Nickel and Lead biosorption by *Curtobacterium* sp. FM01, an indigenous bacterium isolated from farmland soils of northeast Iran

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23 Abstract

24 The *Curtobacterium* sp. FM01 was isolated from farmland soils of northeast Iran and
25 tested for eight heavy metals removal in aqueous solutions. The results showed that the
26 metal removal (%) values for Ni (II) and Pb (II) with FM01 were significantly superior to
27 the corresponding values with other metals. Accordingly, further analyses were concerted
28 on the removal of Ni (II) and Pb (II) from aqueous solution in batch mode using dead
29 biomass of *Curtobacterium* sp. FM01. Basic parameters such as pH (3–7), initial metal ion
30 concentration (0.125–0.5 mM), contact time (5–720 min), varying biomass concentration
31 (1–30 mg) and temperature (10–50°C), were evaluated to determine the best biosorption
32 conditions. The biosorption data are fitted well by the Langmuir biosorption isotherms for
33 Ni (II) and Pb (II). The maximum biosorption capacity was found to be 186.60 for Pb (II)
34 and 140.99 mg g⁻¹ for Ni (II). The biosorption process of Pb (II) onto the FM01 was
35 followed second-order rate kinetics; however, kinetics of biosorption of Pb (II) followed
36 both first- and second-order rate equations, indicating that the process involving the rate-
37 controlling step is complex. Fourier transform infrared (FT-IR) analysis was carried out to
38 characterize involvement of the functional groups of FM01 in the metal ions biosorption.
39 Transmission electron microscopic (TEM) examination of metabolically active bacterial
40 cells revealed surface morphological changed following metal ions biosorption. The
41 observed electron-dense area on the cells is due to the presence of metal ions and contains
42 the biosorbed ions. The study suggests that *Curtobacterium* sp. FM01 could be a promising
43 candidate with a capacity to remove Ni (II) and Pb (II) from aqueous solutions.

44 Key words: Biosorption; Nickel; Lead; *Curtobacterium* sp. FM01; Kinetic

45 **1. Introduction**

46 It is well documented that the presence of heavy metals in the environment can be
47 detrimental to a variety of living things [1]. The main sources of lead pollution in the
48 environment are industrial units, leaded petrol, smoke emissions from coal and gas power
49 stations and use of paints [2]. Accumulation of lead in mammals influences the nervous
50 system by deceleration of neural response. Nickel is a significant environmental inorganic
51 contaminant, with legalized levels lower than 0.04 mg/l in human consumption water.
52 Higher concentrations affect normal flora in ecosystems and are toxic for human beings
53 [3]. The biosorption process is an inactive uptake that exploits cell wall of biomass to
54 confiscate the metal ions from aqueous solutions [4, 5]. A wide variety of living and dead
55 biomass of bacteria, algae, fungi, and plants are capable of sequestering toxic metals from
56 waste streams, which offer an economical alternative for sorption technologies [6, 7]. The
57 capacity of any biosorbent is mainly influenced by biomass characteristics,
58 physicochemical properties of the target metals, and the microenvironment of contact
59 solution including pH, temperature, and interaction with other ions [8, 3]. Mechanisms of
60 cell surface sorption are self-regulating of cell metabolism which is influenced by
61 physicochemical interactions between metal and functional groups of the cell wall.
62 Bacteria express a wide-ranging of complex molecules on their cell wall, which confer
63 anionic net charge to the cell surface at acidic pH values [4]. The *Curtobacterium* genus is
64 a member of the family Microbacteriaceae and aerobic Gram-positive coryneform bacteria
65 with a group B-type peptidoglycan structure and contains well-founded species, including
66 *Curtobacter flaccumfaciens* (a well-established plant pathogen).

67 In the current work, biosorption of Ni (II) and Pb (II) by non-metabolically-active cells of
68 *Curtobacterium* sp. FM01 were examined to evaluate its ability for removing heavy
69 metals. The influence of experimental parameters such as pH, contact time, biosorbent
70 dosage, temperature and initial Nickel and Lead concentrations were studied as well. The
71 kinetic and isotherm data of biosorption studies were processed to gain insight into the
72 biosorption mechanism of the Ni (II) and Pb (II) ions onto this bacterium. Therefore, the
73 innovative aspect of this study was to introduce new bacterial strain with the biosorption
74 capacity for Ni (II) and Pb (II) from aqueous solutions.

