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# Effective removal of antibiotic resistance genes from wastewater using marine waste-derived novel nanocomposites

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ABSTRACT

Wastewater (WW) has been identified as a major hotspot of microbial emerging contaminants (MECs), such as antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). Currently used WW treatment methods cannot efficiently eliminate these pollutants, resulting in passive contamination of adjacent environments receiving undertreated discharge. More effective WW treatment strategies are therefore urgently required. In this study, newly developed and well-characterised semi-interpenetrating polymer network (semi-IPN) hydrogels derived from the valorisation of marine wastes (e.g., shrimp shells) were investigated for their ARG removal potential. The results indicated that multiple ARGs prevalent in WW, such as *ermB*, *qrnS*, *sul1* and *tetO*, were removed by up to 100% after being treated by novel hydrogels. In terms of horizontal gene transfer-associated genetic elements, such as integron-1 *intl1*, transposons *tnpA1* (IS4 group) and *tnpA2* (IS6 group), substantial reduction approaching 99.9% was also achieved. Moreover, up to 97% of efflux pump-associated *qacE*$\Delta$*1* conferring multidrug resistance (MR) was successfully attenuated. To conclude, the semi-INP hydrogels developed exhibited great potential for ARG mitigation towards strengthening WW decontamination, which provides a viable, cost-effective and environmentally friendly novel treatment approach.



## 1. Introduction

Antibiotic resistance (AR) represents a growing threat to environment and public health (Aslam et al., 2021; Larsson and Flach, 2022). As previously documented, various types of wastewater (WW) from domestic, hospital, agricultural and industrial sources constitute a major AR reservoir by harbouring a myriad of microbial emerging contaminants (MECs), such as pathogens, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Li et al., 2022; Magana-Arachchi and Wanigatunge, 2022). Although conventional WW treatment methods (both physicochemical and biological

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approaches) can efficiently remove chemical and organic contaminants, they have a limited effect on dealing with AR-associated pollutants, as they were not originally devised to target antibiotics and ARGs (Hazra et al., 2022; Wang et al., 2020). High variabilities of the chemical structures, thermal stabilities, and hydrophilic/hydrophobic attributes of different antibiotics make it extremely difficult for their removal by the conventional treatment technologies (de Ilurdoz et al., 2022; Hazra et al., 2022; Mathur et al., 2021). In addition, there are identified technical limitations for conventional treatment systems to effectively remove ARB/ARGs, including insufficient screening, low adsorption, ineffective coagulation, releasing and increasing free ARGs, as well as ARB repairing and regeneration (Anthony et al., 2020; Ren et al., 2018; Zheng et al., 2017). Notably, ARGs resistant to all types of antibiotics and mobile genetic elements (MGEs) have been detected in WW treatment plants (WWTPs) all over the world (Igere et al., 2022; Pazda et al., 2019; Wang et al., 2020). An investigation of 12 WWTPs from seven European countries identified multiple ARGs and MGEs which are persistent in effluents (Pärnänen et al., 2019).

The advancement of nanotechnology has opened a new avenue for innovative applications used for water and WW treatment (Cheriyamundath and Vavilala, 2021; Ojemaye et al., 2020; Xu et al., 2022). This takes advantage of the unique chemical, physical and biological properties possessed by the nanomaterials/nanoparticles (Jiang et al., 2018; Yaqoob et al., 2020). For example, nanosilver and silver ions were engineered to remove ARGs from WW (Ma et al., 2016). Venieri et al. (2017) used titanium dioxide ($TiO_2$) nanoparticles in combination with chlorination and UV to target and inactivate ARGs in WW. More recently, using developed functionalised molecularly imprinted polymer (MIP) films and quaternary ammonium salt (QAS)-modified kaolin microparticles, substantial pathogen removal and ARG mitigation in WW has been achieved (Gavrila et al., 2020; Paruch et al., 2021).

In this study, we investigated ARG elimination from WW by using newly developed semi-IPN hydrogels prepared primarily from mineral-enriched chitosan extracted from shrimp shells. Their comprehensive physicochemical properties and distinct pathogen removal effects have been well-characterised in our latest work (Neblea et al., 2023). The focus of the current study is to explore their ARG treatment efficacy in WW through the assessment of genetic marker-based molecular analysis.

