that a part of the denitrifiers was tolerant or resistant towards this antibiotic. In soils amended with manure and sulfadiazine

(SDZ, 40 and 100 mg SDZ kg⁻¹ soil), the community composition of *nirS* nitrite reducers investigated by DGGE did not change despite the observed alterations in abundance (Kleineidam et al, 2010). In contrast, DGGE patterns showed effects of SDZ (10 and 100 mg g⁻¹) on soil bacterial community structures (Hammesfahr et al, 2010). In our study, the richness of the denitrifying community (*nosZ*) was not affected by VA concentrations whereas shifts in the structure of these communities were observed. In upstream sediments, the different VA applications were not correlated to the denitrifying community structures (*nosZ*). In downstream sediment, despite a clear shift in denitrifying community structure compared to the initial sediment, this structure is highly comparable in sediments incubated for 3 weeks with and without VA. This could be explained by the fact that the primers used for the *nosZ* gene amplification targets mainly Gram negative denitrifiers (Jones et al, 2011), which are naturally resistant to vancomycin.

The MIC-E determined from denitrifying enrichments indicated the presence of resistant or non-susceptible denitrifiers in upstream and downstream sediments, which were able to grow at VA concentrations higher than environmental concentrations (> 1 mg L⁻¹). This is consistent with the detection of denitrifying activity in sediments exposed to therapeutic tetracycline concentrations (10 mg L⁻¹; Roose-Amsaleg et al, 2013). Further work will require the characterization of culturable denitrifiers (Gram negative vs Gram positive bacteria) growing at environmental (< 1 mg L⁻¹) and higher VA concentrations. Furthermore, there is a need to confirm the presence of VA resistance genes among these culturable denitrifiers.

In conclusion, our results demonstrated a minor effect of VA towards nitrate reduction rates, partly due to a change in community structure, and due to the resistance of the denitrifiers in these sediments towards VA. Further research regarding the occurrence of VA resistance genes is

required to investigate whether the nitrate reduction rates detected in presence of VA is related to resistant or non-susceptible bacteria.

Acknowledgements

The authors like to thank Benjamin Mercier, Olivier Tronquart for assistance in the field, Tuc Dinh, Fabrice Alliot for the help during antibiotic analysis. This work was financed by the Region Ile de France (RD2S project 2009-07), the program Piren Seine and EC2CO FLASH CNRS.

Table and Figure legends

Table 1. Overview of the characteristics of the sampling sites, upstream and downstream of the WWTP outlet. Concentrations of VA in the sediments were measured in February 2010 (n=2) and expressed per gram dry sediment (gds^{-1}). The total bacterial and denitrifying richness were determined in February 2012.

Table 2. Overview of the nitrate reduction rates in sediments upstream and downstream of the WWTP outlet supplied with different VA concentrations. The effect of VA on the nitrate reduction rates were tested within the sediments of the same site.

Figure 1 Cluster analysis (Bray –Curtis) of the total bacterial community structure of the sediments before (T0) and after VA exposure, upstream (A) and downstream (B) of the WWTP outlet. The number of bands (OTU), representing richness is displayed with a histogram and the similarity of community composition with a dendrogram. T0 stands for initial sediments and 0, 1, 200, 1000 $\mu\text{g/L}$ VA supply during 3 weeks of incubation.

Figure 2. Cluster analysis (Jaccard) of the denitrifying bacterial community structure of the sediments before (T0) and after VA exposure, upstream (A) and downstream (B) of the WWTP outlet. The number of *nosZ* bands (OTU), representing richness is displayed with a histogram and the similarity of community composition with a dendrogram. T0 stands for initial sediments and 0, 1, 200, 1000 $\mu\text{g L}^{-1}$ VA supply during 3 weeks of incubation.

Figure 3. Growth of denitrifiers (gas production) during the MIC-E experiment of the different denitrifying communities from the Charmoise sediment upstream (A) and downstream (B)

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- 462

Table 1

	variable	unit	Upstream	Downstream
Water	pH		8.25	7.98
	T	°C	3.19	3.91
	Conductivity	mS	0.632	0.678
	Turbidity	NTU ⁽¹⁾	7.6	23
	O ₂	mg L ⁻¹	9.83	10.12
Sediment	[VA] ⁽²⁾	ng gds ⁻¹	< 5	62
	<i>16S rRNA</i> bands ⁽²⁾		32	33
	<i>nosZ</i> bands ⁽²⁾		6	6
	<i>16S rRNA</i> similarity	%		65
	<i>nosZ</i> similarity	%		20

(1) NTU : Nephelometric Turbidity Unit

(2) two separate sediment or DNA extractions (n=2)