75 **2. Materials & methods**

76 *2.1. Isolation and identification of bacterial isolate*

77 *Curtobacterium* sp. FM01 was isolated from farmland soils of northeast Iran and
78 identified using biochemical (API 20E Kit) and molecular (16S rRNA gene sequencing)
79 methods. To determine the bacterium growth curve, Erlenmeyer flasks (250 ml) containing
80 50 ml of the lactose broth (LB) were inoculated with 2 ml of *Curtobacterium* sp. FM01
81 and incubated at 30°C for 72 hours. The Bacterial cell growth was determined every 4
82 hours by measuring absorbance at 600 nm using a UV/VIS spectrophotometer
83 (PerkinElmer, Lambda, USA).

84 *2.2. Biosorbent preparation*

85 The strain FM01 was cultivated in 500 ml of LB on the shaker incubator (Kuhner,
86 Germany) at 30°C and 180 rpm for 24 hours (mid-log phase). Following bacterial growth,
87 the produced biomass was separated by centrifugation at 5000 rpm for 10 min (Sigma,

Germany), washed three times with sterile distilled water and to obtain a fine powder, dried cells were freeze dried.

2.3. Selection of best heavy metals for biosorption studies by *Curtobacterium*

A set of 100 ml Erlenmeyer flasks containing 20 ml of tested metal solutions (0.5 mM) including Ni, Cu, Cr, Cd, Zn, Sn, Pb, Co, were used in this experiment. 20 mg of dried powdered dead cells were exposed to these metal solutions for 2 hours at 30°C on a shaker incubator at 150 rpm. After the incubation, the cells were centrifuged at 5000 rpm for 10 min. Supernatant were filtered and residual metal concentration was estimated by using atomic absorption spectrophotometer (AAS, Flame emission, Shimatzo).

2.4. Preparation of Ni and Pb solutions

The heavy metal solutions were prepared from their nitrates salts as Ni (NO₃)₂.6H₂O and Pb (NO₃)₂. Stock solutions were prepared in distilled water separately, sterilized using membrane filter and kept in the refrigerator.

2.5. Biosorption experiments

Biosorption studies were done using various parameters such as time (5-720 min), temperature (10-50°C), pH (3-7), biosorbent dose (1-30 mg) and Ni (II), Pb (II) concentrations (0.125-0.5 mM).

The effect of temperature on biosorption of Ni and Pb by the FM01 dried cells (20 mg) was studied by preparing 20 ml metal solution (0.5 mM, pH=7), with a temperature value between 10-50°C and flasks were agitated at 180 rpm/min. The samples were centrifuged and filtered. The filtrate samples were analyzed for the remaining metal concentrations

with atomic absorption spectrophotometer (AAS). Each experiment was repeated three times to confirm the results. The metal uptake (q) was calculated from the difference between initial (C_i) and final concentration (C_f) of the metal in solution according to the following equation:

$$q = V(C_i - C_f)/M$$

Where V is the liquid sample volume (Litter) and M is the starting sorbent weight (gram) [9].

2.6. Kinetic studies

Kinetic analysis was carried out to correlate the experimental data based on pseudo-first and second order kinetics models. Kinetic of biosorption was determined by analyzing **biosorptive** uptake of the Ni (II) and Pb (II) at different time intervals (5-720 min).

The pseudo-first-order kinetics can be expressed as [10]:

$$q_t = q_e (1 - e^{-k_1 t}) \quad (1)$$

where q_e and q_t are the amount of Pb (II) and Ni (II) biosorbed per unit weight (mg g^{-1} dry weight) of biosorbent at equilibrium and at any time t (min), respectively, and k_1 is the rate constant of pseudo-first-order sorption (min^{-1}).

The pseudo-second-order kinetics can be expressed as [11]:

$$q_t = \frac{q_e^2 K_2 t}{1 + K_2 t q_e} \quad (2)$$

Where q_t is the amount of sorbate on sorbent at time t (mg g^{-1}), k_2 is the equilibrium rate constant of pseudo-second order sorption kinetics ($\text{mg g}^{-1} \text{min}^{-1}$) and q_e is the equilibrium

uptake (mg/g). Thus, the pseudo-first order and pseudo-second order rate constants for the biosorption of Pb (II) and Ni (II) by FM01 cells determined experimentally by plotting q_t versus t using non-linear method according to Eq.s (1) and (2).