## 2. Materials and methods

### 2.1. Semi-interpenetrating polymer network (semi-IPN) hydrogels

Preparation, synthesis, purification and characterisation of the semi-IPN hydrogels tested in the present study have been described in detail in our latest work (Neblea et al., 2023). In brief, commercial chitosan (CC), chitosan synthesised from commercial chitin (CCH), mineral-enriched chitosan extracted from shrimp shells (SHC, as described in the study of Miron et al. (2022)) and vinyl benzyl trimethylammonium chloride (VBTAC) were applied for the synthesis of semi-IPN hydrogels using free radical polymerisation. Three series of nanocomposite samples were prepared as follows: CC-based semi-$IPN_n$ (n = 2, 3, 4), CCH-based semi-$IPN_n$ (n = 2, 3, 4) and SHC-based semi-$IPN_n$ (n = 2, 3, 4). Each series has three subtypes which essentially differ in the content of VBTAC, i.e., 0.3 g in $IPN_2$, 0.5 g in $INP_3$ and 0.7 g in $IPN_4$. In addition, a reference material of polyVBTAC was prepared under the same conditions as all of the other hydrogels but without chitosan (Neblea et al., 2023).

### 2.2. Wastewater

The sampling, filtration and testing of wastewater (WW) followed the same procedure as reported in several of our previous works (Gavrila et al., 2020; Neblea et al., 2023; Paruch et al., 2021). In short, WW samples collected from a homogenisation basin of an operative conventional treatment plant were applied for experiments with the semi-IPN hydrogels and polyVBTAC. Triplicates of both raw (the collected/untreated WW) and processed (treated WW by the nanocomposites) samples underwent vacuum filtration using Labbox systems with PES-membrane filters (PALL 516-0427, pore size 0.45 μm, Ø47 mm). These filters were further processed for DNA extraction and subsequent qPCR assays.

### 2.3. Molecular analyses of ARG markers

Microbial genomic DNA (gDNA) was extracted from each processed PES-membrane filter using the Qiagen DNeasy Powerwater kit. The purified gDNA was used for ARGs examination by applying previously developed panel of ARG markers (Paruch et al., 2021). In addition, two new MGEs, i.e., transposase *tnpA1* (IS4 group) and *tnpA2* (IS6 group) were developed for the extended examination of horizontal gene transfer (HGT). Moreover, efflux pump genes *qacE Δ1* and *qacA/B* were developed to explore the multidrug resistance (MR) profile. All qPCR analyses were carried out using the same assay setup and thermocycling conditions as those which were established and verified previously (Paruch et al., 2021). The qPCR program used for the analyses started with an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturing at 95 °C for 15 s and annealing-extension at 60 °C for 30 s. Sequence information of the new markers is shown in Table 1.

**Table 1**
Sequence information of primers (F – forward primer and R – reverse primer, Zhu et al. (2013)) for the newly developed marker genes in this study.

| Marker gene | Target | Sequences of primes and probes (5′-3′) |
|---|---|---|
| *qacE Δ1* | Efflux pump | F: TCGCAACATCCGCATTAAAA<br>R: ATGGATTTCAGAACCAGAGAAAGAAA |
| *qacA/B* | Efflux pump | F: TTTAGGCAGCCTCGCTTCA<br>R: CCGAATCCAAATAAAACCCAATAA |
| *tnpA1* | Transposon | F: GGGCGGGTCGATTGAAA<br>R: GTGGGCGGGATCTGCTT |
| *tnpA2* | Transposon | F: TGCAGATGGTTTAACCTTGGATATTT<br>R: TCGGTTCATCAAACTGCTTCAC |

## 3. Results and discussion

Among all of the tested ARGs, no signals were detected for *mecA* and *vanA* in the raw WW samples, which coincided with the earlier results from the examination of WW samples from the same WWTP (Paruch et al., 2021). Low/undetectable signals of these two markers indicated undeveloped/less-developed AR in the studied WW, which implied low selective pressure resulting from the limited discharge of the associated antibiotics, i.e., vancomycin (*vanA*) and methicillin (*mecA*), which are often used as the last-resort antibiotics (Koch et al., 2014; Mühlberg et al., 2020). Similar to the findings from the other studies (Pärnänen et al., 2019; Wang et al., 2020), WWTP-prevalent ARGs, e.g., *ermB*, *qnrS*, *sul*1 and *tetO*, were found in high levels in the raw WW samples, at 2.63E+07, 3.93E+09, 3.42E+09 and 8.60E+07 copy numbers (CN)/100 mL, respectively (Table 2a). For dealing with these predominant ARGs, SHC-$IPN_2$/-$IPN_3$ and CCH-$IPN_2$ exhibited exceedingly high removal rates (RR) ranging from 94 to 100%; all outperformed the reference polyVBTAC (61%–69% RR), as shown in Table 2a. Of note, there was no detectable *tetO* left after treatment with SHC-$IPN_2$ and/or SHC-$IPN_3$, indicating the potent degradation potential of SHC-derived hydrogels on targeting this particular ARG.