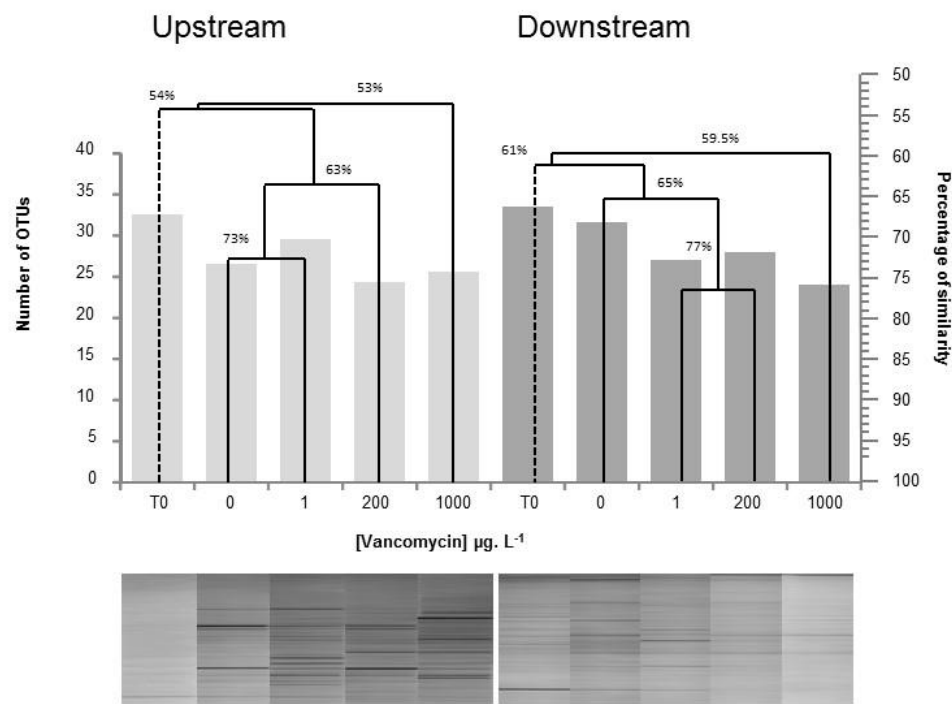
468 Table 2

Site	Vancomycin concentration	Nitrate reduction rates
	$\mu\text{g L}^{-1}$	$\text{nmol cm}^{-3} \text{ h}^{-1}$
Upstream	control	156 ± 16
	1	133 ± 26
	200	122 ± 35
	1000	130 ± 11
Downstream	control	165 ± 21
	1	143 ± 12
	200	184 ± 21
	1000	$130 \pm 15^*$

* indicates significant difference (Tukey , $p < 0.05$)

470

Figure 1



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Figure 2

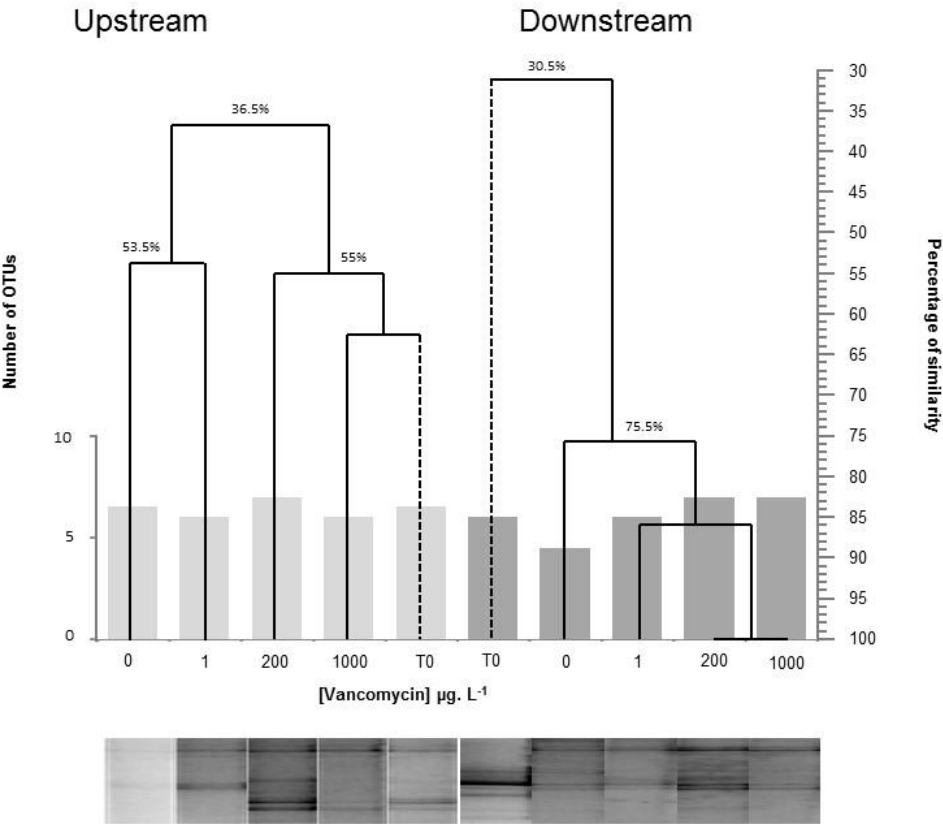


Figure 3