2.7. Isotherm models

Biosorption isotherms are mathematical models that describe the distribution of the adsorbate species among liquid and biosorbent. These isotherms relate metal uptake per unit weight of biosorbent (q) to the equilibrium adsorbate concentration (C_e) such as Langmuir and Freundlich isotherms. The Langmuir sorption model has been applied for the evaluation of maximum metal uptake at different initial metal concentrations and it is based on the assumption that maximum biosorption occurs when a saturated monolayer of solute molecules is present on the biosorbent surface, and the energy of biosorption is constant and there is no migration of biosorbate molecules in the surface plane. The Freundlich isotherm model is describing the biosorption of solutes from a liquid to a solid surface and assumes that different sites with several biosorption energies are involved. The Langmuir isotherm is given by:

$$q_e = \frac{q_{\max} b C_e}{1 + b C_e} \quad (3)$$

q_e is the amount of metal adsorbed per unit weight of biosorbent (mg/g), C_e is the equilibrium metal concentration (mg/ L), q is monolayer biosorption capacity of sorbent, and b is the Langmuir constant (L/mg).

The Freundlich isotherm model assumes neither homogenous site energies nor limited levels of sorption. The model is the earliest known empirical equation and is shown to be

consistent with exponential distribution of active centers, characteristic of heterogenous surfaces [12].

$$q_e = K_F C_e^{1/n} \quad (4)$$

where k_F and n are the distribution coefficient and a correction factor, respectively. Non-linear regression coefficients and parameters are shown in Table 1. Correspondingly, a plot was drawn between C_e versus q_e using the experimental and predicted value by non-linear models.

2.8. FTIR analysis

Fourier Transform Infrared Spectroscopy was performed to determine of the main functional chemical groups present on the bacterial surface which are responsible for biosorption process. The infrared spectra analysis of *Curtobacterium* sp. FM01 were obtained using a Fourier transform infrared spectra (FT-IR, GX 2000) before and after Pb (II) and Ni (II) biosorption, adopting KBr disc technique.

2.9. Transmission Electron Microscopy (TEM)

TEM analysis was performed on metabolically-active cells of *Curtobacterium* sp. FM01 in the presence of Ni and Pb ions. To this end, the cell biomass was incubated with metal solutions under the following conditions; Ni: pH 5, 1 mM Ni solution and Pb: pH 6, 1.5 mM Pb solution. Then samples were centrifuged and washed with deionized water before being prepared for primary fixation. Specimens were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH: 7.2) for 2 hours at 4°C. Then, specimens were washed three times with sodium cacodylate buffer (0.1 M, pH 7.2). To make certain appropriate fixation,

specimens were fixed at 4°C for 1 hour in 1% buffered osmium tetroxide at 4°C for 1 hour. After 1 h, the specimens were once again washed with three changes of 0.1 M cacodylate buffer. Dehydration was carried out with a series of ethanol concentration from 10 to 100%. Specimens were infiltrated with a resin. Thin sections of 70 nm were obtained using diamond knife on a Reichert-Ultracut ultramicrotome, and the sections were mounted on copper grids and coated with carbon for TEM (Philips CM 200) observation.

3. Results and Discussion

3.1. Characterization of Curtobacterium sp. FM01

Based on light-microscopy observation, strain FM01 was obligate aerobic gram-positive non-spore forming bacilli with yellow pigment. The colonies were 2 to 3 mm in diameter after 30 h incubation circular and smooth. Biochemical analysis showed that the isolate was catalase positive, while urease and oxidase were negative. The 16S rRNA gene sequencing showed 98% similarity with *Curtobacterium flaccumfaciens*. The nucleotide sequences described in this study have been submitted to the Gene Bank under the accession number of JQ995283. Bacterial growth profile demonstrated that mid-logarithmic growth phase occurred at 24 h after incubation of the cells into lactose broth.