In the examination of MGEs responsible for HGT (Table 2b), up to 2.55E+08 CN of *int*I1 was detected in 100 mL raw WW sample, suggesting a high tendency for rapid ARG dissemination in WW. In comparison with polyVBTAC (77% RR), CCH-$IPN_2$ and SHC-$IPN_3$ could drastically reduce *int*I1 by 99.9% and 98.1%, respectively. In terms of transposon-mediated HGT, the high content of transposase-encoding genes, e.g., *tnpA1*and *tnpA2*, was found in the raw WW, 1.49E+08 and 4.66E+09 CN/100 mL, respectively (Table 2b). With reference to these findings, SHC-$IPN_2$/-$IPN_3$ and CCH-$IPN_2$ displayed pronounced efficacies to attenuate over 99% of *tnpA1* and *tnpA2*. Notably, SHC-$IPN_2$/-$IPN_3$ almost completely wiped out *tnpA2* (99.9% RR), being evidently superior to polyVBTAC (81%–85%). With respect to MR (Table 2b), the efflux pump is one of essential mechanisms which has evolved by ARB against antibiotic interventions. In our study, *qacA/B* was not detected/quantifiable in the raw WW; however, the other efflux pump gene *qacE Δ1* was found to be abundantly present at up to 4.48E+08 CN/100 mL. CCH-$IPN_2$ and SHC-$IPN_3$ worked the best for its removal, reaching 99.96% and 97% RR, respectively, again surpassing polyVBTAC at 74.0% (Table 2b). The remarkable treatment functionality of the newly engineered semi-IPN hydrogels identified here can be largely attributed to their extraordinary pathogen inactivation in WW, as reported in our previous work (Neblea et al., 2023). Apparently, microbial pathogens and ARGs are inseparably coupled in a co-occurrence manner/hosting relationship (Ju et al., 2016; Shen et al., 2023).

Chitosan, as one of the essential components of the tested hydrogels, has been broadly used as a type of polysaccharide for generating functional biopolymers (Fatullayeva et al., 2022; Karimi-Maleh et al., 2021) and for environmental applications (Goci et al., 2023; Lichtfouse et al., 2019). The increasing applicational popularity of chitosan mainly relies on its intrinsic biodegradability, biocompatibility and bactericidal capabilities (Jiang et al., 2023). In our study, among the tested materials, hydrogels made from commercial chitosan showed the lowest ARG counteracting effects in all tests. In contrast, hydrogels based on chitosan derived from chitin (CCH-$IPN_2$) and extracted from shrimp shells (SHC-$IPN_2$/-$IPN_3$) exhibited the best performance in suppressing various dominant ARGs and their broad dissemination (MGEs-related), as well as inhibiting multi-drug resistance (efflux pump-associated). In fact, this observation coincides with the prominent pathogen inactivation effect of SHC-IPNs ($IPN_2$ and $IPN_3$) and CCH-IPNs ($IPN_2$ and $IPN_3$) revealed in our previous study (Neblea et al., 2023), where high swelling degree and content of native minerals in the chitosan structure were considered to largely justify their remarkable bactericidal capacity. Obviously, chitosan obtained from shrimp shells (SHC), offers a better and more favourable solution than from commercial chitin (CCH), particularly with regards to the marine resource/waste renewing and valorisation, low cost and environmental friendliness (bio-degradable and -compatible).

Concerning the other building component of the novel hydrogels, VBTAC used as the QAS monomer has been well-appreciated for its proven antibacterial activity and low toxicity (Gavrila et al., 2020; Geissen et al., 2015; Gharibi et al., 2019). Notably, the results of this study indicated that the observed ARG removal effects exerted by the novel hydrogels were not VBTAC dose-dependent, meaning that the largest effectiveness was achieved when VBTAC input was lower, such as 0.3 g (CCH-$IPN_2$ and SHC-$IPN_2$) and 0.5 g (SHC-$IPN_3$), but not at the highest content of 0.7 g in any $IPN_4$ series. This is important as VBTAC is costly; thus, it is desirable for low dosages of VBTAC to be sufficient. Also, after being combined with shrimp shell-derived chitosan, the final semi-IPN hydrogels developed can exert satisfactory pathogen and ARG removal synergy.

**Table 2**
(a) The molecular detection of antibiotic resistance genes (ARGs) as quantified in copy numbers (CN) per 100 mL with the standard error (SE) of the means and their removal rate (RR) as a percentage for each treatment test. (b) The molecular detection of mobile genetic elements (MGEs)- and multidrug resistance (MR)-associated genes, as quantified in copy numbers (CN) per 100 mL with a standard error (SE) of means and their removal rate (RR) as a percentage for each treatment test.