3.2. Selection of best heavy metals for biosorption studies by Curtobacterium sp. FM01

The biosorption capacity of Ni, Cu, Cr, Cd, Zn, Sn, Pb, Co by FM01 strain at the same conditions was investigated. The values of metal removal (%) for the various metals studied are: Ni: 38%, Cu: 0%, Cr: 0%, Cd: 0%, Zn: 16%, Sn: 0%, Pb: 37%, Co: 0%, respectively. A higher value implies higher biosorption by the biomass. Therefore,

consideration of metal removal values together established the trend in metal uptake, namely: $\text{Ni} > \text{Pb} > \text{Zn}$. This study showed that the metal removal (%) values for Ni (II) and Pb (II) with FM01 were significantly larger than the corresponding values with other metals. So, Ni and Pb were selected for further biosorption studies.

3.3. Biosorption experiments

3.3.1. Biosorption time

As shown in Fig. 1, the metal biosorption can be divided into two stages: the rate of biosorption is higher at the beginning (the biosorption was increased rapidly during the first 5-10 min) and followed by a second stage with a much lower sorption rate (the biosorption was remained nearly constant after 20 min for Ni (II) and after 40 min for Pb (II). After this equilibrium period, the amount of biosorbed metals ions did not significantly change with time.

3.3.2. Effect of temperature and the activation energy

The biosorption results showed that biosorption capacity between the lowest (10°C) and highest studied temperatures (50°C) was almost constant (Fig. 2). The temperature of the biosorption was less significant when compared with the effect of other physicochemical factor of metals biosorption because the biosorption as energy-independent mechanisms are less expected to be influenced by temperature [13]. This is in agreement with most of the studies reported on the biosorption of heavy metals onto biosorbents [14, 15].

Therefore, it is always preferred to evaluate biosorption at room temperature, as this condition is easy to reproduce [16].

3.3.3. Effect of initial pH on the biosorption capacity

Different pH values of solutions were used to examine the effect of pH on the biosorption by *Curtobacterium* sp. FM01 (pH 3-7). The pH values above 7 are known to result in Ni and Pb precipitation and therefore were not evaluated. Figure 3 demonstrates that biosorption of Ni (II) and Pb (II) was strongly dependent on the pH of the solution. The results are shown the greatest capacity of biosorption by FM01 was obtained at pH 5.0 for Ni (II) ions and pH 6.0 for Pb (II) ions which was rather acidic. These results seem to suggest that the biosorption of Ni (II) and Pb (II) to biomass varies with the type of adsorbate. For both metals, an augmentation of biosorption was observed, as the solution pH increased from 4 to 6 for Pb (II) and from 4 to 5 for Ni (II). This result is in agreement with a previous study, in which biosorption of Pb (II) ions by *Aspergillus fumigates* at pH 6 was higher than other pH values [17]. Similar studies also showed that the maximum nickel biosorption by different microorganisms occurred at pH 5 [13].

At very low pH values (below 3), functional anionic active sites could be protonated at the biosorbent surface so this process could be restricted of cation uptake [3]. At moderate pH values (3-6), linked H^+ is released from the active sites and adsorbed amount of Ni (II) and Pb (II) ions is generally found to increase. At pH values higher than 6, the precipitation might be occurred or both ion exchange and aqueous metal hydroxide formation may turn out to be significant mechanisms in the metal removal process [18].

3.3.4. Effect of dosage of biosorbent on the biosorption

The effect of the biosorbent dose was studied by varying the biosorbent amounts from 1.0 to 30 mg. For all these runs, initial concentration of Ni (II) and Pb (II) was fixed as 0.5

mM. As shown in Figure 4, the uptake of metals decreased with increasing dosage of biosorbent and the highest metals uptake was observed at 1 mg for Ni (II) and Pb (II).

The increased quantity of biosorbed solute per unit weight of biosorbent decreased with increasing biosorbent dosage, which may be due to the complex interaction of several factors. An important factor at high biosorbent dosages is the available solute is insufficient to completely cover the available exchangeable sites on the biosorbent, typically resulting in low solute uptake [19]. In addition, the interference between binding sites due to increased biosorbent dosages cannot be overruled, as this will result in a low specific uptake. This result is in good agreement with those obtained in other studies [13, 14, 15].

3.3.5. Initial Ni and Pb concentrations

Present studies indicated that the biosorption increased with increasing initial concentration of Ni (II) and Pb (II) (Fig. 5). This is because the ratio of the initial moles of solute to the available surface area is low at lower initial solute concentrations. However, at higher concentrations, the sites available for sorption become fewer compared to the moles of solute present. Hence, the removal of solute is strongly dependent upon the initial solute concentration. It is always necessary to identify the maximum saturation potential of a biosorbent, for which experiments should be conducted at the highest possible initial solute concentration [16]. As shown in Fig 5, higher biosorption in these conditions occurred for Ni (II) in 1 mM and for Pb (II) in 1.5 mM. Similar results reported that an increase in the initial metal concentration increased the mass transfer driving force of the

metal ions between the aqueous solution and biosorbent, which lead to increase in metal ions uptake [13, 14, 15].

3.3.6. *Ni (II) and Pb (II) biosorption kinetics*

Non-linear regression method has been used to predict the best sorption kinetics and also to obtain the kinetic parameters (Table 1). The R^2 values as a coefficient of determination indicated that the R^2 values for pseudo-second-order were higher than the pseudo-first order (Fig. 6 (a & b)). The biosorption process of Pb (II) onto the FM01 was followed second-order rate kinetics; however, kinetics of biosorption of Ni (II) followed both first- and second-order rate equations, indicating that the process concerning the rate-controlling step is complex including boundary layer as well as intraparticle diffusion processes.

3.3.7. *Biosorption isotherm*

At various initial biomass concentrations, batch biosorption equilibrium was reached within 20 min for Ni (II) and after 40 min for Pb (II) and the biosorption isotherms were interpreted in terms of the Langmuir and Freundlich non-linear models (Table 1). The biosorption capacity of Langmuir and Freundlich constants calculated from the corresponding isotherms with the correlation coefficients are presented in Table 1. The comparison of R^2 values for both metals shows that the biosorption process for Ni (II) and Pb (II) could be described by the Langmuir model equation (Figure 7 (a & b) (Table. 1). These values indicated that the metal ions were favorably adsorbed by strain FM01 in the present study. The applicability of Langmuir isotherms to the biosorption of Ni (II) and Pb(II) ions shows that monolayer biosorption of active sites on the surface of biosorbent conditions exist under the experimental conditions employed [8].

3.3.8. FTIR analysis

The FTIR spectra for the metals-loaded and metal-free biomass and biomass after biosorption (Ni and Pb ions-loaded) in the range of 750–3500 cm^{-1} have been shown in the Fig. 8. The FT-IR spectrum displays a number of absorption peaks, reflecting a complex nature of *Curtobacterium* sp. FM01 cell surfaces.

A peak at 3300 cm^{-1} corresponds to the stretching band of the N–H from amino group and indicates a banded hydroxyl group. After the contact with Ni and Pb, the cell surface exhibited spectra with clear shifts of the N–H and OH stretching band to lower frequencies, two bands in the region of 2925 cm^{-1} corresponds to the symmetrical –CH– vibrations in lipids. Two strong bands at 1650 and 1540 cm^{-1} are attributed respectively to the amide I (–CO–) and amide II (–NH–) in proteins. The significant shifts in these peaks indicates the binding of Ni and Pb with amides I and II in cell surfaces of *Curtobacterium* sp. FM01. A single band near 1400 cm^{-1} due to the vibration deformation δ (CH), region of 1205 contains band characteristics of C–C bonds and an sorption band at the location 1080 cm^{-1} corresponds to the –COC– group vibrations in the cyclic structures of carbohydrates. The above observations indicated that the involvement of these functional groups such as carboxylic, hydroxyl and carbonyl groups of saccharides and amino groups of proteins in the Ni (II) and Pb (II) biosorption process.

3.3.9. Transmission electron microscopy (TEM) analysis

Electron microscopic examination of metabolically-active cells of *Curtobacterium* sp. FM01 before and after metals uptake was undertaken. The electron micrograph (Fig. 9a) of the control FM01 cells showed a normal appearance, whereby it does not show any

obvious electron-dense areas. The electron micrograph (Figure 9 b & c) of the FM01 cells which had biosorption Ni (II) and Pb (II) revealed a visible strongly electron-dense area on the middle section of the cell wall. It is suggested that the observed electron-dense area on the cells is due to the presence of metal ions and contains the biosorbed ions.