(a)

| Sample | *ermB* | | *qrnS* | | *sul1* | | *tetO* | |
|---|---|---|---|---|---|---|---|---|
| | CN ± SE · 100 $mL^{-1}$ | RR | CN ± SE · 100 $mL^{-1}$ | RR | CN ± SE · 100 $mL^{-1}$ | RR | CN ± SE · 100 $mL^{-1}$ | RR |
| $WW_1$ | 1.66E+06 ± 4.16E+00 | | 1.68E+09 ± 3.80E+03 | | 1.52E+07 ± 5.24E+01 | | 4.16E+07 ± 5.97E+02 | |
| CC-$IPN_2$ | 4.21E+05 ± 2.17E+01 | 74.6% | 5.14E+08 ± 5.36E+02 | 69.4% | 3.78E+06 ± 2.85E+02 | 75.2% | 7.78E+06 ± 3.74E+02 | 81.3% |
| CC-$IPN_3$ | 1.58E+06 ± 1.78E+01 | 4.8% | 2.01E+09 ± 3.39E+03 | 0.0% | 1.54E+07 ± 1.02E+03 | 0.0% | 3.31E+07 ± 1.33E+03 | 20.5% |
| CC-$IPN_4$ | 1.53E+06 ± 6.49E+00 | 7.7% | 2.34E+09 ± 2.01E+03 | 0.0% | 1.80E+07 ± 9.15E+02 | 0.0% | 4.32E+07 ± 1.06E+03 | 0.0% |
| $WW_2$ | 3.56E+06 ± 1.52E+02 | | 3.93E+09 ± 3.75E+03 | | 3.22E+07 ± 7.14E+02 | | 6.33E+07 ± 7.91E+02 | |
| CCH-$IPN_2$ | 1.30E+04 ± 2.09E+00 | 99.6% | 9.35E+06 ± 3.35E+02 | 99.8% | 6.67E+04 ± 7.73E+00 | 99.8% | 1.92E+05 ± 1.73E+01 | 99.7% |
| CCH-$IPN_3$ | 4.45E+05 ± 4.47E+00 | 87.5% | 3.74E+08 ± 7.75E+03 | 90.5% | 4.36E+06 ± 3.44E+01 | 86.4% | 7.55E+06 ± 9.71E+01 | 88.1% |
| CCH-$IPN_4$ | 2.00E+06 ± 1.88E+00 | 44.0% | 2.19E+09 ± 2.61E+04 | 44.3% | 2.12E+07 ± 5.80E+00 | 34.1% | 4.41E+07 ± 1.31E+03 | 30.4% |
| $WW_3$ | 2.63E+07 ± 2.32E+02 | | 2.98E+08 ± 9.37E+02 | | 3.42E+09 ± 5.29E+03 | | 8.60E+07 ± 1.30E+03 | |
| SHC-$IPN_2$ | 4.61E+05 ± 3.48E+01 | 98.2% | 1.88E+07 ± 1.71E+03 | 93.7% | 8.52E+07 ± 1.65E+03 | 97.5% | 0.00E+00 ± 0.00E+00 | 100.0% |
| SHC-$IPN_3$ | 2.50E+05 ± 5.88E+00 | 99.0% | 8.32E+06 ± 2.13E+02 | 97.2% | 3.76E+07 ± 5.11E+02 | 98.9% | 0.00E+00 ± 0.00E+00 | 100.0% |
| SHC-$IPN_4$ | 1.12E+06 ± 2.32E+01 | 95.7% | 3.26E+07 ± 3.36E+02 | 89.1% | 1.60E+08 ± 3.55E+03 | 95.3% | 2.44E+06 ± 7.25E+01 | 97.2% |
| polyVBTAC | 2.32E+06 ± 3.79E+02 | 67.9% | 6.35E+07 ± 3.68E+02 | 69.4% | 1.15E+08 ± 2.37E+01 | 60.7% | 4.60E+07 ± 5.37E+03 | 61.0% |

(b)

| Sample | *intI1* | | *tnpA1* | | *tnpA2* | | *qacE Δ1* | |
|---|---|---|---|---|---|---|---|---|
| | CN ± SE · 100 $mL^{-1}$ | RR | CN ± SE · 100 $mL^{-1}$ | RR | CN ± SE · 100 $mL^{-1}$ | RR | CN ± SE · 100 $mL^{-1}$ | RR |
| $WW_1$ | 1.45E+07 ± 3.86E+02 | | 1.45E+06 ± 7.12E+01 | | 1.88E+06 ± 7.60E+01 | | 2.02E+07 ± 1.42E+01 | |
| CC-$IPN_2$ | 4.51E+06 ± 2.26E+02 | 68.9% | 5.63E+05 ± 8.08E+00 | 61.1% | 5.48E+05 ± 1.47E+01 | 70.8% | 9.23E+06 ± 7.00E+02 | 54.3% |
| CC-$IPN_3$ | 1.47E+07 ± 2.79E+02 | 0.0% | 1.16E+06 ± 6.51E+01 | 19.8% | 1.69E+06 ± 1.27E+02 | 10.1% | 2.34E+07 ± 1.74E+03 | 0.0% |
| CC-$IPN_4$ | 1.55E+07 ± 1.02E+03 | 0.0% | 1.32E+06 ± 8.53E+01 | 8.7% | 1.50E+06 ± 7.31E+01 | 19.9% | 2.03E+07 ± 1.84E+03 | 0.0% |
| $WW_2$ | 7.58E+07 ± 2.88E+03 | | 2.58E+06 ± 1.07E+02 | | 3.31E+06 ± 1.07E+02 | | 1.35E+08 ± 3.65E+03 | |
| CCH-$IPN_2$ | 9.12E+04 ± 1.85E+01 | 99.9% | 9.61E+03 ± 1.36E+00 | 99.6% | 1.86E+04 ± 1.57E+00 | 99.4% | 5.49E+04 ± 9.79E+00 | 99.96% |
| CCH-$IPN_3$ | 4.50E+06 ± 6.64E+00 | 94.1% | 4.23E+05 ± 5.91E+00 | 83.6% | 4.66E+05 ± 4.93E+01 | 85.9% | 1.14E+07 ± 3.16E+01 | 91.6% |
| CCH-$IPN_4$ | 2.40E+07 ± 1.06E+03 | 68.4% | 9.44E+05 ± 2.95E+01 | 63.5% | 1.39E+06 ± 3.00E+01 | 57.9% | 5.49E+07 ± 3.11E+03 | 59.3% |
| $WW_3$ | 2.55E+08 ± 3.90E+03 | | 1.49E+08 ± 9.73E+03 | | 4.66E+09 ± 9.73E+03 | | 4.48E+08 ± 7.35E+03 | |
| SHC-$IPN_2$ | 9.81E+06 ± 8.34E+01 | 96.2% | 1.14E+06 ± 3.01E+01 | 99.2% | 5.18E+04 ± 7.26E+00 | 99.99% | 3.05E+07 ± 4.05E+03 | 93.2% |
| SHC-$IPN_3$ | 4.81E+06 ± 2.25E+02 | 98.1% | 6.11E+05 ± 5.10E+00 | 99.6% | 3.78E+04 ± 1.42E+00 | 99.99% | 1.36E+07 ± 7.77E+02 | 97.0% |
| SHC-$IPN_4$ | 1.74E+07 ± 5.29E+02 | 93.2% | 1.86E+06 ± 7.04E+01 | 98.8% | 9.95E+04 ± 2.55E−01 | 99.99% | 5.22E+07 ± 1.97E+03 | 88.3% |
| polyVBTAC | 2.71E+07 ± 1.11E+03 | 77.0% | 1.70E+06 ± 1.63E+02 | 80.6% | 1.09E+06 ± 5.77E+02 | 84.50% | 4.38E+07 ± 2.70E+03 | 74.0% |

## 4. Conclusions

In summary, the newly developed semi-IPN hydrogels demonstrated high efficiency in WW treatment with regard to the specific MEC removal. The production of novel materials from the valorisation of marine waste (shrimp shells) has proven to be a cost-effective, feasible and new value generating method for the recycling of marine resources. Hydrogels prepared from shrimp shell-derived chitosan and VBTAC illustrated superior ARG mitigation efficacies in WW. Together with the previously demonstrated significant pathogen removal ability, novel materials have exhibited profound application potential for WW treatment, specifically in tackling MECs, such as ARGs.

## CRediT authorship contribution statement

**Lisa Paruch:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. **Adam M. Paruch:** Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Iulia Elena Neblea:** Nanocomposite preparation, Formal analysis, Writing – review & editing. **Tanta-Verona Iordache:** Supervision, Project administration, Resources, Writing – review & editing. **Andreea G. Olaru:** Wastewater sampling and preparation, Funding acquisition, Writing – review & editing. **Anita-L. Chiriac:** Funding acquisition, Project administration, Resources, Writing – review & editing. **Andrei Sarbu:** Supervision, Resources, Writing – review & editing.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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