4. Conclusions

The results obtained in this study showed that the native strain *Curtobacterium* sp. FM01 is a potential biosorbent, with a high biosorption capacity, for the efficient removal of Ni (II) and Pb (II) from wastewaters. According to the parameters of the Langmuir isotherms, the maximum biosorption capacities of Pb (II) and Ni (II) for FM01 were 186.60 and 140.99 (mg g⁻¹), respectively. The excellent applicability of Langmuir isotherm to the metal ions biosorption showed the occurrence of maximum biosorption of active sites on the surface of biosorbent. The inappropriate disposal of heavy metal containing waste or products, as a result of fast-growing industrial activities, is indeed a global concern. Accordingly, the use of biomass-based biosorbents appears attractive alternative approach for the removal of water pollutants. The bacteria biosorption technologies are still being developed, however, more detailed investigations is required to reveal exact mechanisms involved in the heavy metal uptake by metabolically-active and non-active cells at the cellular and molecular levels.

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Figure Captions

Fig. 1. Effect of contact time on biosorption of Ni (II) and Pb (II) by *Curtobacterium* sp. FM01 (Error bars show standard deviation).

Fig. 2. Effect of temperature on biosorption of Ni (II) and Pb (II) by *Curtobacterium* sp. FM01 (Error bars show standard deviation).

Fig.3. Effect of pH values on biosorption of Ni (II) and Pb (II) by *Curtobacterium* sp. FM01 (Error bars show standard deviation).

Fig.4. Effect of biosorbent dose (mg) on biosorption of Ni (II) and Pb (II) by *Curtobacterium* sp. FM01 (Error bars show standard deviation).

Fig.5. Effect of metals concentration on biosorption of Ni (II) and Pb (II) by *Curtobacterium* sp. FM01 (Error bars show standard deviation).

Fig. 6. The non-linear form of pseudo-first (a) and pseudo-second-order model (b) for Ni (II) and Pb (II) biosorption by lyophilized cells of *Curtobacterium* sp. FM01.

Fig. 7. The non-linear form of Langmuir (a) and Freundlich (b) biosorption isotherms of lead by *Curtobacterium* sp. FM01.

Fig. 8. Fourier transform infrared (FTIR) spectra of *Curtobacterium* sp. FM01 cells before and after metal-loading.

Fig. 9. Transmission electron micrographs (TEM) of metabolically-active cells of *Curtobacterium* sp. FM01 before (a) and after Ni (b) and Pb stress (c).

Table 1. Isotherm and kinetic parameters obtained for the biosorption Pb (II) and Ni (II) by *Curtobacterium* sp. FM01 using the non-linear methods.

Metal	Biosorbent	Langmuir isotherm			Freundlich isotherm		
		$Q_{\max}$	B (1/mg)	R^2	K_f	n	R^2
Pb	FM01	186.6063	0.0060	0.8937	8.9276	2.2614	0.8087
Ni	FM01	140.9994	0.0123	0.9659	17.5687	3.1776	0.9112

Metal	Biosorbent	Pseudo-first-order model			Pseudo-second-order model		
		q_e	K_1	R^2	q_e	K_2	R^2
Pb	FM01	96.4802	0.1741	0.9827	101.337	0.0032	0.9942
Ni	FM01	88.1078	0.85965	0.9994	88.2496	0.18826	0.9994

Table 2. Comparison of maximum biosorption capacity of Lead and Nickel by various biological sorbents.

Metal	Species name	$q_{\max}$ (mg g ⁻¹)	References
Pb (II)	<i>Bacillus</i> sp. (ATS-1)	92.3	[21]
	<i>Enterobacter</i> sp.J1	50.9	[10]
	<i>Aspergillus fumigates</i>	43.66	[17]
	<i>Pseudomonas stutzeri</i>	153.3	[20]
	<i>Curtobacterium</i> sp. FM01	180.60	Present study
Ni (II)	<i>Pseudomonas aeruginosa</i>	5.7	[3]
	<i>Geobacillus toebii</i> sub.sp. <i>decanicus</i>	21	[13]
	<i>Geobacillus thermoleovorans</i> sub.sp. <i>stromboliensis</i>	42	[13]
	<i>Curtobacterium</i> sp. FM01	140.99	Present study

Figure 1

494 **Figure 2**

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521 **Figure 3**

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548 **Figure 4**

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575 **Figure 5**

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Figure 6 (a, b)

Figure 7 (a, b)

665 **Figure 8**

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683 **Figure 9 (a, b, c)**

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